REGULATION OF THE U3 SMALL SUBUNIT PROCESSOME AND ASSOCIATED RNA-BINDING PROTEINS

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Declaration

I certify that this thesis contains my own work, except where acknowledged, and that no part of this material has been previously submitted for a degree or any other qualification at this or any other University.

Abstract

The rate of ribosome biogenesis regulates the growth rate of the cell and is believed to be linked to the cell's proliferative potential. Moreover, ribosome production is down-regulated in terminally differentiated cells and up-regulated in the majority of cancers. rRNA transcription is regulated in these processes although much remains unclear about the regulation of rRNA processing. In eukaryotes, 18S ribosomal RNA (rRNA) processing is mediated by the small subunit (SSU) processome. This is composed of the U3 small nucleolar (sno)RNP, many sub-complexes and a range of putative rRNA binding and modifying proteins. It is not clear however, which proteins bind or cleave the pre-rRNA, with the exception of NOB1. Moreover, the majority of research to date has been conducted in the yeast *Saccharomyces cerevisiae* whereas the human SSU processome remains largely unstudied.

Here we report that U3 snoRNP accumulation and function are regulated through the U3-specific hU3-55k protein. We demonstrate that U3 snoRNP levels are specifically down-regulated during human lung (CaLu-3) and colon (CaCo-2) epithelial cell differentiation and that this is likely mediated through regulating hU3-55k levels. Moreover, CaCO-2 adenocarcinoma cells are believed to revert to their pre-cancerous phenotype during differentiation, suggesting that U3 snoRNP levels increase during tumourogenesis. We also show that phosphorylation of hU3-55k is likely to be essential for U3 snoRNP function; being required for the initial cleavage of the pre-rRNA. We therefore demonstrate two independent mechanisms that may regulate ribosome biogenesis through hU3-55k. We also demonstrate that the human and yeast SSU processomes contain many orthologous proteins. However, components responsible for 3' pre-18S rRNA processing may function at temporally and spatially different points to their counterparts in yeast. Nonetheless, PNO1 and NOB1 are closely associated, with their nucleocytoplasmic shuttling affected by blocking pre-rRNA transcription, CRM1 mediated export and the mTOR pathway, likely preventing pre-40S export to the cytoplasm. This suggests yet another level of regulation to ribosome biogenesis through pre-rRNA processing.

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Abbreviations and Acronyms

A	Alanine
ABT1 (ESF2)	An enhance of basal transcription
ActD	Actinomycin D
AdoMet	S-adenosyl-L-methionine
AMV	Avian myeloblastosis virus
ATP	Adenosine triphosphate
BCD1	Box C/D snoRNA accumulation protein
BPTE	Bis-Tris- PIPES- EDTA
BSA	Bovine serum albumin
C	Cytosine (DNA/RNA)
C	Cysteine (Protein)
CAB	Cajal Body box
cDNA	Complementary Deoxyribonucleic Acid
CK2	Casein Kinase 2
CMV	Cytomegalo virus
CRM1 (XPO1)	Chromosomal Region Maintenance 1
CTD	Carboxyl-terminal domain
C-terminal	Carboxyl-terminal
CTP	Cytidine triphosphate
D	Aspartic Acid
DAPI	4’,6-diamidino-2-phenylindole
DFC	Dense fibrillar component
DIM1	Di-Methyltransferase 1
DIM2 (PNO1)	DIM1-binding protein
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol

E	Glutamic Acid
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EMG1	Essential for mitotic growth 1
EtBr	Ethidium bromide
ETS	External transcribed spacer
F	Phenylalanine
FBS	Foetal bovine serum
FC	Fibrillar Centre
FISH	Fluorescence *in situ* hybridisation
x – FLAG	2 x FLAG 6 x His tagged protein (where x is the protein)
FRT	Flp Recombination Target
G	Glycine (Protein)
G	Guanosine (DNA/RNA)
G1	Gap 1
G2	Gap 2
GC	Granular component
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GST	Glutathione S-Transferase
GTP	Guanosine triphosphate
H	Histidine
His	Histidine
His tag or 6xHis	6 x Histidine tag
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulphonic acid
HRP	Horseradish peroxidase
H. sapiens	*Homo sapiens*
HSC70	Heat Shock Protein 70
HSP90	Heat Shock Protein 90
hU3-55k	Human U3-associated 55 kDa Protein
I	Isoleucine

IF	Immunofluorescence
IMP3	Interacts with MPP10, 3
IMP4	Interacts with MPP10, 4
IP	Immunoprecipitation
ITS	Internal transcribed spacer
K	Lysine
Kb	Kilobase
KCl	Potassium chloride
kDa	KiloDalton
KOAc	Potassium acetate
KOH	Potassium hydroxide
L	Leucine
LMB	Leptomycin B
LSU	Large subunit
LSm	Like Sm Protein
M	Methionine
m_3G	2,2,7-trimethylguanosine cap
m^7G	7-monomethylguanosine cap
$MgCl_2$	Magnesium chloride
MgOAc	Magnesium acetate
miRNAs	MicroRNAs
M. jannaschii	*Methanocaldococcus jannaschii*
M. musculus	*Mus musculus*
M-Phase	Mitotic phase
MPP10	M-phase phosphoprotein 10
mRNA	messenger RNA
MRD1	Multiple RNA-binding domain-containing protein 1
N	Asparagine
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NaH_2PO_4	Sodium phosphate, monobasic

Na_2HPO_4	Sodium phosphate, dibasic
NEP1	Nucleolar essential protein 1
NES	Nuclear export signal
NLS	Nuclear localisation signal
NOP17	Nucleolar localisation protein 17
NOR	Nucleolar organizer region
NMD3	Nonsense-mediated mRNA decay protein 3
NPC	Nuclear pore complex
nt	Nucleotide
N-terminal	Amino-terminal
NTP	Nucleoside triphosphate
NUFIP	Nuclear FMRP interacting protein
P	Proline
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-buffered saline
pcDNA5-FLAG	Modified pcDNA5 vector containing a 2xFLAG – PreScission Protease Site – 6xHis Tag sequence
PCR	Polymerase chain reaction
PHAX	Phosphorylated adaptor for RNA export
PIC	Pre-initiation complex
PIN	PilT N terminus
PIPES	1,4-Piperazinediethanesulphonic acid
PNB	Prenucleolar bodies
PNK	Polynucleotide kinase
PNO1 (DIM2)	Partner of NOB1
pre-rRNA	Precursor rRNA
pre-snoRNA	Precursor-small nucleolar ribonucleic acid
ψ	Isomerization of uridine to pseudouridine
PWP2 (UTP1)	Periodic tryptophan protein 2
R	Arginine
RCL1	RNA terminal phosphate cyclase like
rDNA	Ribosomal DNA

RNA	Ribonucleic acid
RNAi	RNA interference
RNP	Ribonucleoprotein(s)
Rplx	LSU ribosomal protein x (where x is a number)
Rpm	Revolutions per minute
Rpsx	SSU ribosomal protein x (where x is a number)
rRNA	Ribosomal RNA
RRP	Ribosomal RNA-processing protein
S	Serine
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulphate
siRNA	small interfering RNA
SL1	Selectivity factor 1 protein complex
snoRNA	small nucleolar RNA
snoRNP	small nucleolar ribonucleoproteins (snoRNA-protein complexes)
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoproteins
SOF1	Suppressor of fibrillarin
S-Phase	Synthesis Phase
SSC	Sodium chloride- sodium citrate
SSU	Small subunit
SV40	Simian vacuolating virus 40
T	Threonine (Protein)
T	Thymidine (DNA /RNA)
TBE	Tris- boric acid- EDTA
TBP	TATA binding protein
Tet.	Tetracycline
TGS1	Methyltransferase trimethlguanosine 1
TPR	Tetratricopeptide repeats
TIP48	TBP (TATA binding protein) - interacting protein of 48kDa

TIP 49	TBP (TATA binding protein) - interacting protein of 49kDa
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA
TTP	Thymidine triphosphate
UBF1	Upstream binding factor 1
tUTP	Transcriptionally required U three protein
UTP	U three (3) protein
U	Uridine
µg	Microgram
µl	Microlitre
µm	Micrometer
UV	Ultra violet
V	Valine
v/v	Volume per volume
W	Tryptophan
w/v	Weight per volume
X. Laevis	*Xenopus laevis*
XPO1 (CRM1)	Exportin 1
Y	Tyrosine
$ZnSO_4$	Zinc sulphate

Chapter One

Introduction

1.1 Ribosomes and rDNA Organisation

The ribosome is a universally conserved cellular machine, composed of ribosomal RNA (rRNA) and many more ribosomal proteins (r-proteins). Ribosomes are responsible for the translation of coding RNA and protein production through peptide bond formation. The cytoplasmic eukaryotic ribosomes and prokaryotic ribosomes sediment at 80S (Svedberg units) and 70S, respectively. They are composed of a large subunit (LSU; 50S in prokaryotes and archaea, 60S in eukaryotes); responsible for peptidyl transferase activity, and a small subunit (SSU; 30S in prokaryotes and archaea, 40S in eukaryotes); responsible for the decoding function of the ribosome (Lafontaine and Tollervey, 2001). The SSU is composed of a single rRNA (16S in prokaryotes and archaea, 18S in eukaryotes) whereas the LSU is composed of either two (5S and 23S in prokaryotes) or three (5S, 5.8S and 25S/28S in eukaryotes) rRNAs. Each subunit also has many associated r-proteins; 32 in the SSU and 46 in the LSU in *Saccharomyces cerevisiae*, although numbers vary with species (Mager et al., 1997; Planta and Mager, 1998). These are thought to be important in maintaining the rRNA structure (Lafontaine and Tollervey, 2001).

In almost all instances, much of the mature rRNA is processed from polycistronic precursors (pre-rRNA), with the structural organisation of the rRNA genes generally conserved between species (Figure 1.1). Similarly, the key steps are also well conserved, these include; transcription of the pre-rRNA; covalent modification of the pre-rRNA; processing of the rRNA to the mature species; and assembly of the r-proteins with the rRNA. In eukaryotes, the 18S, 5.8S and 28S (25S in yeast) rRNAs are transcribed by RNA polymerase (pol.) I as a single precursor which contains two internal transcribed sequences (ITS1 and ITS2), and two external transcribed sequences (5’ ETS and 3’ ETS), all of which need to be removed to

generate the mature rRNA through processing events. The 5S rRNA is transcribed separately by RNA pol. III. The prokaryotic and archaeal pre-rRNA generally contain the 16S, 23S and the 5S in a single precursor. These are similarly flanked by 5' and 3' ETS with internal ITS motifs. In addition, the ITS region of bacterial and archaeal pre-rRNAs contains a transfer RNA (tRNA).

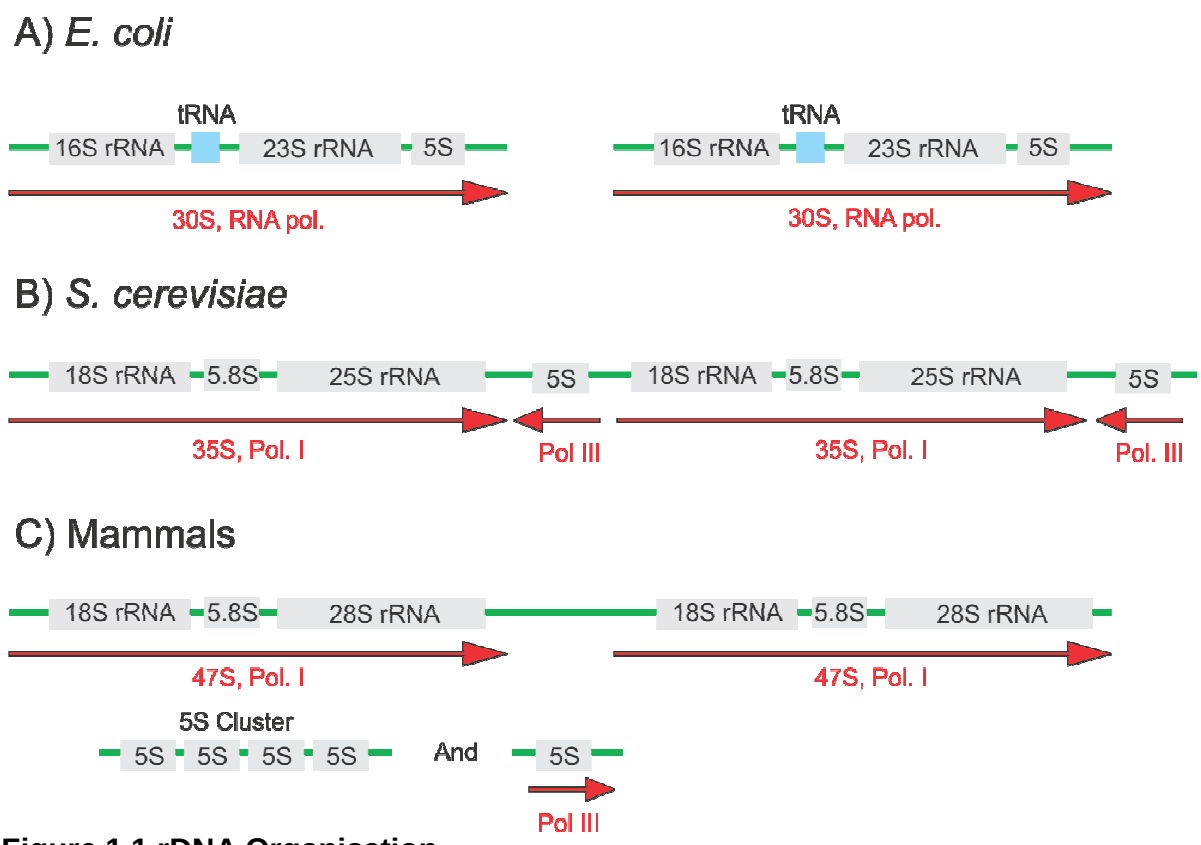


Figure 1.1 rDNA Organisation
A) In prokaryotes, the small subunit (16S) and large subunit (23S and 5S) rRNAs are transcribed from a single operon as a single 30S precursor by RNA polymerase. There is often a tRNA gene located between the 16S and 23S rRNAs. **B)** In the yeast *Saccharomyces cerevisiae* the small subunit (18S) and large subunit (5.8S and 25S) rRNAs are transcribed as a single 35S precursor by RNA pol. I The 5S rRNA gene is located at the 3' end of the 28S rRNA gene and is transcribed by RNA pol. III. **C)** In mammals, the small subunit (18S) and large subunit (5.8S and 28S) rRNAs are transcribed as a single 47S precursor by RNA pol. I. The 5S rRNA gene is present either separately, or in clusters elsewhere in the genome. These are transcribed by RNA pol. III.

Bacterial rRNA genes are present in single operons, with *Escherichia coli* only containing 7 copies and prokaryotes on an average containing just 3 or 4 copies per genome (Turova, 2003). Contrastingly, in the yeast *S. cerevisiae*, there are ~150 copies of the 35S rDNA gene arranged in tandem repeats on chromosome 12, transcribed by RNA pol. I (Kobayashi et al., 1998). Moreover, there are believed to be 300-400 rRNA tandemly repeated rDNA genes in human cells. Each of these repeats is approximately 43 kb in size and contains the 47S rDNA sequence, although this is only 13 kb. These are distributed on the short arms of acrocentric chromosomes (chromosomes 13, 14, 15, 21 and 22) (Prieto and McStay, 2005). Head-to-tail tandemly repeated gene clusters, representing on average 3Mb of DNA, are termed NORs (nucleolar organizer regions), with several rDNA clusters from different chromosomes often forming a single nucleolus (Prieto and McStay, 2005). It is believed that only around half of these repeats are transcriptionally active at any one time; with the rest potentially being important instead, in maintenance of nucleolar organisation and chromatin structure, independent of ribosome biogenesis (Dammann et al., 1993; Jordan et al., 1996; Sanij and Hannan, 2009).

1.2 rDNA Transcription in Eukaryotes

In eukaryotes the 47S pre-rRNA (35S in yeast) is transcribed by RNA pol. I in the nucleolus. This is in contrast to the 5S rRNA which is transcribed by RNA pol. III in the nucleoplasm of the cell (Lee and Nazar, 2003; Nazar, 2004). Yeast (*S. cerevisiae*) RNA pol. I has been highly studied and is known to be made up of 14 subunits: 10 core proteins and 2 heterodimers (Russell and Zomerdijk, 2005; Yamamoto et al., 2004). Contrastingly, less is known about human pol. I. It is however, known that it contains 13 subunits with the subunits PAF67 and RPA43 thought to recruit RNA pol. I to the promoter through interaction with the initiation factor TIF-IA (Yuan et al., 2002).

Efficient transcription of rDNA by RNA pol. I requires the formation of a pre-initiation complex (PIC) on the rDNA promoter. In mammalian cells, the 47S rDNA

gene promoter contains an upstream control element (UCE) and core promoter sequence. This enables the formation of a transcriptionally competent complex (Raska et al., 2004). The upstream binding protein (UBF) is believed to associate with the promoter elements as a dimer, aiding recruitment of RNA pol. I and the promoter selectivity factor SL1 (which comprises the TATA-binding protein (TBP), and at least five TBP-associated factors (TAFs) (Denissov et al., 2007; Gorski et al., 2007; Lin et al., 2002)). Also required are the transcription initiation factors TIF-IA and TIF-IC (Raska et al., 2004). UBF is believed to function in maintaining under-condensed r-chromatin of active NORs (required for active transcription) whereas SL1 is known to interact with promoter DNA in a highly sequence-specific manner, aiding further formation of the PIC (Mais et al., 2005; Prieto and McStay, 2007). The PIC in turn recruits an initiation-competent subfraction of Pol I; allowing transcription of the rRNA genes (Prieto and McStay, 2005). The polymerase II transcription factor TFIIH complex is then required for pol. I driven elongation, and the transcription termination factor TTF-1 required for terminating transcription and ribosomal chromatin remodeling (Raska et al., 2004).

During metaphase, NORs which were transcriptionally active in the previous interphase, form secondary constrictions. The chromatin in these structures is less condensed and contains many factors involved in ribosome biogenesis, including UBF, despite rDNA transcription being shut off (Prieto and McStay, 2005; Prieto and McStay, 2007; Sanij and Hannan, 2009). The association of UBF and other factors are thought to allow active NORs to remain under-condensed during mitosis, giving the metaphase chromosomes concerned a distinct secondary constriction structure; facilitating the rapid onset of rDNA transcription and nucleolar reformation as cells exit mitosis (Prieto and McStay, 2005; Prieto and McStay, 2007; Sanij and Hannan, 2009)

1.3 rRNA Modifications and snoRNPs

During ribosome maturation, the pre-ribosomal RNA (rRNA) is covalently modified, guided by a myriad of small nucleolar ribonucleoproteins (snoRNPs); protein

complexes on a snoRNA (small nucleolar RNA) backbone. These snoRNAs contain conserved structural and sequence motifs which are important for RNA-RNA and protein-RNA interactions. Subsequently, the pre-rRNA is cleaved into mature rRNA (Gallagher et al., 2004; Terns and Terns, 2002). It has recently been demonstrated that in *S. cerevisiae*, the pre-rRNA is co-transcriptionally modified and processed (Kos and Tollervey, 2010). Although similar modifications and processing steps are known to occur to the nascent pre-rRNA in humans, it is not entirely clear if these steps also take place co-transcriptionally.

Regardless of this, the first maturation steps for both yeast and human pre-rRNAs are a range of covalent modifications, guided by the snoRNA sequence of the snoRNP. These function by correctly positioning the modifying enzymes on the pre-rRNA by base-pairing to the target site (Henras et al., 2004b; Kiss-Laszlo et al., 1996; Kiss-Laszlo et al., 1998; Terns and Terns, 2002). In mammals, more than 200 rRNA nucleotides are modified by either the Box C/D snoRNAs (responsible for methylation of specified rRNA ribose moieties) or Box H/ACA snoRNAs (converting uridines to pseudouridines; Figure 1.2). The snoRNAs are defined by the associated proteins and conserved RNA sequence elements / Box motifs (Gerbi et al., 2001; Krogan et al., 2004). Modifications occur in highly conserved sequences of rRNA and may be found on newly transcribed rRNAs prior to cleavage, occurring co-transcriptionally in yeast (Kos and Tollervey, 2010). These modifications cluster to functionally important domains of the mature rRNA; important to the secondary structure of the rRNA and possibly modifying interactions with ribosomal proteins (Decatur and Fournier, 2003).

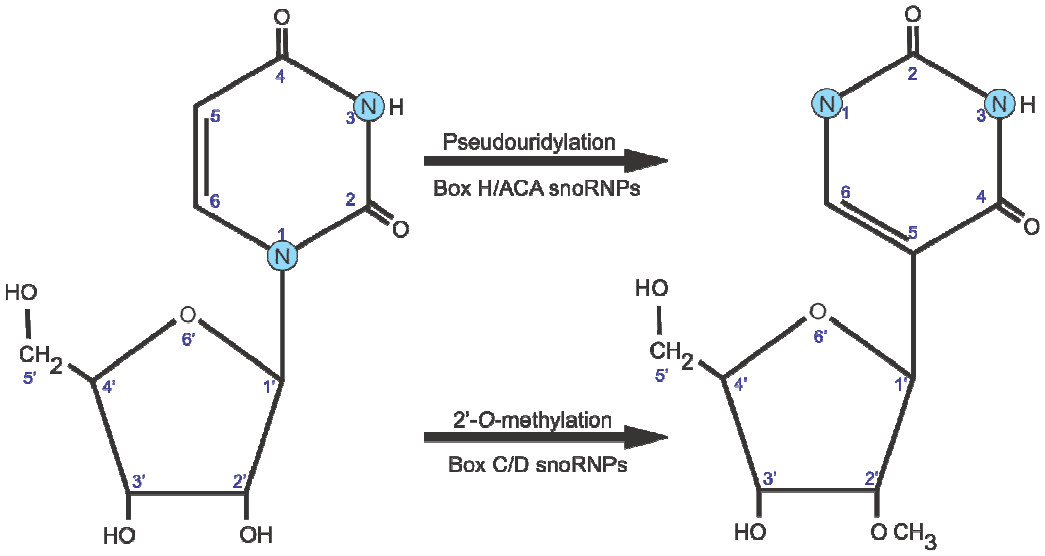


Figure 1.2 Covalent rRNA Modifications Guided by Box H/ACA and Box C/D snoRNPs
Conversion of uridines into pseudouridine (guided by BOX H/ACA snoRNPs, top) and 2'-*O*-methylation (guided by Box C/D snoRNPs, bottom) are shown. Nitrogen atoms are shown as blue circles. Only the sugar moiety of the ribose is shown, without associated phosphates at the 3' and 5' carbons. The numbering of the carbon atoms is shown in dark blue. Figure based on previous work (Henras et al., 2008).

1.3.1 Box H/ACA snoRNPs

The Box H/ACA snoRNPs direct pseudouridylation (the isomerisation of uridine to pseudouridine) of target rRNA residues. They are composed of snoRNA and the common core proteins NHP2, GAR1, NOP10 and Dyskerin, whereby Dyskerin (Cbf5 in yeast) catalyses the pseudouridylation of target rRNA residues (Henras et al., 1998; Lafontaine et al., 1998a; Zebarjadian et al., 1999).

The Box H/ACA snoRNAs are characterised by conserved sequence elements termed Boxes H and ACA. In eukaryotes these snoRNAs typically form a secondary structure consisting of two hairpin units, separated by a single-stranded hinge that contains the Box H motif (ANANNA, with N being any nucleotide; Figure 1.3). The second hairpin is followed by a single-stranded segment with the Box ACA tri-nucleotide sequence, located three nucleotides upstream of the mature 3'-end of the snoRNA (Matera et al., 2007; Reichow et al., 2007).

These guide RNAs are responsible for the selection of the nucleotides that will be modified through specific Watson-Crick base-pairing interactions with the pre-rRNAs (Henras et al., 2008). Box H/ACA guide snoRNAs contain small sequences of complementarity with pre-rRNAs located on both sides of internal loops (pseudouridylation pockets) within the stem-loop structures that bracket the H box. The antisense sequences of the pseudouridylation pocket bind immediately upstream and downstream from the targeted pre-rRNA uridine (Figure 1.3). This uridine is thus exposed and unpaired at the centre of the RNA junction (Ganot et al., 1997; Ni et al., 1997). This allows the uridine in one or both of the pockets to be modified by Dyskerin with the modification(s) occurring 14–16 nucleotides upstream of the H or ACA box motifs (Ganot et al., 1997; Ni et al., 1997). Accordingly, it is believed that a set of each of the core proteins are capable of associating with each hairpin of eukaryotic Box H/ACA snoRNPs (Figure 1.3).

It is worth noting that some eukaryotic Box H/ACA (and Box C/D) snoRNPs also contain a Cajal body (CAB)-box sequence element (UGAG) for Cajal body retention. This subset of RNAs act to guide the modification of small nuclear RNAs rather than rRNA (Matera et al., 2007). These are therefore called small Cajal-body-specific (sca) RNAs due to their retention in the Cajal bodies of the cell (Henras et al., 2004b). Furthermore, telomerase RNA (required for the replication of telomeres), also contains an H/ACA motif, a CAB-box, associates with the same core proteins and localises to the Cajal bodies, so may also be considered a scaRNA (Jady et al., 2004; Mitchell et al., 1999; Wang and Meier, 2004).

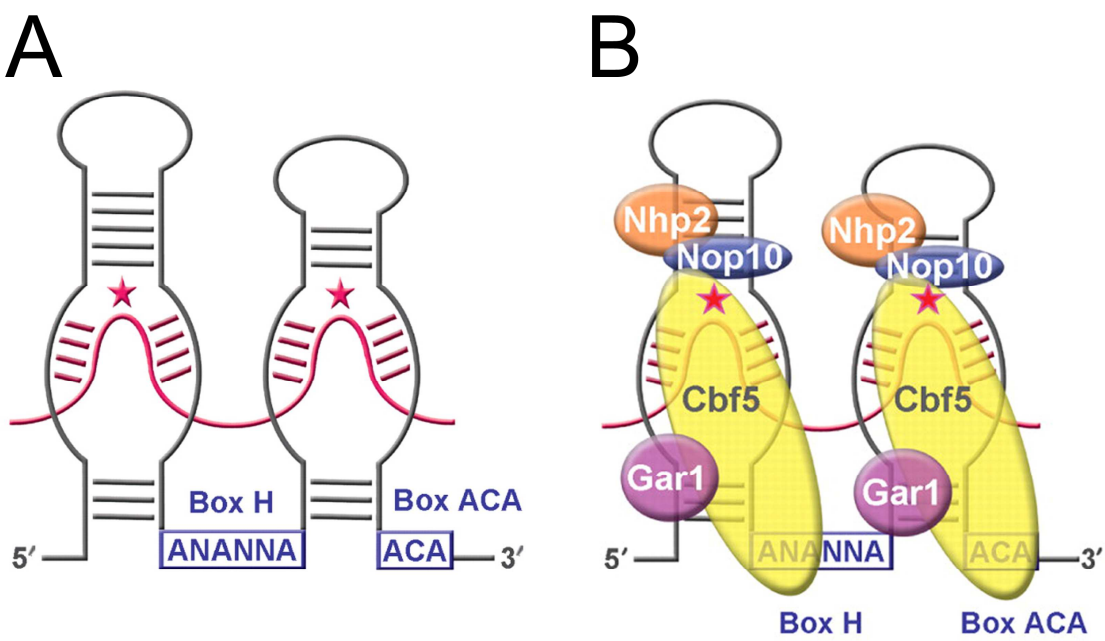


Figure 1.3 Eukaryotic Box H/ACA snoRNPs
A) The Box H/ACA snoRNPs are proposed to form two stem-loop structures (grey) with conserved sequence motifs (blue). The snoRNA forms Watson-Crick base pairs with specific sites of rRNA to enable pseudouridylation (red stars). **B)** Each stem-loop of the H/ACA snoRNA is proposed to associate with a set of the core proteins (GAR1, Cbf5/Dyskerin, NOP10 and NHP2). Cbf5/Dyskerin is proposed to catalyse the isomerisation of uridine to pseudouridine. Figure adapted from (Reichow et al., 2007).

1.3.2 Box C/D snoRNPs

The Box C/D snoRNAs direct the site-specific 2'-*O*-methylation of ribose moieties in rRNA and snRNAs. They contain the core proteins NOP56, NOP58, 15.5K (Snu13 in yeast) and the methyltransferase fibrillarin (Nop1 in yeast) (Baserga et al., 1991; Galardi et al., 2002; Lafontaine and Tollervey, 1999; Lafontaine and Tollervey, 2000; Lyman et al., 1999; Schimmang et al., 1989; Tyc and Steitz, 1989; Watkins et al., 2000; Wu et al., 1998).

The Box C/D snoRNPs contain conserved box C (RUGAUGA, at the 5' end of the snoRNA) and a Box D (CUGA, 3' end of the snoRNA) sequence elements. The 5' and 3' ends of the snoRNA are often base-paired, forming a stem–loop structure (Figure 1.4). The majority of Box C/D snoRNAs also contain a related, although less highly conserved, Box C'/D' motif (Kiss-Laszlo et al., 1996; Kiss-Laszlo et al., 1998).

The site of rRNA modification is specified by the formation of base pairs between the rRNA and snoRNA over 10–20 nucleotides. The base pairing occurs upstream of the D / D' box motifs with rRNA flanking the site of 2'-*O*-methylation. The methylation reaction takes place exactly 5 nucleotides upstream of the conserved CUGA motif, catalysed by the methyltransferase fibrillarin (Galardi et al., 2002; Kiss-Laszlo et al., 1996; Kiss-Laszlo et al., 1998). Fibrillarin is believed to transfer the methyl group from the co-factor S-adenosyl-L-methionine (AdoMet) to the ribose 2'-OH of the RNA substrate of the target rRNA residue (Galardi et al., 2002).

The related common core proteins NOP56 and NOP58 are highly homologous and belong to a family of proteins which contain a conserved NOP domain; proposed to function in RNP association (Gautier et al., 1997). Although both NOP56 and NOP58 are essential for ribosome biogenesis and NOP58 is thought to be required for snoRNP stability in yeast, their exact function is not clear (Gautier et al., 1997; Lafontaine and Tollervey, 1999). The 15.5K protein belongs to the L7Ae protein family that share a homologous RNA binding motif. This family includes the box H/ACA snoRNP core protein NHP2 (Henras et al., 1998; Watkins et al., 1998). The association of 15.5K to the box C/D snoRNA is essential for the binding of the other common core proteins (Watkins et al., 2002). The protein is believed to act as nucleating factor, the binding of which has been proposed to result in conformational changes in the snoRNA, generating binding sites for the remaining snoRNP proteins (Watkins et al., 2002).

The stoichiometry of the snoRNP proteins is not definitively known although it is thought that most eukaryotic box C/D snoRNAs adapt a pseudo-symmetric architecture, containing an additional, related C'/D' motif; thus the snoRNPs may contain two active guide sequences (Figure 1.4), giving rise to the model of NOP56 and NOP58 bridging the two box C/D motifs, with a fibrillarin protein associated with each D (or D') box motif (Reichow et al., 2007; Venema and Tollervey, 1999). In yeast, the core proteins NOP1 (fibrillarin), NOP56, and NOP58 have been shown to directly cross-link to the 3' end of the U3 Box C/D snoRNA, near the

conserved box D motif (Granneman et al., 2009). It has been demonstrated through crosslinking of the U25 snoRNP in *Xenopus* oocytes, that NOP56 and NOP58 recognise the C' and C Boxes, respectively, whilst fibrillarin interacts with D and D' Boxes, supporting the idea of an asymmetric snoRNP (Cahill et al., 2002). Furthermore, 15.5K has been shown to only associate directly with the box C/D motif of a composite archetypal box C/D snoRNA *in vitro* (Szewczak et al., 2002).

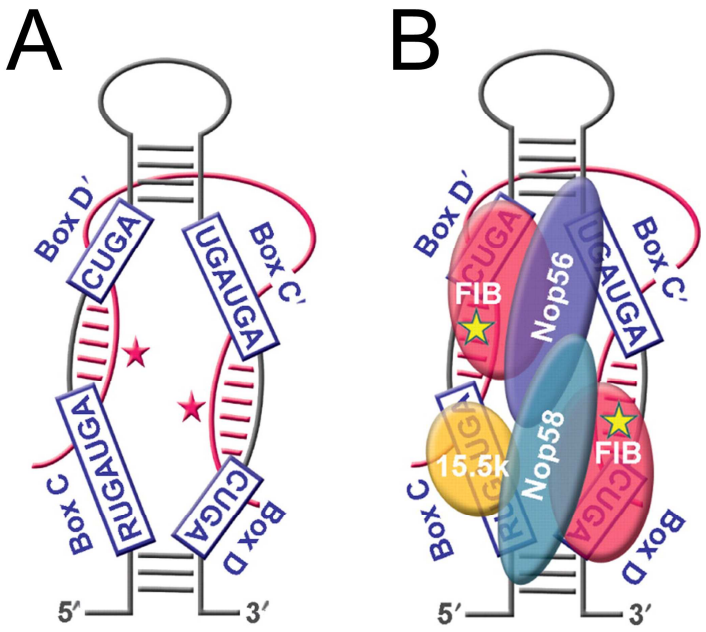


Figure 1.4 Eukaryotic Box C/D snoRNP
A) The Box C/D snoRNPs are proposed to form a stem-loop structure (grey) with conserved sequence motifs (blue). The snoRNA forms Watson-Crick base pairs with specific sites of rRNA (red) to enable methylation (red stars). **B)** The Box C/D snoRNP is proposed to form a pseudo-symmetric structure. The 15.5K protein is thought to bind solely at the C/D site of the snoRNA. NOP56 and NOP58, recognize the C' and C Boxes, respectively although may also recognise the Box D motif, as shown in yeast. A copy of fibrillarin (Fib) interacts with each D and D' Box, with the catalytic centre and ribosome methylation indicated by a yellow star. Figure adapted from (Reichow et al., 2007).

It should be noted that recently, an alternative model of the box C/D snoRNP has been proposed based on the analysis of an sRNP from the archaeon *Methanocaldococcus jannaschii*. This was achieved by single-particle electron microscopy of reconstituted box C/D sRNPs and suggests that they form a dimeric structure composed of two sRNPs, each containing two sets of the core-proteins

(Bleichert et al., 2009). This was however, achieved *in vitro* and it is unclear if this reflects the state *in vivo* in archea, or indeed in mammalian cells.

1.4 Box C/D snoRNP Biogenesis

The U3 snoRNA belongs to the box C/D snoRNA family, of which there are over 100 different members in humans (Lestrade and Weber, 2006; Ono et al., 2010). The majority of box C/D snoRNAs function as sequence-specific guides to direct 2'-*O*-methylation of rRNA whilst U3 and a small number of others act to guide cleavages of the pre-rRNA (including U22, U8, U14 and U13; see section 1.6).

Many Box C/D snoRNAs are encoded within the introns of protein-coding genes. These intronic Box C/D snoRNAs are excised from the pre-mRNA in the nucleus by either the splicing dependent, or the splicing independent pathway. The majority of intronic Box C/D snoRNAs are processed by the canonical / lariat (lasso-like) splicing pathway in which, the spliceosome brings the two exon-intron junctions into proximity, removes the intron and ligates the two exons together (Terns and Terns, 2002). The splicing independent pathway relies upon endonucleolytic cleavages of the flanking intronic sequences to release the snoRNA from the pre-mRNA (Caffarelli et al., 1996). The pre-snoRNAs are then processed further to form the mature snoRNA.

In contrast, a subset of box C/D snoRNAs, including U3, U8 and U13, are independently transcribed by RNA polymerase II and contain a co-transcriptionally added 5'- 7-methylguanosine (m^7G) cap which is hypermethylated to give a trimethylguanosine cap structure ($m^{2,2,7}G$ / m_3G cap), an important part of committing the transcripts to the nucleolar localisation pathway, most likely added in the Cajal bodies by TGS1 (Jacobson and Pederson, 1998; Terns and Terns, 2002; Verheggen et al., 2002).

In yeast the majority of box C/D snoRNA genes are encoded as independent genes, being both monocistronic (U3 snoRNA) and polycistronic (U14 snoRNA). In

both cases, the nascent Box C/D snoRNAs contain 5' and 3' extended sequences. In yeast, the 5' extended sequences of Box C/D snoRNAs are processed by the 5'-3' exonucleases RAT1 and XRN1. These are also involved in processing at the 5' end of the 5.8S rRNA, with their functions thought to be conserved in mice (Petfalski et al., 1998).

This is in comparison to the 3' extended sequences of many yeast Box C/D snoRNAs (including U3 snoRNA) which are cleaved by the endonuclease RNT1 (Chanfreau et al., 1998; Kufel et al., 2003; Kufel et al., 1999). The 3' extended sequences of Box C/D snoRNAs terminate in poly(U) tracts which are normally stabilised by binding of the yeast La homologue, LHP1 and in the case of pre-U3, the association of the LSM proteins (Kufel et al., 2003). The Box C/D snoRNAs are subsequently processed by the 3'-5' exonuclease complex, the exosome (Allmang et al., 1999). It is thought that the core proteins then displace LHP1 and define the 3' end of the mature snoRNA, with their association allowing snoRNA accumulation and nucleolar localisation (Kufel et al., 2003; Lafontaine and Tollervey, 1999; Lange et al., 1998a; Lange et al., 1998b; Narayanan et al., 1999; Samarsky and Fournier, 1998; Speckmann et al., 1999; Verheggen et al., 2002; Watkins et al., 2002).

Less is known about processing of Box C/D pre-snoRNAs in higher eukaryotes however, processing at the 5' end of Box C/D pre-snoRNAs in has been shown to require an endonuclease, XendoU. This was identified in *Xenopus* and was found be required for a 5' cleavage event which releases the U16 and U86 Box C/D snoRNAs from their respective host introns (Laneve et al., 2003). Furthermore, homologs of a number of yeast snoRNA processing factors have also been shown to interact with the box C/D pre-snoRNP in human cells. These include La, LSM4 and RRP46 (an exosome component) which associate with U3 and U8 box C/D pre-snoRNPs (Watkins et al., 2004; Watkins et al., 2007). It has been shown that the La protein interacts with an early U8 pre-snoRNA whereas RRP46 and LSM4 only associate later in the biogenesis pathway (Watkins et al., 2007). In contrast to

yeast, the core proteins also associate with an early U8 precursor indicating that snoRNA biogenesis may vary between yeast and humans.

In both humans and yeast, snoRNP biogenesis involves the dynamic, sequential association of numerous factors as part of a large multiprotein pre-snoRNP complex, believed to partly form at the site of transcription, with subsequent protein recruitment and processing within Cajal bodies in higher eukaryotes or the nucleolus/nucleolar body in yeast (Verheggen et al., 2002; Watkins et al., 2004). These factors include proteins linked to snoRNP assembly (TIP48, TIP49, NUFIP, TAF9, NOP17 and BCD1), molecular chaperones (HSP90 and HSC70), nucleocytoplasmic transport factors (PHAX, CRM1, CBC, Nopp140, Ran and Snurportin1) and proteins implicated in snoRNA maturation (TGS1, La, LSm proteins and the exosome) (Boulon et al., 2008; Boulon et al., 2004; McKeegan et al., 2007; Verheggen et al., 2002; Watkins et al., 2004; Watkins et al., 2007; Zhao et al., 2008). Theses pre-snoRNP factors have been proposed to form a scaffold that drives core snoRNP protein assembly, with several of these factors also predicted to function in the assembly of the U3-specific box B/C snoRNP complex (McKeegan et al., 2007; McKeegan et al., 2009).

1.5 rRNA Processing

As mentioned previously, during pre-rRNA transcription in yeast, many of the covalent modifications and nucleolytic cleavages occur co-transcriptionally; separating the mature rRNAs from the long (13 kb human) precursor in the majority of cases (Kos and Tollervey, 2010), although dependant on growth conditions (Osheim et al., 2004). Whilst the majority of research has focused on the yeast system, it is thought likely that similar modifications and processing steps may also occur co-transcriptionally in human cells (Granneman and Baserga, 2005; Henras et al., 2004a; Peculis, 2001).

1.5.1 Pre-rRNA Processing in *S. cerevisiae*

Pre-rRNA processing has been most extensively studied in the yeast *S. cerevisiae* (Granneman and Baserga, 2004), in which the 35S transcript is processed co-transcriptionally (Kos and Tollervey, 2010). The first cleavage occurs in the fibrillar structures of the nucleolus, at the A_0 site (5'ETS) followed by A_1 (5' ETS / 18S 5' boundary) which occur to remove the 5'ETS, producing the 32S pre-rRNA (Figure 1.5). Cleavage at A_2 subsequently occurs within ITS1, separating the pre-18S (20S) from the 25S and 5.8S precursors ($27SA_2$). The large and small subunit precursors are then processed by different pathways. The 20S precursor is transported to the cytoplasm where cleavage at the D site produces the mature 18S rRNA (Fatica et al., 2003; Schafer et al., 2003). This linear pathway is in contrast to the two alternative nucleolar and nuclear processing pathways of the large subunit precursor. Approximately 85% of the pre-rRNA is cleaved at A_3 in ITS1 which is followed by 5' to 3' exonuclease activity to site B_{1S}. The remaining 15% of $27SA_2$ is cleaved at B_{1L}. After this, the processing events are believed to be the same with cleavage at B_2, within the 3' end of the pre-rRNA, occurring simultaneously to the B_1 cleavage steps. This is followed by cleavage at C_2 (within ITS2) which corresponds with movement to the nucleus. Subsequent exonuclease digestion in both 5' to 3' and 3' to 5' directions (to sites C_1 and E respectively) produce the mature 25S and 5.8S rRNAs which are then exported to the cytoplasm (Henras et al., 2008). It should be noted that long and short forms of 5.8S rRNAs are produced; $5.8S_L$ and $5.8S_S$, dependent on the processing at the 5' end of the $27SA_2$ precursor at either site $B1_L$ or $B1_S$.

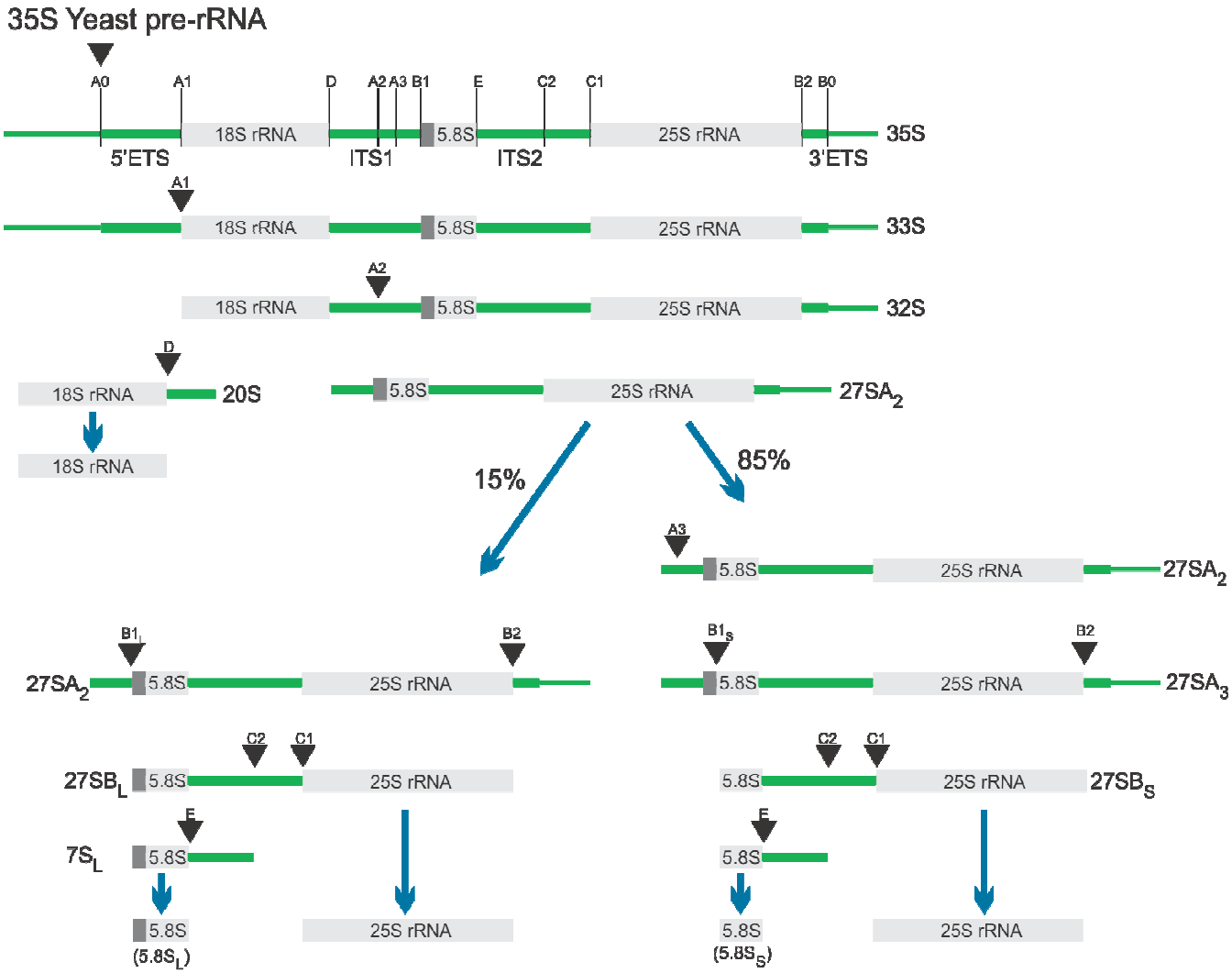


Figure 1.5 Pre-rRNA Processing in *S. cerevisiae*
In *S. cerevisiae* 18S, 5.8S and 25S rRNA are transcribed as a single 35S pre-rRNA transcript which is shown at the top of the panel. RNA is shown in green (cleaved from the mature product; named beneath) and grey (constituting the mature rRNA; named within the Box). Cleavage sites so far identified are indicated by vertical black lines with the cleavage site named above for each. ETS; External transcribed sequence, ITS; Internal transcribed sequence. Processing of yeast pre-rRNA is shown with blue arrows indicating processing steps, black triangles indicating the sites of cleavage. Based on previous work (Fatica and Tollervey, 2002; Granneman and Baserga, 2004; Henras et al., 2008; Rouquette et al., 2005).

1.5.2 Pre-rRNA Processing in *H. sapiens*

In higher eukaryotes pre-rRNA processing is thought to be largely similar to that of yeast with the A' cleavage and covalent modifications thought to occur co-transcriptionally in *Xenopus laevis* and mammalian cells (Granneman and Baserga, 2005; Henras et al., 2004a; Lazdins et al., 1997; Peculis, 2001). In human pre-rRNA, the primary cleavage step has been shown to occur at A' (also known as site 01). This occurs within the 5' ETS and is upstream of the yeast A_0 site (Enright et al., 1996; Henras et al., 2008; Kass et al., 1990). Processing at A' occurs at two adjacent sites, with processing at site 02 (3'ETS), leading to two discrete 45S pre-rRNAs that differ by only a few nucleotides at the 5' end (Kass et al., 1987).

Recently, it has been demonstrated that human pre-rRNA also contains an A0 site (Rouquette et al., 2005). Note, for clarification, "A0" is used to denote the site in humans whereas "A_0" is used in yeast. This cleavage occurs downstream of site A' in human pre-rRNA. It has also been demonstrated that *Xenopus laevis*, *Trypanosoma brucei* and Mice can cleave pre-rRNA at a site comparable to that of site A_0 in yeast (Borovjagin and Gerbi, 2001; Hartshorne et al., 2001; Kent et al., 2009; Rouquette et al., 2005). In human pre-rRNA, it would appear that there are 3 alternative pathways for 45S processing; cleavage at site A0 in the 5' ETS, leading to a 43S product; cleavage at site 1 at the 5'ETS/18S border leading to a 41S product; or cleavage at site 2 within ITS1, leading to a 32S (5.8S and 28S) and 30S (18S) product (Figure 1.6) (Hadjiolova et al., 1993; Rouquette et al., 2005). These pathways then reconverge upon cleavages at sites 1 (5'ETS) and 2 (ITS1), separating the 18S precursor (21S) from the 5.8S and 28S precursor (32S). The 21S pre-rRNA is then cleaved 24 nucleotides into ITS1 (called site 2' here) to produce 18S-E, a step not present in yeast. This is then cleaved at site 3, producing mature 18S rRNA (Rouquette et al., 2005). The 32S pre-rRNA is cleaved at site 3' to produce mature 28S rRNA and the 5.8S precursor 12S. This is subsequently cleaved at site 4', producing mature 5.8S rRNA.

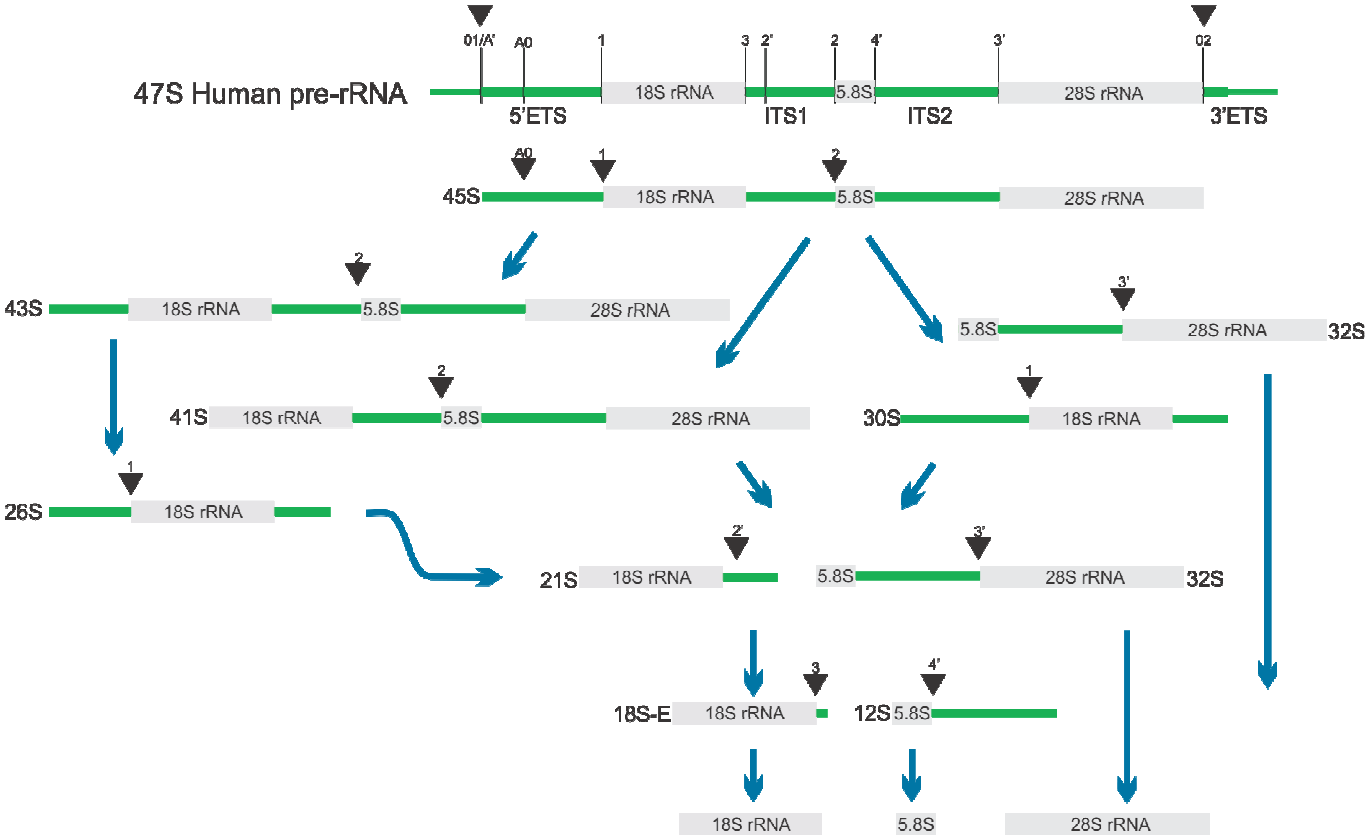


Figure 1.6 Pre-rRNA Processing in *H. sapiens*
Full length human pre-rRNA transcript is shown at the top. RNA is shown in green (cleaved from the mature product; named beneath) and grey (constituting the mature rRNA; named within the Box). Cleavage sites so far identified are indicated by vertical black lines with the cleavage site named above for each. ETS; External transcribed sequence, ITS; Internal transcribed sequence. Processing of human pre-rRNA is shown with blue arrows indicating processing steps, black triangles indicating the sites of cleavage. Derived from previous work (Hadjiolova et al., 1993; Henras et al., 2008; Rouquette et al., 2005).

1.5.3 A Comparison of 18S processing in Humans and Yeast

Many of the processing steps appear similar between yeast and humans. Significant exceptions are the A' and site 2' cleavage steps which have only been observed in higher eukaryotes. In human cells, cleavages at sites 1 and 2 in the pre-rRNA lead to the occurrence of a 21S pre-rRNA which is cleaved again at site 2' (around nucleotide 24 of ITS1) to produce 18S-E pre-rRNA (Figure 1.7). This is then exported to the cytoplasm and the subsequent site 3 cleavage produces the mature 18S rRNA (Hadjiolova et al., 1993; Rouquette et al., 2005). In contrast,

yeast pre-rRNA is cleaved at A_1 and A_2, releasing a 20S pre-rRNA which is exported directly to the cytoplasm where it is cleaved at site D to produce 18S rRNA (Fatica and Tollervey, 2002; Henras et al., 2008). This demonstrates that there is at least one additional step in 18S pre-rRNA processing in human cells. When we consider that human pre-rRNA is also processed at both A0 and A' sites (Figure 1.7), in contrast to the A0 site alone in yeast pre-rRNA, it becomes clear that pre-rRNA processing in higher eukaryotic cells may be more complex than in their lower eukaryotic counterparts (Rouquette et al., 2005).

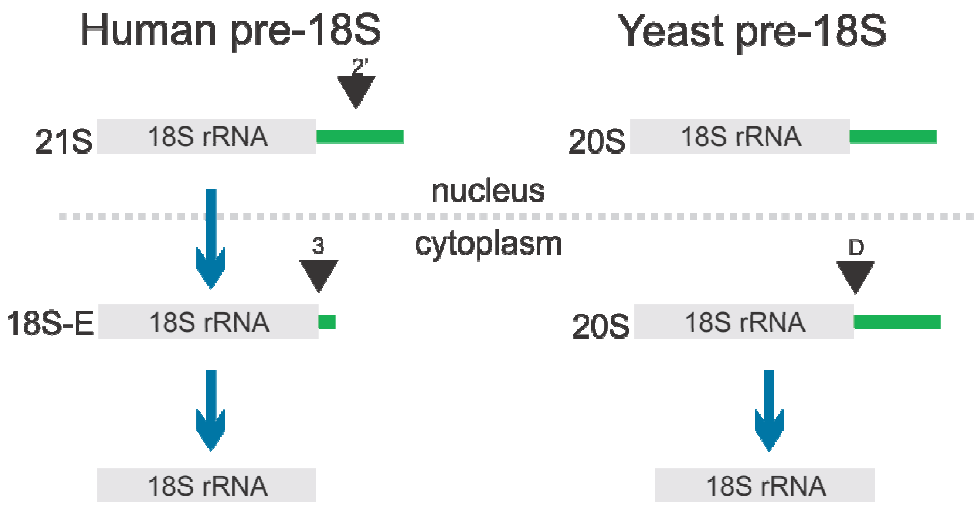


Figure 1.7 18S rRNA Processing in Yeast and Humans
Processing of human and yeast rRNA (grey) from 21S and 20S precursors are shown. Blue arrows indicate processing steps, black triangles indicating the sites of cleavage. Steps in the nucleus and cytoplasm are indicated. Figure derived from previous work (Fatica and Tollervey, 2002; Hadjiolova et al., 1993; Henras et al., 2008; Rouquette et al., 2005)

1.6 snoRNPs Required for pre-rRNA Processing

A number of snoRNPs are involved in regulating pre-rRNA processing rather than directly facilitating base modifications (Maxwell and Fournier, 1995; Terns and Terns, 2002). Unlike most other Box C/D snoRNPs, the U3 snoRNA is not involved in methylation. Instead, it is required for the initial cleavage steps of pre-rRNA; crucial for production of the 18S rRNA. It functions as part of a multi-protein component complex termed the small subunit (SSU) processome. This is required

for cleavages at sites A', 1 and 2 (A_0, A_1 and A_2 in yeast). Other non-base modifying snoRNAs are also required for pre-rRNA processing. These include the Box C/D snoRNA U8 which is found throughout vertebrates, although not found in yeast. In *Xenopus* it has been shown to be essential for 5.8S and 28S rRNA maturation (Peculis and Steitz, 1993). The U8 snoRNA is thought to base pair with the 28S pre-rRNA in a region where the 5.8S interacts in the mature ribosome. It was thought that U8 may be displaced from pre-28S rRNA by 5.8S rRNA for processing to occur (Peculis, 1997). However, more recently the helicase DDX51 has been shown to promote U8 displacement from the pre-rRNA, which is necessary for the removal of the 3' ETS from 28S rRNA (Srivastava et al., 2010).

A host of other snoRNAs have also been implicated in pre-rRNA processing. This includes the box C/D snoRNPs U14 and U22, and the box H/ACAs E1 (U17 / snR30), E2, E3, and to a lesser extent snR10 although not all RNAs have been observed in all species. In yeast, the Box C/D U14 snoRNA has been shown to base-pair with 18S pre-rRNA and to be required for rRNA processing at sites A_1 and A_2 (Li et al., 1990; Liang and Fournier, 1995). U14 is observed across many species including yeast, plants and mammals, although in *Xenopus*, it is required for methylation but is not essential for processing whereas in mice it is known to play a role in the primary cleavage steps (Dunbar and Baserga, 1998; Enright et al., 1996; Liang and Fournier, 1995; Maxwell and Fournier, 1995). In contrast, the Box C/D snoRNA U22 is only found in vertebrates and has been shown in *Xenopus* to be essential for cleavages either side of the 18S pre-rRNA (Tycowski et al., 1994; Tycowski et al., 1996).

The Box H/ACA snoRNAs E1 (Xenopus) / U17 (mammals) / snR30 (Yeast), E2 and E3 have also been shown to be important to pre-rRNA processing. In *Xenopus*, E1 snoRNA has been shown to be required for cleavage in the 5' ETS, E2 at the 3' end of 18S rRNA, and E3 at the 5' end of 5.8S rRNA (Mishra and Eliceiri, 1997). Similarly, U17 and E3 have been shown to be required for 5'ETS processing in mammals (Enright et al., 1996). The H/ACA snoRNP snR30 is also required for 18S production in yeast (Atzorn et al., 2004) whereas depletion of the

H/ACA snoRNP snR10 only leads to a mild defect in processing although both also pseudouridylate the pre-rRNA (Torchet and Hermann-Le Denmat, 2002). It would therefore appear that U3, U14 and U17 are conserved between yeast and human cells. In contrast, snR10 has only been found in yeast, and U8, U22, E2 and E3 only found in vertebrates. Interestingly, many of these snoRNPs are devoid of nucleotide modification functions, therefore playing dedicated roles in pre-rRNA processing. In contrast, U14, snR30 and snR10 are known to have roles in both processing and modification (Atzorn et al., 2004; Dunbar and Baserga, 1998; Henras et al., 2008; Torchet and Hermann-Le Denmat, 2002).

1.7 Maturation and Export of the Ribosomal Subunits

The vast majority of work examining ribosome maturation has focused on the processes in yeast (*S. cerevisiae*) however, as suggested previously, significant differences may exist in human cells. In yeast, sucrose gradient analysis identified a 90S particle containing the 35S pre-rRNA and a much higher ratio of protein to RNA than was observed in the mature ribosome (Trapman et al., 1975; Udem and Warner, 1973). This was discovered to be due to the presence of many non-ribosomal processing factors and some ribosomal proteins (Bernstein et al., 2004; Dragon et al., 2002). Within the 90S pre-ribosomal particle the majority of processing factors are associated with the biogenesis of the small subunit, therefore the complex was termed the small subunit (SSU) processome (Bernstein et al., 2004; Dragon et al., 2002; Grandi et al., 2002). In yeast, the SSU forms co-transcriptionally, starting with the compaction of the transcript. This can be observed as 5' terminal balls by electron microscopy on chromatin spreads (Miller and Beatty, 1969; Mougey et al., 1993). In some instances (~30% of cases) transcription continues to the 3' ETS with cleavage here releasing the 35S pre-rRNA (Kos and Tollervey, 2010). It has however been suggested that when growth conditions are favourable, the RNA pol. I transcript is cleaved co-transcriptionally, releasing pre-40S particles without prior 90S particle formation (Osheim et al., 2004), with recent evidence from yeast demonstrating that the majority of pre-rRNA is cleaved in this way (Kos and Tollervey, 2010).

The initial pre-ribosomal particle is composed of the nascent pre-rRNA and many factors linked to processing of the small subunit, in agreement with co-transcriptional processing of the SSU (Figure 1.8). In contrast, only a small number of factors at this point are linked to processing of the large subunit (Grandi et al., 2002; Schafer et al., 2003). It has been shown that the U3 snoRNP is a crucial part of this SSU processome along with many associated factors (Borovjagin and Gerbi, 2000; Dragon et al., 2002; Grandi et al., 2002). The SSU processome is required for cleavages surrounding the pre-18S rRNA, after which the majority of the factors are believed to dissociate. Other factors however, are believed to associate with the complex at this point and aid translocation to the cytoplasm where the pre-40S final maturation steps occur (Brand et al., 1977; Fatica et al., 2004; Lafontaine et al., 1994; Lamanna and Karbstein, 2009; Pertschy et al., 2009; Rouquette et al., 2005; Vanrobays et al., 2003; Vanrobays et al., 2001; Zemp et al., 2009).

In contrast, the bulk of pre-60S factors are thought to assemble onto the 27S rRNA after the A_2 site cleavage (Tschochner and Hurt, 2003). This indicates that there is clear separation of the biogenesis factors associated with the pre-40S and pre-60S particles, suggesting distinct biogenesis mechanisms (Grandi et al., 2002; Liang and Fournier, 1997). The pre-60S particles are dynamic but through extensive study have been categorised into nucleolar particles, nucleolar/nucleoplasmic particles, nucleoplasmic particles, nucleoplasmic/cytoplasmic particles and cytoplasmic particles; with transport correlating with the association of different processing factors and maturation of the pre-rRNA (Nissan et al., 2002; Tschochner and Hurt, 2003). Around 50 non-ribosomal proteins are thought to associate with the earliest nucleolar pre-60S ribosomes, with factors linked to pre-rRNA modification, GTPases, nucleases, and nucleocytoplasmic transport factors all thought to bind sequentially, although significantly fewer processing factors are present on the most mature pre-60S subunits after export to the cytoplasm (Nissan et al., 2002; Tschochner and Hurt, 2003). This demonstrates that the pre-60S particle undergoes a complicated set of processing, assembly and maturation

steps although these have been more widely studied than those of the pre-40S ribosomal subunit. It should also be noted that as with the pre-40S, the vast majority of work examining the pre-60S has been carried out in the yeast *S. cerevisiae*.

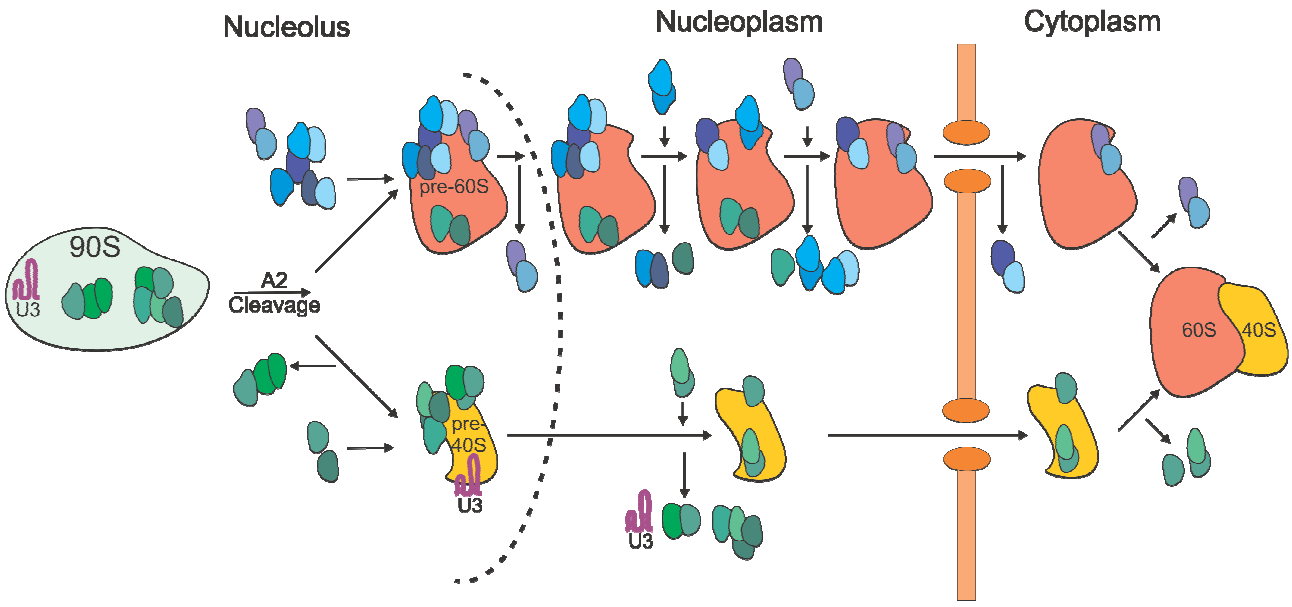


Figure 1.8 An Overview of Maturation and Export of the 40S and 60S Ribosomal Subunits The yeast 35S pre-rRNA is incorporated into a 90S pre-ribosomal particle containing small subunit processing factors (green) the U3 snoRNP (purple) and only a few 60S processing factors. Site A_2 cleavage separates the 40S and 60S precursors in the nucleolus giving rise to the $27SA_2$ rRNA, containing 5.8S and 25S pre-rRNAs, and 20S rRNA containing the 18S pre-rRNA. At this point, the majority of SSU processome proteins and U3 snoRNP dissociate which coincides with movement to the nucleoplasm. Chaperone factors are believed to associate to enable translocation to the cytoplasm where 20S pre-rRNA is cleaved and modified to mature 18S rRNA. In contrast, after A_2 cleavage the majority of large subunit processing factors associate (blue) and the pre-60S particle moves to the nucleoplasm. A succession of processing and modification steps coincides with the association and dissociation of a host of factors. Similarly to the pre-40S particle, chaperone-type factors are believed to associate with the pre-60S particle to facilitate movement to the cytoplasm, where final maturation of the rRNA occurs. Finally the ribosomal subunits associate at the site of mRNA translation. Figure based on previous work (Tschochner and Hurt, 2003).

1.8 The Small Subunit Processome

The SSU processome was previously purified from the yeast *S. cerevisiae* and shown to contain the U3 snoRNP, a key component of the complex, and a host of associated proteins (Dragon et al., 2002). The SSU processome is essential for production of the small subunit of the ribosome, modulating cleavages surrounding the 18S pre-rRNA, A_0-A_2 in yeast, A' - site 2 in humans. It is thought that the terminal balls observed at the 5' end of transcribed pre-rRNA represent the initial SSU processome complex in yeast (Miller and Beatty, 1969; Mougey et al., 1993). Whilst several proteomic studies have investigated the composition of the SSU processome in *S. cerevisiae*, the results show some variation (Bernstein et al., 2004; Dragon et al., 2002; Grandi et al., 2002; Krogan et al., 2004; Schafer et al., 2003). This is thought to be due to the dynamic nature of the complex, with factors associating and dissociating throughout the processing of the pre-40S rRNA. Similarly, a difference in experimental methods is likely to cause some of the apparent differences in the composition of the complex.

It should be noted that, in contrast to yeast, large-scale proteomic analysis has not been performed on human cells. Therefore, the composition of the human SSU processome is relatively unknown, outside of studies examining specific homologous proteins (Gerus et al., 2010; Granneman et al., 2003; Granneman et al., 2002; Prieto and McStay, 2007; Rouquette et al., 2005; Turner et al., 2009). It would appear however, that the small subunit precursor can be processed at A' and A0, leading to a more complex pathway for production of the small subunit (Rouquette et al., 2005). Furthermore, an additional processing step is required at the 3' end of human 18S pre-rRNA (Rouquette et al., 2005). It is therefore possible that the pre-40S complex associated with 21S pre-rRNA may be different in humans to that in yeast. As this study focuses on the human SSU processome, the associated proteins are written using human nomenclature to maintain consistency and clarity. However, the majority of work so far has been conducted on the yeast SSU processome.

In yeast, the SSU processome is composed of more than 40 proteins, many of which associate with and / or modify the rRNA such as RNA helicases, GTPases, RNA annealing factors, methyltransferases, and potential chaperones (Bernstein et al., 2004; Dragon et al., 2002; Henras et al., 2008). It should also be noted that 5 ribosomal proteins (RPS4, RPS6, RPS7, RPS9 and RPS14) are also associated with the complex but remain as part of the mature ribosome (Bernstein et al., 2004; Dragon et al., 2002) however, these factors were not identified as SSU processome components by other groups (Grandi et al., 2002; Krogan et al., 2004; Perez-Fernandez et al., 2007). Although many of the proteins present with the SSU processome have been identified, relatively little is known about their function. Nonetheless, five main sub-complexes have been identified within the SSU processome. These are the U3 snoRNP, MPP10 (M phase phosphoprotein 10) complex, tUTP (transcriptionally associated U three protein) complex, UTP-B complex and UTP-C complex. These complexes have been shown to be capable of forming independently of the SSU processome. It is believed that they may associate as pre-formed sub-complexes, although if they perform discrete or overlapping functions within the SSU processome is not clear.

In humans, it is has been shown that the U3 snoRNP, tUTP, UTP-B and MPP10 sub-complexes are present in the SSU processome and it has been suggested that these may function in a similar way to their counterparts in yeast (Gerus et al., 2010; Granneman et al., 2003; Granneman et al., 2002; Prieto and McStay, 2007; Rouquette et al., 2005; Turner et al., 2009). It should be noted however, that studies have been limited to only a few SSU processome proteins in human cells.

1.8.1 The Major Complexes of the SSU Processome

1.8.1.1 U3 snoRNP

The U3 snoRNP has been shown to sediment as both a 12S monoparticle and 80S component of the SSU processome in both yeast and humans (Billy et al., 2000; Granneman et al., 2003; Granneman et al., 2004; Turner et al., 2009; Tyc and

Steitz, 1989). In contrast to conventional Box C/D snoRNPs, it is not involved in guiding the methylation of pre-rRNA, but is required for the initial cleavages surrounding the 18S pre-rRNA in all eukaryotes examined (Borovjagin and Gerbi, 2001; Granneman et al., 2004; Kass et al., 1990; Savino and Gerbi, 1990)

The U3 snoRNA is the most abundant of all snoRNAs. It contains two distinct functional domains. The 5' end of the snoRNA contains an A'/A motif responsible for pre-rRNA base-pairing. The U3 snoRNA is however, classed as a Box C/D snoRNA (Tyc and Steitz, 1989). The 5' region of the snoRNA is the protein-binding domain and in both yeast and mammals contains the Box C/D motif and the associated core Box C/D proteins NOP58, NOP56, 15.5K (Snu13p in yeast) and fibrillarin (Nop1 in yeast) (Granneman et al., 2009; Lubben et al., 1993; Watkins et al., 2002; Watkins et al., 2004; Watkins et al., 2000). The U3 snoRNA does not however contain a C'/D' motif but instead has a U3-specific box B/C motif to which an additional 15.5K protein and the U3-specific hU3-55k (Rrp9 in yeast) are associated (Figure 1.9; (Granneman et al., 2002; Lubben et al., 1993; Pluk et al., 1998; Venema et al., 2000)).

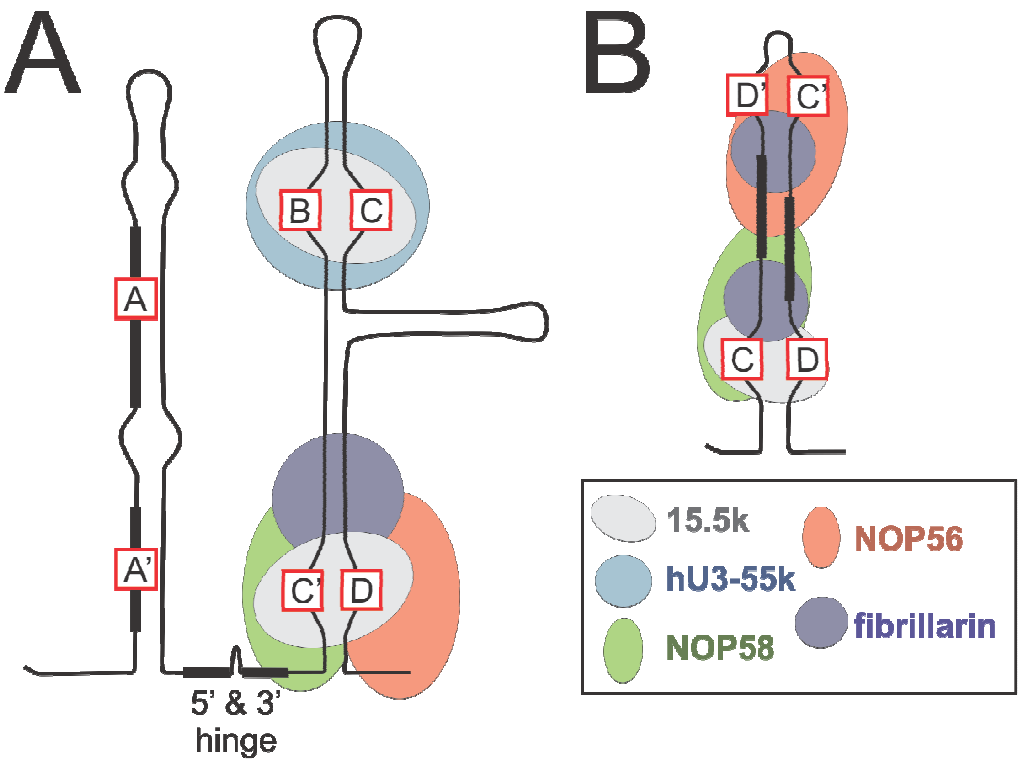


Figure 1.9 U3 snoRNP and a Conventional Box C/D snoRNP
A) A model of the U3 box C/D snoRNP with core-proteins associated; essential for 18S pre-rRNA processing **B)** A conventional box C/D snoRNP with core proteins associated; required for methylation of pre-rRNA. Solid black line indicates RNA sequence; thick black lines indicate sections believed to base-pair with target pre-rRNA sequences (not shown). Conserved boxes are indicated by red boxes, with the name of the conserved motif shown inside. Proteins are as indicated within the key at the bottom right. Models based on previous work (Granneman et al., 2002; Reichow et al., 2007; Watkins et al., 2002; Watkins et al., 2000).

The 5' half of the U3 snoRNA contains a unique A'/A motif helical sequence (Figure 1.9). When this is unfolded, it base-pairs with the 5'ETS /18S pre-rRNA, forming a 5'-18S pre-rRNA / U3 snoRNA duplex (Figure 1.10). Similarly, the 3' hinge of the U3 snoRNA base-pairs close to the A' cleavage site and the 5' hinge base-pairs near the A0 cleavage site, suggesting that these interactions may be important in exposing residues to be cleaved (Borovjagin and Gerbi, 2001). Interestingly, the 3' hinge is more important for pre-rRNA processing in *Xenopus* (Borovjagin and Gerbi, 2000), whilst pre-rRNA processing in yeast is more dependent on the 5' hinge sequence (Beltrame and Tollervey, 1995).

Nonetheless, in both humans and yeast the U3 snoRNA - rRNA duplex is required for the pre-rRNA processing steps surrounding the pre-18S rRNA (Granneman et

al., 2004; Hughes, 1996). Furthermore, the base-pairing between the U3 snoRNA and the 5' ETS / 18S pre-rRNA is believed to prevent premature pseudo-knot structure formation within the small-subunit rRNA; a long-range interaction crucial in the overall folding of the mature rRNA (Hughes, 1996; Sharma and Tollervey, 1999). This supports a model whereby U3 snoRNA acts as a bridge to draw together the 5' and 3' ends of the 18S rRNA pre-rRNA to coordinate their cleavage and aid SSU biogenesis (Borovjagin and Gerbi, 2001; Gerbi et al., 2003).

The base pairing between the U3 snoRNA and pre-rRNA is not however, required for incorporation of the U3 snoRNP into the 80S complex in humans, whereas the B/C motif of the snoRNA is crucial (Granneman et al., 2004). It would therefore seem that the box B/C associated protein, hU3-55k, may be vital to U3 snoRNP recruitment of SSU processome factors and in its association with the pre-rRNA. This suggests U3 snoRNA – pre-rRNA base-pairing in human cells may occur after initial recruitment of the SSU processome to the pre-rRNA, aided by other protein factors (Granneman et al., 2003; Granneman et al., 2004). Although it is not clear if this is also the case in yeast, core U3 snoRNP proteins in *S. cerevisiae* have been shown to crosslink directly to the pre-rRNA, suggesting they may play a role in U3 snoRNA – pre-rRNA association (Granneman et al., 2009). It should however, be noted these factors alone are insufficient for complex formation in humans (Granneman et al., 2003; Granneman et al., 2004) and most likely do not associate specifically with the pre-rRNA independently of other factors.

Interestingly, in *E. coli*, the biogenesis of the SSU also involves structural isomerisation that controls the formation of the central pseudo-knot. In this instance however, there is no U3 snoRNP. Instead, the 5′ region of the 16S pre-rRNA is base paired to an adjacent sequence in the 5′ ETS region, preventing premature pseudo-knot formation (Dennis et al., 1997). This demonstrates that preventing premature folding of the pre-rRNA is a highly conserved requirement of ribosome biogenesis.

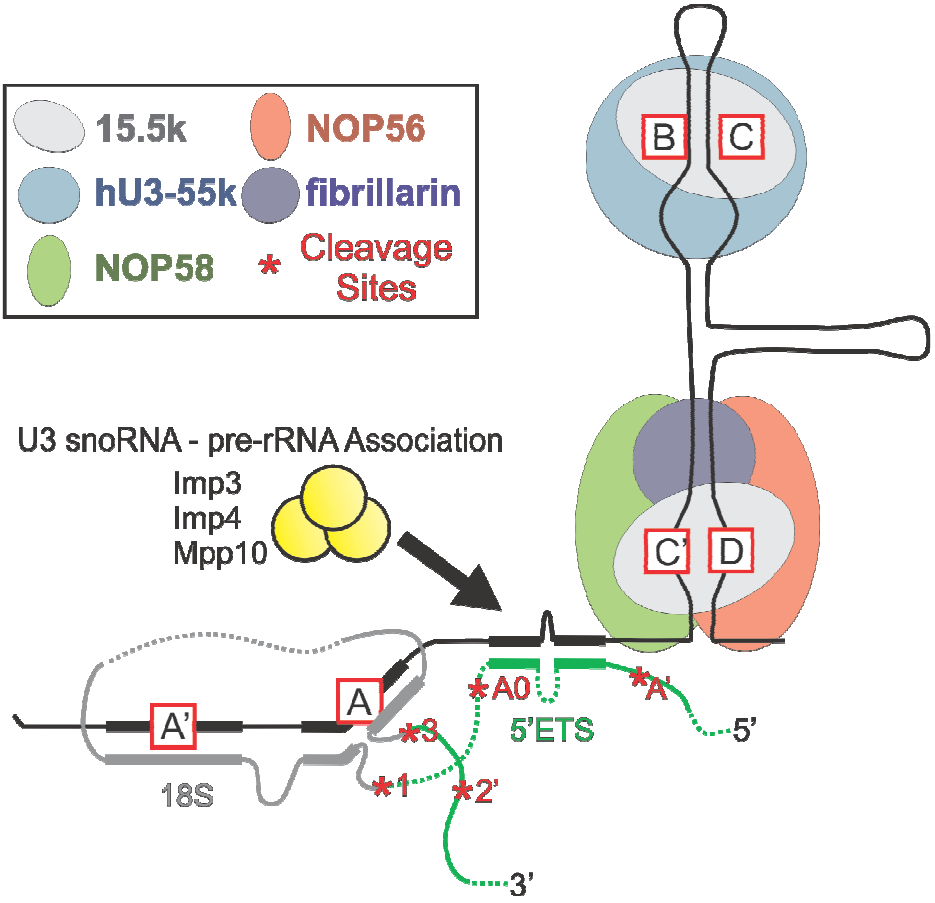


Figure 1.10 U3 snoRNP Base Pairing with 18S pre-rRNA
A simplified model illustrating the base pairing interactions between the U3 snoRNA and 18S region of pre-rRNA, bringing together cleavage sites 1 and 2 in human pre-rRNA at the A Box of the U3 snoRNA, and exposing site 01. Solid black lines indicate U3 snoRNA sequence; thick black lines indicate sections believed to base-pair with target pre-rRNA sequences, dashed lines indicate sections that have been significantly shortened. Conserved Boxes are indicated by red Boxes, with the name of the conserved motif shown inside. U3 snoRNP proteins are as indicated within the key at the top left, IMP3, IMP4 and MPP10 proteins indicated as yellow circles. The pre-rRNA is shown in green for the untranslated regions, in grey for the 18S pre-rRNA, labelled accordingly, with thick lines indicating base-pairing to the U3 snoRNA sequences. Asterisks indicate cleavage sites required for mature 18S rRNA production. Model derived from previous data (Granneman et al., 2002; Granneman et al., 2004; Watkins et al., 2000).

1.8.1.2 MPP10 Complex

Base-pairing between the U3 snoRNA and pre-rRNA has been shown to be required for efficient production of the 18S rRNA in both yeast and humans (Beltrame and Tollervey, 1995; Granneman et al., 2004; Hughes, 1996; Sharma and Tollervey, 1999). In all eukaryotes, premature formation of the central 18S

rRNA pseudo-knot is believed to be prevented by binding of the 5′ end of the 18S rRNA to the U3 snoRNA, with the MPP10/IMP3/IMP4 complex thought to aid the RNA base-pairing (Gerczei and Correll, 2004; Gérczei et al., 2009; Granneman et al., 2003; Granneman et al., 2004; Lafontaine and Tollervey, 2001).

MPP10 was originally discovered in human cells as a protein phosphorylated during mitosis (Matsumoto-Taniura et al., 1996). In both humans and yeast the protein has been show to contain potential protein-protein interacting coiled-coil domains and to associate with the U3 snoRNA as part of the SSU processome (Dunbar et al., 1997; Westendorf et al., 1998). Furthermore, in yeast, MPP10 has been shown to be required for cleavages surrounding the 18S pre-rRNA (Dunbar et al., 1997; Lee and Baserga, 1997).

In both yeast and humans MPP10 has been shown to associate with IMP3 and IMP4 (Granneman et al., 2003; Lee and Baserga, 1999). IMP3 contains a putative S9 (yeast, S4 in prokaryotes) RNA binding domain (Lee and Baserga, 1999). In *E. coli*, S4 has been shown to be an early SSU associated protein, capable of RNA binding and important in RNA folding (Lee and Baserga, 1999; Stern et al., 1986). Furthermore, due to the homology with the ribosomal protein RPS9, it has been suggested IMP3 may be replaced by RPS9 in the mature small ribosomal subunit (Fromont-Racine et al., 2003). IMP4 contains a σ^{70}-like motif, first identified in the σ^{70}-like family of prokaryotic RNA polymerase transcription factors; capable of interaction with the DNA promoter sequence and nascent RNA (Wehner and Baserga, 2002).

In *S. cerevisiae*, IMP3 and IMP4, most likely with MPP10, are believed to unfold the U3 snoRNA A'/A helical sequence and accelerate the formation of a 5'-18S pre-rRNA / U3 snoRNA duplex; required for pre-rRNA processing (Gerczei and Correll, 2004; Gérczei et al., 2009). Specifically, IMP3 and IMP4 have been shown to stabilise the duplex between the U3 snoRNA hinge region and the 5'ETS of the pre-rRNA, whereas only IMP4 mediates duplex formation between the U3 snoRNA A stem and 18S pre-rRNA (Figure 1.10; (Gerczei and Correll, 2004). By mediating

formation of both essential U3-pre-rRNA duplexes, IMP3 and IMP4 may help the SSU processome to dock onto the pre-rRNA (Gerczei and Correll, 2004).

In humans, IMP3 and IMP4 are mainly found to interact with the U3 snoRNA in the SSU processome, with binding to MPP10 correlating with nucleolar accumulation of the proteins (Granneman et al., 2003). The pre-rRNA base-pairing sequences of U3 snoRNA (where these proteins associate; A' Box and the 3' and 5' hinges) are not required for incorporation of the U3 snoRNP into the SSU processome, but the B/C motif of the snoRNA (where hU3-55k is associated) is crucial (Granneman et al., 2004). Collectively this suggests that neither MPP10, IMP3 or IMP4 are required for incorporation of the U3 snoRNA into the SSU processing complex.

1.8.1.3 tUTP Complex

The tUTP (transcriptional U three proteins) complex is composed of tUTP4, tUTP5, tUTP10, tUTP15 and tUTP17. In yeast the complex also contains tUTP8 and tUTP9 although these have not been found in humans (Gallagher et al., 2004; Krogan et al., 2004; Prieto and McStay, 2007). The tUTP complex has been shown to be required for optimal rRNA transcription in both *S. cerevisiae* (Gallagher et al., 2004) and HeLa cells (Prieto and McStay, 2007).

In both yeast and humans, the tUTP complex has been shown to assemble on pre-rRNA and function as a distinct complex, independently of the other SSU processome components (Krogan et al., 2004; Perez-Fernandez et al., 2007; Prieto and McStay, 2007). In yeast, data from large-scale proteomic studies indicate that tUTP proteins may associate with the rDNA to enable efficient transcription and subsequently associate with the nascent pre-rRNA (Gallagher et al., 2004; Perez-Fernandez et al., 2007). This is believed to facilitate the association of subsequent SSU processome components (Figure 1.11), therefore co-ordinating pre-rRNA transcription and processing (Gallagher et al., 2004; Perez-Fernandez et al., 2007; Prieto and McStay, 2007).

In humans, it has been shown that the tUTPs are recruited directly to the rDNA independently of transcription, the underlying DNA sequence, and location within the nucleolus. Their association with the pre-rRNA is however strictly dependent on binding of the RNA pol. I transcription factor UBF at nucleolar organiser regions, where the tUTP complex subsequently associates (Prieto and McStay, 2007). It has also been shown in HeLa cells that the tUTP factors and active transcription are both required for assembly of the 80S SSU processome. Depletion of tUTP proteins lead to the accumulation of a 50S SSU processome intermediate, shown not to contain the tUTP, UTP-B, MPP10 proteins or pre-rRNA; believed to represent an 80S SSU processome precursor (Turner et al., 2009). It has not however, been examined in human cells how the tUTPs aid the association of subsequent SSU processome components, in which order they assemble, or when the tUTP complex dissociates from the processome.

1.8.1.4 UTP-B Complex

The UTP-B complex in yeast is composed of PWP2 (UTP1), UTP6, UTP12 (DIP2), UTP13, UTP18 and UTP21 and is required for cleavages at A_0, A_1 and A_2 (Dosil and Bustelo, 2004; Krogan et al., 2004). The tUTP complex is required for its association with the pre-rRNA (Perez-Fernandez et al., 2007), although the UTP-B complex is capable of directly binding the 5' end of nascent 35S pre-rRNAs (Dosil and Bustelo, 2004)

In yeast, depletion of the UTP-B component PWP2 prevents IMP3, IMP4 and the U3 snoRNP from associating with the 90S pre-rRNA particle (Dosil and Bustelo, 2004). Conversely, blocking U3 snoRNP assembly is not sufficient to prevent UTP-B association with the 35S pre-rRNA, suggesting that the UTP-B complex associates discretely and is required for subsequent association of both the U3 snoRNP and MPP10 complex (Dosil and Bustelo, 2004). The UTP-B proteins have been observed to contain WD or TPR repeat motifs. These motifs are known to act as protein-protein interaction domains, suggesting that the UTP-B complex may be a structural component of the SSU processome; aiding in the recruitment of

subsequent processing factors. This however, raises the interesting question of how the UTP-B complex itself associates with the rRNA, as this is currently unknown.

In contrast, very little is known about the role of the UTP-B proteins in human cells. UTP13 has been shown to localise to the nucleolus of HeLa cells, to associate with the U3 snoRNA in the 80S SSU processome, and to associate with pre-rRNA before and after the initial cleavage event (Turner et al., 2009). Furthermore, tUTP proteins are required for the association of UTP-B proteins with the SSU processome (Turner et al., 2009). Interestingly, as in yeast, UTP12 and UTP13 are believed to associate with the pre-rRNA independently of the U3 snoRNP (Turner et al., 2009). It is not however, known if other UTP-B proteins are associated, if they are orthologous to those in yeast or what role they may play in human cells.

1.8.1.5 UTP-C Complex

The UTP-C complex in yeast is composed of RRP7, UTP22 and the casein kinase II (CKII) subunits CKA1, CKA2, CKB1, CKB2 (Krogan et al., 2004). RRP7 has been shown to be required for the processing steps leading to the production of 20S pre-rRNA and it has been suggested that it is required for correct assembly of the ribosomal protein RPS27 into the pre-ribosomal particle (Baudin-Baillieu et al., 1997). Similarly, UTP22 mutants in yeast have also been shown to reduce pre-18S rRNA processing (Peng et al., 2003), with the protein known to contain at least seven phosphoserine/threonine residues (Albuquerque et al., 2008; Chi et al., 2007). CKII is a multifunctional kinase, thought to phosphorylate many different proteins. Interestingly, though it has been implicated in the modification of ribosomal proteins in the yeast *Trichosporon cutaneum* (Wojda et al., 2002).

This is intriguing as phosphorylation is thought to be important in the regulation of ribosome biogenesis although little is known about how this is achieved. Nonetheless, HRR25 (a casein kinase 1 isoform) has been implicated in late remodeling steps of the pre-40S subunit and correct formation of the beak

structure *in vitro* (Schafer et al., 2006) and RIO2 (right open reading frame, an atypical protein kinase) in 3'-end processing of 18S rRNA and the recycling of processing factors PNO1, LTV1, and NOB1 in human cells *in vivo* (Zemp et al., 2009). The importance of these kinases therefore suggests that protein phosphorylations play an important role in 40S biogenesis (Zemp and Kutay, 2007).

It is known that the tUTP complex is required for association of the UTP-C complex with the 35S pre-rRNA in yeast (Perez-Fernandez et al., 2007). Similarly, the RNA-binding protein RRP5 is also required for incorporation of the UTP-C complex into the pre-ribosomes, but not for the incorporation of the U3 snoRNP, the tUTP or the UTP-B complexes (Perez-Fernandez et al., 2007; Vos et al., 2004a). This further demonstrates that these factors appear to form and associate in a discrete manner, most likely associating as pre-formed sub-complexes. In contrast, none of the UTP-C proteins have been studied in human cells. It has however, been demonstrated that RRP5 associates with the U3 snoRNP as a component of the 50S SSU processome precursor (Turner et al., 2009), potentially suggesting an alternative assembly pathway in human cells.

1.8.2 Additional Components of the SSU Processome

1.8.2.1 RRP36

RRP36 has recently been demonstrated as a novel SSU processome component in yeast. It localises to the nucleolus and is associated with both 90S and 40S pre-ribosomal particles (Gerus et al., 2010). Its association into the SSU processome complex requires the incorporation of the tUTP, UTP-B, and UTP-C sub-complexes, although not RRP5. It is also shown that RRP36 depletion does not impair the incorporation of these sub-complexes or the U3 snoRNP into pre-ribosomes, suggesting that the protein most likely associates after these major sub-complexes associate. The requirement for this protein for early pre-rRNA cleavages was also shown to be conserved in humans (Gerus et al., 2010).

1.8.2.2 The RCL1/BMS1 Complex

In yeast, the GTPase BMS1 has been shown to be required for processing of the 18S pre-rRNA (Gelperin et al., 2001). It is thought that BMS1 forms a stable sub-complex with the putative endonuclease RCL1 and the U3 snoRNA; potentially to control an early step in the assembly of the pre-40S particle (Karbstein and Doudna, 2006; Karbstein et al., 2005).

RCL1 (RNA terminal phosphate cyclase like) displays sequence homology to RNA 3'-terminal phosphate cyclases however, it is said not to have cyclase activity. Instead it has been proposed to be a pre-rRNA endonuclease or a structural component of the SSU processome (Billy et al., 2000; Wegierski et al., 2001).

The N-terminus of BMS1 contains a G-domain (similar to the GTP-GDP binding G proteins' α-subunit) (Wegierski et al., 2001). Indeed, it has been demonstrated that RCL1 may activate BMS1 by promoting GDP/GTP exchange, therefore acting as a guanine exchange factor (Karbstein and Doudna, 2006). It has also been suggested that the BMS1 complex may act as a molecular switch with SOF1 (putative β-subunit) and DHR1 (putative RNA-helicase) to initiate pre-rRNA cleavages (Wegierski et al., 2001), with DHR1 shown to associate with RCL1 in yeast (Colley et al., 2000).

From data in *S. cerevisiae*, a model has been proposed whereby GTP associates with BMS1, increasing its affinity for RCL1. This is thought to increase the affinity of the BMS1-GTP complex for U3 snoRNP. This U3 snoRNP/BMS1/RCL1 complex is in turn, believed to associate with pre-ribosomes via the U3 snoRNP. It is suggested that a signal from the pre-ribosome may induce a conformational change in BMS1 which facilitates GTP hydrolysis, contributing to the release of RCL1. Therefore, RCL1 is thought to be deposited on the pre-ribosome whilst BMS1-GDP dissociates (Karbstein and Doudna, 2006; Karbstein et al., 2005).

In humans significantly less is known about this complex. It has been shown that BMS1 and RCL1 are capable of interacting in human cells, suggesting that their role may be evolutionarily conserved, although this is not demonstrated (Wegierski et al., 2001). It has also been shown in HeLa cells that BMS1 is associated with the U3 snoRNP and localises to the nucleolus in both the presence and absence of RNA pol. I transcription, suggesting that the protein naturally localises here (Turner et al., 2009). It is however unknown if BMS1 acts as a GTPase here, or if RCL1 is a component of the SSU processome.

1.8.2.3 Helicases

RNA helicases are believed to play an important role in rRNA biogenesis as they are responsible for unwinding RNA duplexes and RNP complex remodeling (Jankowsky and Bowers, 2006). They usually utilise energy derived from ATP hydrolysis to break the hydrogen bonds formed between opposite bases to allow unwinding of small RNA duplexes but can also target RNA-protein interactions to dissociate RNP complexes (Jankowsky and Bowers, 2006). Through modulating RNA-RNA and RNA-protein interactions helicases are thought to allow structural rearrangements within the rRNA (Granneman et al., 2006a). Moreover, both U3 snoRNA hybridization to and dissociation from pre-rRNA is almost certainly modulated by factors possessing RNA-RNA annealing and RNA helicase activities (Henras et al., 2008)

The helicases are members of the DEAD/H box protein family. The DEAD box family contain nine conserved motifs (Q-motif, motif 1, motif 1a, motif 1b, motif II, motif III, motif IV, motif V, and motif VI) whereby motif II (also known as the Walker B motif) contains the amino acid sequence DEAD (Linder et al., 1989). In the related DEAH box proteins, the Q-motif is not found, but 8 similar motifs (I – VI) are present (Linder, 2006). It should be noted that DEAD/H box proteins play a role in many aspects of RNA metabolism including pre-mRNA splicing and the initiation of translation (Linder, 2006). This suggests that that RNA helicases may have distinct biochemical properties and diverse roles in ribosome biogenesis, potentially

outside that of RNA unwinding (Garcia and Uhlenbeck, 2008; Granneman et al., 2006a).

In *S. cerevisiae*, there are 19 putative RNA helicases predicted to play a crucial role in ribosome biogenesis (Bohnsack et al., 2009; Granneman et al., 2006a; Granneman et al., 2006b). Seven of these proteins (DHR1, DHR2, DBP8, ROK1, FAL1, RRP3, and DBP4) have been shown to be specifically involved in 18S rRNA synthesis. Five of these helicases (DHR1, DHR2, DBP8, ROK1, RRP3) have been shown to interact with the SSU processome and are therefore proposed to be components of the complex (Granneman et al., 2006a). In comparison, HAS1 and PRP43 are the only helicases demonstrated to be required for both SSU and LSU synthesis (Bohnsack et al., 2009; Emery et al., 2004; Granneman et al., 2006a).

PRP43 has been shown to associate at sites in the 18S and 25S rRNA and interestingly, is important for both the release and association of various modifying snoRNAs (Bohnsack et al., 2009). Moreover, it has been shown to be required for maturation of the SSU in yeast (Pertschy et al., 2009). It was demonstrated *in vivo* that the helicase PRP43 and its co-factor PFA1 were required to act together with the late processing factor LTV1 to facilitate site D cleavage by the endonuclease NOB1 (Pertschy et al., 2009). This suggests that helicase activity may be required to expose the pre-rRNA site to be cleaved.

The helicase activity of DBP4 in yeast has been shown to be required for unwinding of the U14 and snR41 snoRNAs from the pre-rRNA (Kos and Tollervey, 2005). Similarly, DHR1 has been shown to associate with the U3 snoRNP, with both DHR1 and DHR2 required for cleavages A_1 and A_2, and A_0, A_1, and A_2, respectively. It has therefore been suggested that the U3 targets DHR1 to the pre-rRNA where it functions in the formation of the central pseudo-knot (Colley et al., 2000).

It has also been shown in yeast that the nucleolar protein ESF2 interacts directly with the DEAD/H box RNA helicase DBP8 to stimulate ATP hydrolysis

(Granneman et al., 2006b). Furthermore, ESF2 can bind to pre-rRNAs and is therefore suggested that it may function to bring DBP8 to the pre-rRNA; regulating both its enzymatic activity and site of action (Granneman et al., 2006b). It is likely, therefore, that DBP8 is also an SSU processome interacting protein.

Again, little is known about the function of the helicase proteins or potential protein co-factors in human cells. Nonetheless, in HeLa cells it has been shown that DBP4 can associate with pre-rRNA and the U3 snoRNP, and is a component of the 50S pre-SSU processome complex observed upon RNA pol. I inhibition (Turner et al., 2009). This suggests that in humans, the helicase DBP4 is also a component of the SSU processome. Furthermore, it has recently been demonstrated in mammalian cells that the helicase DDX51 interacts with the GTP-binding protein NOG1 (associated with 60S ribosome maturation). The helicase can also bind the pre-60S complex and promote the displacement of U8 snoRNA from the pre-rRNA; necessary for cleavage of the 3' ETS (Srivastava et al., 2010). This process is exclusive to metazoans as U8 is not thought to exist in yeast cells.

1.8.2.4 Nucleases

The SSU processome is responsible for the cleavages surrounding the 18S pre-rRNA, with the U3 snoRNP thought to be a key component in orchestrating these events (Henras et al., 2008). The U3 snoRNP however, is not itself an endonuclease and despite the identification of many of the SSU processome components in yeast, the identification of the endonucleases has remained elusive.

1.8.2.4.1 NOB1

NOB1 is the only identified endonuclease, responsible for cleavage at site D in yeast (Lamanna and Karbstein, 2009; Pertschy et al., 2009). The protein stably associates with late 40S particles (which also contain the kinase RIO2 and LTV1) after cleavages at sites A_0–A_2 in yeast (Schafer et al., 2003). The protein contains a PINc (PilT N-terminus) domain (Pertschy et al., 2009). These domains are

thought to be responsible for nucleotide-binding and RNase activity (Schneider et al., 2009). Only a few PINc domain proteins have been previously characterised in yeast, including NMD4; involved in nonsense-mediated mRNA decay, RRP44; an exosome component capable of acting as an exo- and endonuclease; and the potential endonucleases UTP23 and UTP24 (Bleichert et al., 2006; He and Jacobson, 1995; Lebreton et al., 2008; Schneider et al., 2009). The PINc domain of NOB1 is essential to its processing of the 20S pre-rRNA to 18S rRNA, with interactions between NOB1 and the RNA helicase PRP43 also required for the cleavage event in yeast (Pertschy et al., 2009). Furthermore, it has been shown that the primary binding sites for NOB1 are distinct from its cleavage site and that the PINc domain is not required for rRNA association (Granneman et al., 2010). The cleavage step has been shown to occur cytoplasmically in yeast although the protein has been observed in both the nucleus and nucleolus (Fatica et al., 2003; Pertschy et al., 2009). This suggests that the protein associates with the complex early in the processing pathway and that its activity is suppressed until the pre-rRNA reaches the cytoplasm. This is however, in contrast to other observations that suggest that NOB1 is mainly associated with later-stage, cytoplasmic pre-40S particles (Schafer et al., 2003).

1.8.2.4.2 UTP23 & UTP24

UTP23 and UTP24 were identified in *S. cerevisiae* by their interaction with FAF1 (another ribosome biogenesis factor); also known to interact with KRR1 (Karkusiewicz et al., 2004). UTP23 and UTP24 are essential proteins in yeast and are required for the cleavages surrounding the 18S pre-rRNA (Bleichert et al., 2006; Rempola et al., 2006). These proteins contain PINc domains and have been implicated as potential nucleases. Specifically, point-mutations within UTP24's PINc domain prevent cleavages at sites A_1 and A_2. It is therefore believed UTP24 may be an endonuclease whilst UTP23 may act as a co-factor in yeast, although this has not been directly demonstrated (Bleichert et al., 2006). One possibility is that, as PINc domain proteins form tetrameric complexes, the active nuclease may be a hetero-tetrameric complex consisting of UTP23 and UTP24.

1.8.2.4.3 RCL1

It has been suggested in yeast that the potential 3'-terminal phosphate cyclase RCL1 may function as an endonuclease. It has the potential to modify RNA and is required for cleavages A_0, A_1 and A_2 (Billy et al., 2000). RCL1 requires the GTPase BMS1 for association with U3 snoRNP in the 90S pre-ribosome (Karbstein and Doudna, 2006; Karbstein et al., 2005). It is believed that hydrolysis of GTP acts as a molecular switch to deposit RCL1 on the pre-rRNA, potentially where it may cleave the pre-rRNA (as previously discussed) although its activity as a nuclease has not yet been demonstrated experimentally. Interestingly, like the endonuclease NOB1, RCL1 also associates with a helicase, in this instance DHR1 (Colley et al., 2000).

1.8.2.4.4 Small Subunit Nucleases in Human Cells

In human cells, little is known about UTP23, UTP24 and RCL1. NOB1 has been observed to localise cytoplasmically, where the final cleavage step is also believed to occur and it has been shown to be a component of late 40S particles (Rouquette et al., 2005; Zemp et al., 2009). Furthermore, nuclear localisation of the protein is observed under depletion of the export factor CRM1 (chromosomal region maintenance) / exportin 1 (XPO1)) or the kinase RIO2 (Zemp et al., 2009). This suggests that NOB1 may be shuttled between the nucleus and cytoplasm in human and yeast cells. It is not however known how this is controlled, when NOB1 associates with the pre-rRNA or if its endonuclease activity is conserved from yeast.

1.8.2.5 RNA Binding Proteins

The SSU processome contains a number of other factors which are believed to interact with the pre-rRNA. In yeast, this includes KRR1 (Sasaki et al., 2000), MRD1 (Jin et al., 2002), LTV1 (Seiser et al., 2006), PNO1 (Vanrobays et al., 2004;

Vanrobays et al., 2008), ESF2 (Hoang et al., 2005), RRP5 (de Boer et al., 2006), RRP12 (Oeffinger et al., 2004) and PRP43 (Gerus et al., 2010).

1.8.2.5.1 PNO1

Two NES-containing trans-acting factors, LTV1 and PNO1, have been implicated as potential CRM1 adapter proteins in pre-40S export in *S. cerevisiae* (Seiser et al., 2006; Vanrobays et al., 2008). LTV1 has been shown to associate with CRM1 and shuttle between the cytoplasm and nuclease in a CRM1 dependent manner. The protein co-sediments with 40S particles and upon depletion of LTV1, SSU export is reduced (Seiser et al., 2006). This suggests that LTV1 may be one of several adapter proteins that link the nuclear export machinery to the small subunit.

It is possible that PNO1 plays a similar role in ribosome biogenesis to that of LTV1. The NOB1 binding partner PNO1 (Partner of NOB1, also known as DIM2) contains a conserved KH-domain (Tone and Toh, 2002). This is a motif of 70-100 residues allowing direct protein-RNA interactions; residues 173-225 of PNO1. The PNO1 protein has been shown to directly bind the pre-rRNA through this KH domain at the 5'-end of ITS1, with conserved residue G207 essential for the association (Vanrobays et al., 2008). Upon depletion of PNO1, a 22S (A_0-A_3) product is described in yeast (Peng et al., 2003). However, similarly to LTV1, the PNO1 protein shuttles between the nucleolus and the cytoplasm in yeast. The trafficking of PNO1 is tightly regulated by growth, most likely under the control of the TOR signalling cascade (target of rapamycin; a serine / threonine kinase, related to ATM and a member of the PI 3-kinase superfamily); suggesting a role beyond that of the initial cleavage steps (Vanrobays et al., 2004; Vanrobays et al., 2008). Furthermore, PNO1 in yeast is required for co-transcriptional ribosome assembly, pre-40S intranuclear transport and nucleocytoplasmic export of the pre-ribosomal subunit (Vanrobays et al., 2008).

Interestingly, it has been demonstrated in yeast that although NOB1 and PNO1 are consistently found in pre-ribosomes containing the kinase RIO2, they do not

associate with the newly described pre-rRNA-free RIO2 protein module(s) containing ENP1, LTV1, TSR1, KRR1, HRR25 and DIM1 (Merl et al., 2010). It is therefore suggested that the interaction of at least two discrete protein modules; the NOB1-PNO1 module; and the RIO2 module(s), with nascent 40S ribosomal subunits, is necessary to allow efficient processing at the 3' end of 18S rRNA (Merl et al., 2010).

In human cells, LTV1 and PNO1 are known to be components of the late 40S particles and to shuttle between the nucleus and cytoplasm (Zemp et al., 2009). The kinase RIO2 has been shown to be required for the final maturation of 20S pre-rRNA in HeLa cells with depletion leading to cytoplasmic accumulation of PNO1 and LTV1, and an inability to dissociate from the cytoplasmic pre-40S particle (Zemp et al., 2009). Conversely, inhibition of CRM1 was shown to result in a nuclear accumulation of LTV1, although PNO1 localisation was not examined (Zemp et al., 2009). Nonetheless, these proteins may be important co-factors that enable translocation of pre-40S particles to the cytoplasm in eukaryotes.

1.8.2.5.2 KRR1

KRR1 is another highly conserved KH-domain (residues 154-206) protein, possibly derived from a common ancestor to PNO1 and is known to interact with KRI1 (KRR1 interaction protein) in yeast, to form a complex which is required for 40S ribosome biogenesis (Bernstein et al., 2004; Gromadka and Rytka, 2000; Sasaki et al., 2000; Vanrobays et al., 2004). It is observed to co-sediment with 90S pre-ribosomal particles and late 40S particles and is essential for production of the 18S rRNA in yeast (Grandi et al., 2002; Sasaki et al., 2000). KRR1 is highly expressed in dividing cells, with a host of potential phosphorylation sites, but expression is largely down regulated when entering stationary phase, with transcription possibly coordinated with that of the ribosomal proteins (Gauci et al., 2009; Gromadka et al., 2004; Gromadka and Rytka, 2000; Imami et al., 2008; Wang et al., 2008). Moreover, it may also be involved in the nuclear transport of ribosomes in *S. cerevisiae* as KRR1 mutants displayed specific nucleolar accumulation of pre-40S

particles (Grandi et al., 2002). The protein may also play a role in the S phase check point, potentially linking cell cycle and cell growth post-transcriptionally in *S. pombe* (Kondoh et al., 2000).

In *S. cerevisiae*, KRR1 has been demonstrated to be a component of an RNA-free module containing RIO2, ENP1, LTV1, TSR1, HRR25 and DIM1 (Merl et al., 2010). This suggests that it may be part of a pre-formed protein complex which may potentially associate / dissociate with pre-rRNA in a modular fashion. In contrast, the protein KRR1 has not been studied in human cells.

1.8.2.5.3 MRD1

In *S. cerevisiae*, MRD1 is required for pre-rRNA cleavages at A_0-A_2, has been shown to bind to the pre-rRNA early during transcription and to be required for compaction of the pre-18S rRNA into SSU processome (Jin et al., 2002; Segerstolpe et al., 2008). When associated with the pre-rRNA, MRD1 has been observed to also interact with UTP-B, MPP10 and U3 snoRNP sub-complexes (Segerstolpe et al., 2008). MRD1 contains five distinct RNA binding domains (RBDs), each of which are functionally distinct and required during different steps of pre-rRNA processing in yeast (Lundkvist et al., 2009). MRD1 is not required for U3 snoRNA association with pre-rRNA but mutations of the RBDs trap U3 snoRNA in pre-ribosomal complexes, both in base-paired and non-base-paired interactions, suggesting it is required for dissociation of the U3 snoRNP from pre-rRNA (Lundkvist et al., 2009). Interestingly, MRD1 is not thought to be a helicase or helicase cofactor, suggesting the mechanism of controlling U3 snoRNP release is indirect (Lundkvist et al., 2009).

In human cells relatively little is known about MRD1. It has however, been shown to associate with the U3 snoRNP in the SSU processome and is present both before and after the initial pre-rRNA cleavage event (Turner et al., 2009).

1.8.2.5.4 ESF2

The yeast homolog of murine ABT1 (an enhancer of basal transcription (Oda et al., 2000)), ESF2 has been shown to associate with the sequences within the 5'ETS pre-rRNA, 18S and 25S rRNA and U3 snoRNA (Granneman et al., 2006b; Hoang et al., 2005). Interestingly, depletion leads to SSU processome inhibition, with the U3 snoRNA remaining associated to 35S pre-rRNA; demonstrating that that ESF2 is important for pre-rRNA cleavage and the release of the U3 snoRNP from the pre-ribosome (Hoang et al., 2005). Furthermore, ESF2 has been shown to co-purify with PWP2 (UTP-B protein) and MPP10 (Hoang et al., 2005), and also act as a binding partner and cofactor for the DBP8 helicase to aid ATP hydrolysis; with speculation that it may bring DBP8 to the pre-rRNA and stimulate its enzymatic activity (Granneman et al., 2006b).

The murine homolog mABT1 associates with the TATA binding protein (TBP); a component of the transcription factor TFIID responsible for binding the TATA box (Oda et al., 2000). Exogenous expression of mABT1 in mammalian cells was observed to stimulate gene expression regardless of cis-regulatory elements and was therefore shown to promote basal transcription (Oda et al., 2000). Furthermore, the protein was observed to mainly localise within the nucleolus of COS7 cells although some nuclear localisation was also observed (Oda et al., 2000). The role of this protein in ribosome biogenesis in humans however, is unknown.

1.8.2.5.5 RRP5

In *S. cerevisiae*, RRP5 is essential to biogenesis of both the 40S and 60S ribosomal subunits (Dragon et al., 2002; Grandi et al., 2002; Schafer et al., 2003; Venema and Tollervey, 1996). The N-terminus of RRP5 contains 12 S1 RNA-binding motifs whilst the C-terminus has 7 tetratricopeptide repeats (TPRs), believed to form scaffolds to aid protein-protein interactions (Blatch and Lassle, 1999; Eppens et al., 2002; Torchet et al., 1998). Several of the TPR motifs are

required for cleavages at sites A_0, A_1 and A_2, whereas the S1 domains are required for cleavages at sites A_3 or A_2 (Eppens et al., 2002; Vos et al., 2004b). It has been suggested that RRP5 binds close to the A_2 cleavage site, in ITS1 of yeast pre-rRNA (de Boer et al., 2006). It has however, been demonstrated to also be capable of non-specific RNA-binding, suggesting that associated proteins may specify the locus of association (de Boer et al., 2006).

The RRP5 protein is also required for incorporation of the UTP-C complex into the pre-ribosomes, but not for the incorporation of the U3 snoRNP, the tUTP or the UTP-B complexes (Perez-Fernandez et al., 2007; Vos et al., 2004a). Assembly of RRP5 into the processing complex independently of the U3 snoRNP may suggest that it directly binds the pre-rRNA. It is also possible however, that it associates as part of another complex. It has been shown very recently that RRP5 is a member of an RNA-free, NOC1-NOC2 protein module. In agreement with this, it is believed that NOC1 and NOC2 are required for biogenesis of the large subunit, with both NOC1 and NOC2 components of 90S and 66S (60S precursor) pre-ribosomes (Merl et al., 2010; Milkereit et al., 2001).

In humans, RRP5 has been observed to be a component of the pre-50S SSU processome complex, remaining associated with the U3 snoRNP, but not the pre-rRNA, after RNA pol. I depletion (Turner et al., 2009). RNA pol. I transcription is required for U3 snoRNA – pre-rRNA association, therefore RRP5 is most likely recruited to the pre-rRNA through the U3 snoRNP-containing SSU processome complex (Turner et al., 2009).

1.8.2.5.6 ENP1

ENP1 has been shown to be a SSU processome component in yeast (Schafer et al., 2003) and as such, is required for efficient production of 20S, and therefore 18S rRNA (Chen et al., 2003). The protein is localised in the nucleus and concentrated in the nucleolus of yeast cells (Chen et al., 2003).

In agreement with this, ENP1, DIM1 and RRP12 are components of the SSU processome that remain associated with the 18S rRNA precursor after the initial cleavages, forming part of the intermediate pre-40S particles in the nucleolus (Grandi et al., 2002; Schafer et al., 2003). The most prominent structural reorganisation of the pre-40S particle is thought to occur in the nucleolus. This is the formation of the beak; a structural feature of the mature subunit that is absent from earlier precursors (Schafer et al., 2006). The formation of this structure involves phosphorylation of ENP1 and LTV1 by the kinase HRR25 and dephosphorylation of the ribosomal protein RPS3 which is thought to ensure its incorporation (Granneman et al., 2010; Schafer et al., 2006). Late pre-40S particles contain a different protein kinase, RIO2 which is required for the final processing steps (Schafer et al., 2006; Schafer et al., 2003), which is most likely also the case in human cells (Zemp et al., 2009).

1.8.2.6 RNA Modifying Proteins

A number of the SSU processome components are believed to be involved in modifying the rRNA. These include DIM1 (Lafontaine et al., 1994; Vanrobays et al., 2004) and EMG1 (Eschrich et al., 2002; Leulliot et al., 2008). These modifications are thought to aid the proper folding, stability and function of the target rRNA.

1.8.2.6.1 EMG1

EMG1 (essential for mitotic growth 1), also known as NEP1 (nucleolar essential protein 1) is a conserved protein from archaea to eukaryotes (Eschrich et al., 2002). In yeast, EMG1 is a component of the SSU processome (Bernstein et al., 2004) and is required for production of the 18S rRNA (Liu and Thiele, 2001). The protein is observed throughout the cell, although nuclear localisation depends on its association with NOP14/UTP2 (Liu and Thiele, 2001). It is thought that EMG1 acts as a methyltransferase during ribosome biogenesis through interaction with the methyl donor, S-adenosyl-methionine (SAM) (Eschrich et al., 2002; Leulliot et al., 2008). Furthermore, it has been shown that the protein is capable of binding

RNA directly and that RNA association, as opposed to the catalytic activity of EMG1, is essential for ribosome biogenesis (Leulliot et al., 2008). The protein has been shown to associate at nucleotides 913–918 in the archaea *Methanocaldococcus jannaschii* 16S rRNA (equivalent to 1190-1195 in yeast 18S rRNA) and to methylate the base of a pseudouridine at nucleotide 913 in *M. jannaschii* (1191 in yeast) (Wurm et al., 2010).

In humans, it has been shown that a mutation of EMG1 is responsible for the severe developmental disorder Bowen-Conradi syndrome (Armistead et al., 2009; Wurm et al., 2010). This is believed to occur through a reduction in the level of the protein due to aggregation of EMG1 (Armistead et al., 2009). There is however, no direct evidence of the protein functioning in humans as a methyltransferase or for its role in ribosome biogenesis, although it is said to be likely conserved (Wurm et al., 2010).

1.8.2.6.2 DIM1

In yeast, PNO1 is known to associate with the di-methyltransferase DIM1; responsible for methylation of adenosine residues A1779 and A1780 (in *S. cerevisiae*) at the 3' terminus of 18S pre-rRNA, with both PNO1 and DIM1 required for this methylation event (Lafontaine et al., 1994; Vanrobays et al., 2004). It is believed that this methylation may represent a quality control mechanism as its enzymatic function can be separated from its involvement in pre-rRNA processing, such that methylation is not a pre-requisite for cleavage at sites A_1, A_2 or D in yeast, although the presence of DIM1 protein is required (Lafontaine et al., 1995; Lafontaine et al., 1998b). It has been demonstrated that this di-methylation is a late event in pre-rRNA processing of yeast, occurring in the cytoplasm, prior to the site D cleavage (Brand et al., 1977; Lafontaine et al., 1998b). In yeast, the majority of the protein has been found to localise to the nucleolus although some cytoplasmic localisation has been noted (Lafontaine et al., 1998b; Schafer et al., 2003). Similarly, DIM1 has been found to associate with both early and intermediate 40S pre-ribosomes (Lafontaine et al., 1998b; Schafer et al., 2003), binding rRNA

sequences involved in tRNA interactions and mRNA decoding in the mature ribosome, indicating that DIM1 association is incompatible with translation (Granneman et al., 2010).

It is therefore thought that in yeast, DIM1 binds to pre-rRNA in the nucleolus at an early stage of ribosome biogenesis. It is possible that a component of the processing machinery then senses this interaction and cleavage at sites A_1 and A_2 proceeds; otherwise processing is blocked. This may prevent the formation of mature but unmodified, and therefore aberrant, 40S ribosomal subunits which may be detrimental to the cell (Lafontaine et al., 1998b). This implies that DIM1 may have a role in both early and late 18S rRNA processing. In yeast, the majority of the SSU processome components dissociate from the pre-rRNA with, or shortly after cleavage at site A_2 (Grandi et al., 2002). ENP1, DIM1 and RRP12 however, remain associated with the 18S rRNA precursor; forming part of the intermediate 40S pre-particles in the nucleolus (Grandi et al., 2002; Schafer et al., 2003). This is consistent with DIM1 playing a role in both early and late 18S rRNA processing.

In humans, TSR1, LTV1, RIO2, ENP1, HRR25, DIM1, NOB1, and PNO1 were all found in late 40S precursors (Zemp et al., 2009). This is in agreement with data from yeast (Schafer et al., 2003) and most likely suggests that the two distinct modules (PNO1-NOB1, and TSR1, LTV1, RIO2, KRR1, ENP1, HRR25 and DIM1) recently described in yeast, may be co-purified from human cells (Merl et al., 2010; Zemp et al., 2009). It is however, not known if DIM1 is present in early pre-ribosomal particles or if / when it is responsible for the di-methylation at the 5' end of 18S rRNA in humans. Similarly, the localisation of the protein or its ability to associate with pre-rRNA in human cells is unknown.

1.8.2.7 SSU Processome Hierarchical Assembly

In *S. cerevisiae* a hierarchical model for the assembly of the SSU processome has been proposed (Figure 1.11) (Perez-Fernandez et al., 2007). It is believed that a number of factors associate as discrete pre-formed protein sub-complexes; work

further supported recently by the observation of similar sub-complexes required for late 40S pre-rRNA processing (Merl et al., 2010). Initially, the tUTP complex is thought to assemble onto the 35S pre-rRNA, essential for the assembly of a number of subsequent factors via two separate, and mutually independent, assembling routes. One of these involves the formation of an assembly intermediate composed of the U3 snoRNP, the PWP2/UTP-B, subunit and the MPP10 complex. A number of proteins (MPP10, IMP4, UTP20, BMS1, KRE33, ENP2, NOC4, KRR1, and NOP1) have been shown to assemble onto these pre-ribosomes independently of UTP-C and RRP5, although it is not known if this occurs at the same time, or subsequent to UTP-B and U3 snoRNP assembly. The other route involves the initial incorporation of RRP5 which is thought to facilitate the subsequent binding of the UTP-C module. The significance of the two different pathways is however, unclear and it is not known if the MPP10 complex and other factors subsequently associate similarly with the UTP-C assembly pathway although it would be assumed that they must for efficient pre-rRNA processing.

Whilst little is known about the assembly of the sub-complexes in human cells, it has been shown that the tUTP assembles to sites bound by UBF to aid transcription. It is then thought that the tUTP complex associates with the nascent pre-rRNA to aid recruitment of subsequent processing factors (Prieto and McStay, 2007). Such factors may be part of a 50S SSU processome, thought to be a precursor to the mature 80S processome, which is recruited to the pre-rRNA as a pre-formed sub-complex (Turner et al., 2009).

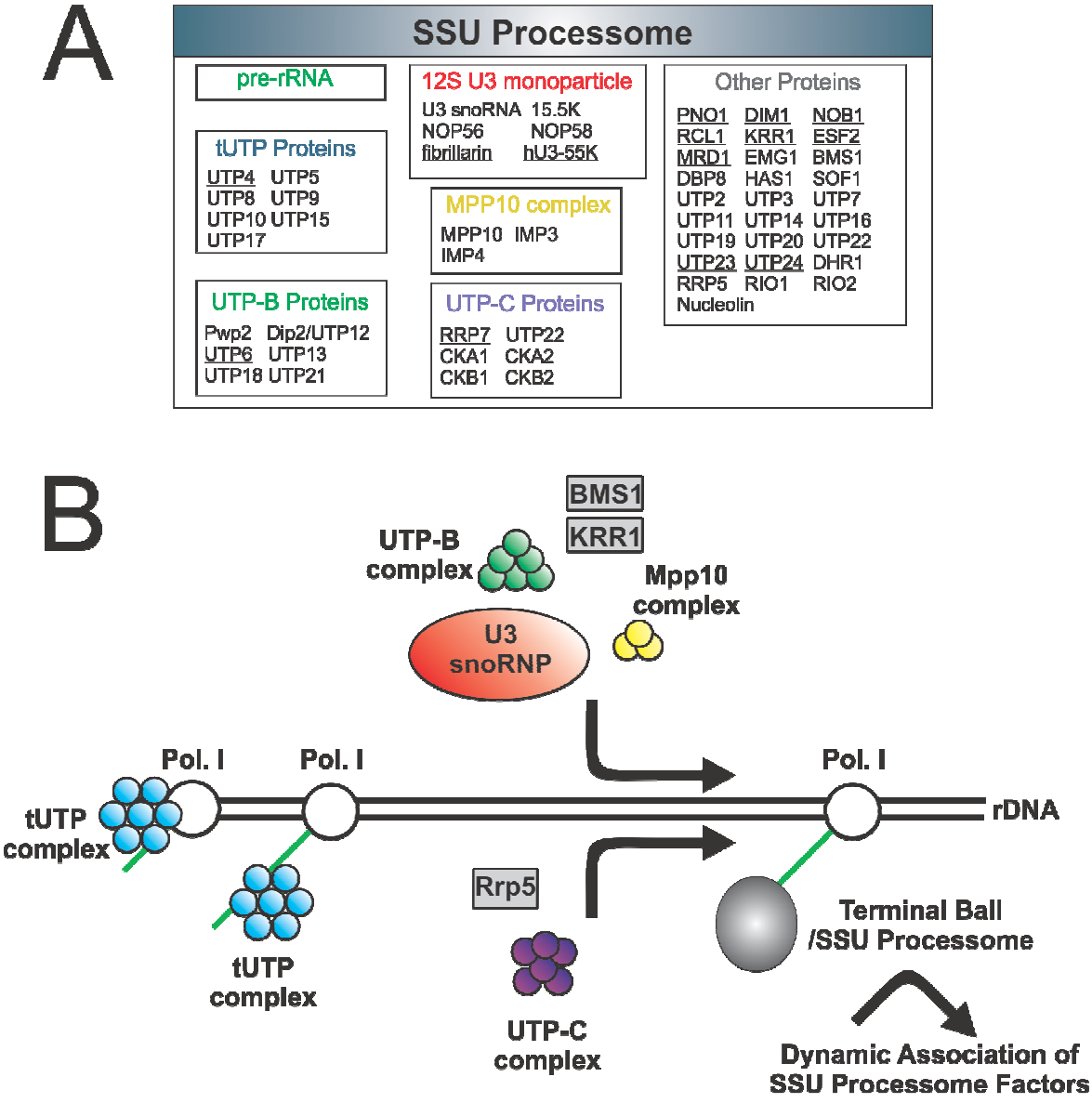


Figure 1.11 Pre-rRNA Transcription and SSU Processome Assembly
A) Components of the SSU processome, demonstrated mainly through work in yeast, colouring as in (B). Not all factors are present at the same time. Proteins to be examined in this study are underlined. **B)** A model for the initial steps of SSU processome assembly, forming the 90S in *Saccharomyces cerevisiae*, most likely highly similar in human cells. Shown is RNA polymerase I (white circle) transcribing pre-rRNA (green line), the association of the tUTP complex (blue) and subsequent assembly of the U3 snoRNP (red), UTP-B (green), MPP10 (yellow) and UTP-C (purple) complexes, along with RRP5, BMS1, KRR1 (grey) and other factors not shown. These are believed to form the initial 90S complex in yeast (visualised as the terminal ball by EM) with association and dissociation of subsequent SSU processome factors. Figure (B) based on previous figure (Perez-Fernandez et al., 2007), (A) and (B) based on data discussed (Billy et al., 2000; Bleichert et al., 2006; Borovjagin and Gerbi, 2001; Colley et al., 2000; Gallagher et al., 2004; Granneman and Baserga, 2004; Granneman et al., 2003; Henras et al., 2008; Krogan et al., 2004; Liang and Fournier, 2006; Lundkvist et al., 2009; Perez-Fernandez et al., 2007; Rempola et al., 2006; Turner et al., 2009; Wegierski et al., 2001; Wehner et al., 2002).

1.8.2.8 Nuclear Export Factors

Following nucleolar assembly and nucleolar and nucleoplasmic maturation, pre-ribosomes are exported seperately to the cytoplasm through the nuclear pore complex (NPC) before undergoing final maturation steps and achieving translational competence (Zemp and Kutay, 2007). Export of each ribosomal subunit is an active process that requires a karyopherin of the β-family (CRM1 / XPO1), a nuclear export sequence (NES)-containing adaptor(s), the RanGTP cycle, and specific nucleoporins (NUPs) (Askjaer et al., 1999; Fornerod et al., 1997; Zemp and Kutay, 2007).

Export of 60S subunits was shown to involve the RanGTPase system which controls the directionality of nuclear transport pathways, relying on RanGTP-binding transport receptors (Zemp and Kutay, 2007). In the nucleus, RanGTP binds to the karyopherin CRM1 along with the cargo protein to be translocated to the cytoplasm. The complex is transported through the nuclear pore into the cytoplasm and upon hydrolysis of RanGTP to RanGDP by Ran GTPase activating protein, the complex dissociates and export cargo is released.

The protein CRM1 is required for nuclear export of proteins containing a nuclear export sequence (NES) (Fornerod et al., 1997; Fukuda et al., 1997), Snurportin 1 (a protein involved in snRNP import) (Paraskeva et al., 1999), adapter proteins such as PHAX (phosphorylated adapter for RNA export) (Ohno et al., 2000) and HIV Rev (Askjaer et al., 1998) through the nuclear pore complex (NPC). Export of 60S subunits requires the RanGTPase system to control directionality of the export (Zemp and Kutay, 2007). Moreover, translocation is most likely mediated by the NES-containing co-factor NMD3, a trans-acting factor associated with late pre-60S particles which is thought to act as an adapter protein for CRM1-mediated export (Ho et al., 2000; Zemp and Kutay, 2007). In addition, it is highly likely that a host of other factors such as PRP12 (a potential RanGTP dependent nuclear transport receptor) and MTR2 (part of the mRNA export receptor MEX67/MTR2 heterodimer)

are also required for cytoplasmic export and factors such as LSG1 (GTPase) and SQT1 for subsequent NMD3 recycling (Zemp and Kutay, 2007).

Similar to export of the large ribosomal subunit, export of the small subunit is also thought to require CRM1, a nuclear export sequence (NES)-containing adaptor, the RanGTP cycle, and specific nucleoporins (NUPs) (Askjaer et al., 1999; Fornerod et al., 1997; Zemp and Kutay, 2007).

In yeast, late-stage pre-40S particles have been shown to be exported to the cytoplasm via a CRM1-mediated pathway (Moy and Silver, 2002), which can be specifically and effectively disrupted by LMB (Fukuda et al., 1997). It has also been demonstrated that LTV1 shuttles between nucleus and cytoplasm in a CRM1-dependent manner (Seiser et al., 2006). Moreover, both LTV1 and PNO1 have been suggested to act as pre-40S adapter proteins, providing the NES (Nuclear export signal) motif required for localisation to the cytoplasm (Seiser et al., 2006; Vanrobays et al., 2008). As is the case for export of the 60S pre-ribosome, export of the 40S particle also requires the HEAT repeat / Armadillo RRP12 protein. RRP12 participates in the export of both subunits, most likely through interactions with nucleoporins and has been shown to be an SSU processome component in yeast (Grandi et al., 2002; Oeffinger et al., 2004; Schafer et al., 2003; Vanrobays et al., 2008).

In human cells, CRM1 is believed to be required for the nucleolar localisation and nuclear export of various snoRNPs including U3 (Boulon et al., 2004; Watkins et al., 2004) and U8 (Watkins et al., 2007). CRM1 inhibition and depletion has also been shown to lead to RIO2 and NOB1 localisation shifting from the cytoplasm to the nucleus, whereas ENP1 shifts from the nucleolus to the nucleus, suggesting the accumulation of a nucleoplasmic 40S precursor (Rouquette et al., 2005; Zemp et al., 2009). Furthermore, the kinase activity of RIO2 has been shown to be essential for the recycling of PNO1, LTV1, and NOB1 as well as for 18S-E pre-rRNA processing in HeLa cells (Zemp et al., 2009). The role that PNO1, LTV1, NOB1 and DIM1 play is however, undocumented in humans.

1.9 The Nucleolus, Structure and Regulation

1.9.1 Structure of the Nucleolus

In eukaryotes, pre-ribosomal RNA (rRNA) transcription occurs within the nucleolus - a nuclear sub-compartment formed around nucleolar organiser regions (NORs) at the end of mitosis. Each human cell contains approximately 400 copies of the 43 kb human ribosomal gene repeat (Nazar, 2004; Prieto and McStay, 2005). These converge to form the nucleoli, with the NORs acting as the site from which the rDNA genes are transcribed by RNA pol. I during interphase. It is believed that not all of these repeats are transcriptionally active at any one time; potentially being important instead, in maintenance of nucleolar organisation and chromatin structure, independent of ribosome biogenesis (Rempola et al., 2006; Sanij and Hannan, 2009).

It is within the nucleolus that not only pre-rRNA transcription, but also the majority of pre-rRNA processing and ribosome assembly steps occur. Nucleoli vary depending on their activity, species and cell type. The nucleolus is formed as an outcome of ribosome biogenesis, such that the structure of the nucleolus and the pre-rRNA processing steps are linked. The nucleolus is neither surrounded by a membrane, nor anchored by an underlying scaffold but is comprised of three distinct structures in higher eukaryotes, visualised by EM, which correlate to the stages of biogenesis (Figure 1.12).

There are however notable distinct differences between pre-rRNA processing in humans and yeast (Granneman and Baserga, 2004; Henras et al., 2008; Rouquette et al., 2005; Terns and Terns, 2002). One major difference is the spatial organisation of pre-rRNA processing. In contrast to yeast nucleoli, higher eukaryotes contain three distinct nucleolar compartments, fibrillar centres (FC), dense fibrillar component (DFC) and granular component (GC) where processing has been shown to occur in a vectorial fashion (Thiry and Lafontaine, 2005).

With the exception of 5S rRNA, pre-ribosomal RNAs (rRNAs) are transcribed by RNA pol. I at the FC / DFC border of mammalian nucleoli, as a single large precursor (Cheutin et al., 2002; Huang, 2002; Puvion-Dutilleul et al., 1997). Elongating rRNAs subsequently move rapidly into the surrounding DFC (Cheutin et al., 2002) where initial processing and cleavage steps around pre-18S rRNA (at the A' site and within the 5'ETS) are thought to occur (Derenzini et al., 1990; Granneman et al., 2004) before movement to the granular component (GC). It is here that removal of the core 5'ETS sequence (site 1 cleavage) is then thought to take place (Gerbi and Borovjagin, 1997; Lazdins et al., 1997). The pre-rRNAs then move into the nucleoplasm, before finally being exported through nuclear pores to the cytoplasm (Lei and Silver, 2002). Similarly, in plant cells, removal of the 5'ETS is believed to occur in the DFC whereas removal of ITS1 occurs in the GC (Beven et al., 1996).

The localisation profiles of both the U8 and U3 Box C/D snoRNAs are in agreement with these observations. The U8 snoRNA has been observed to localise to the DFC region of the nucleolus, consistent with an early role in pre-rRNA processing (Granneman et al., 2004). In contrast, the U3 snoRNA is known to localise throughout the DFC and GC regions of the nucleolus, consistent with roles in both the initial and later cleavage steps of pre-rRNA processing (Gerbi and Borovjagin, 1997; Granneman et al., 2004). This suggests that in the DFC, U8 snoRNA mediates the cleavage at site 02 within the 3'ETS and U3 snoRNA mediates cleavage at A' within the 5' ETS. The U8 snoRNA is then thought to dissociate whilst U3 remains associated to facilitate cleavages at sites 1 and 2; either side of the 18S rRNA.

For efficient movement of the U3 snoRNA from the DFC to the GC, both the Box B/C motif and the hinge region are essential in human cells. The B/C motif is required for association of hU3-55k and for the formation of the 80S SSU processome (Granneman et al., 2004). In contrast, the U3 snoRNA hinge region is required for base-pairing to 5'ETS pre-rRNA sequences (Granneman et al., 2004). Moreover, both the B/C motif and the hinge region are required for association of

MPP10 and it is suggested that MPP10 association may aid initiation of the A' cleavage step and translocation of the 80S complex to the GC (Granneman et al., 2004).

The pre-rRNA is processed as it moves from the DFC of the nucleolus to the cytoplasm in a vectorial fashion, therefore the localisation profiles of the various processing factors can be informative as to their temporal role in rRNA production. Accordingly, the localisation of fibrillarin has been widely studied, and as a core component of Box C/D snoRNPs is observed to localise to the DFC (Ochs et al., 1985), where the majority of Box C/D snoRNPs are believed to function. Accordingly, core proteins NOP56 and NOP58 were observed to co-localise with fibrillarin in the DFC whereas hU3-55k has been observed in the DFC and GC, consistent with its association with the U3 snoRNP (Leary et al., 2004). Moreover, the majority of SSU processome components appear to localise to both the DFC and GC components of the nucleolus. This includes MPP10, IMP3, IMP4, SOF1 and RCL1 (Leary et al., 2004), nucleolin and B23 (Biggiogera et al., 1989), tUTP4 and tUTP10 (Prieto and McStay, 2007), UTP13 and BMS1 (Turner et al., 2009).

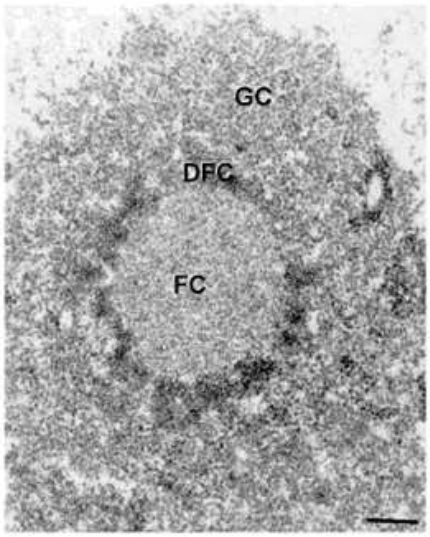


Figure 1.12 EM of Mammalian Nucleolar Structures
Electron micrograph illustrating the morphology of the nucleolus in mouse Ehrlich ascites tumour cells. The nucleolus is composed of three sub-compartments: fibrillar centers (FC), the dense fibrillar component (DFC) and granular component (GC). Scale Bar (bottom right) is 0.2µm. Figure modified from Scheer & Hock (Scheer and Hock, 1999).

1.9.2 The Nucleolus During Mitosis

The nucleolus is a dynamic structure and during mitosis in mammalian cells, the nucleoli disassemble and their components disperse (Leung et al., 2004). Nucleolar disassembly begins in prophase with the block in RNA pol. I activity which continues to the end of telophase (Sirri et al., 2000). This is achieved through the phosphorylation of the transcriptional machinery by cdc2–cyclin B kinase activity (Sirri et al., 2000).

It was previously believed that the complete rDNA transcription machinery remained assembled with rDNA sequences during mitosis. This included RNA pol. I, upstream binding factor (UBF), the TATA-binding protein (TBP)-containing selectivity factor SL1 and the transcription termination factor TTF-1, which facilitates initiation and mediates termination of rDNA transcription (Sirri et al., 2000). More recently however, it has been demonstrated in HeLa cells that components of RNA pol. I dissociate briefly from the rDNA during metaphase, although many components of the machinery such as UBF, remain associated (Leung et al., 2004). This is thought to occur prior to breakdown of the nuclear lamina, also facilitated by cdc2–cyclin B kinase activity (Burke and Ellenberg, 2002; Pines and Rieder, 2001).

It has also been shown that disruption of rDNA transcription can be functionally separated from the inhibition of pre-rRNA processing (Sirri et al., 2000). Unlike the rDNA transcription machinery, which largely remains associated with the rDNA throughout mitosis, the processing factors of the DFC and GC are thought to dissociate. At early prophase, the pre-rRNA processing machinery of the DFC and GC begins to move out of the nucleolus and by the end of prophase, chromosomes are condensed and the nucleolus is no longer visible (Gautier et al., 1992). In particular, the DFC marker fibrillarin, and GC marker B23 have been shown to leave the nucleolus at approximately the same time, although after that of RNA pol. I (Leung et al., 2004). It is therefore believed that nucleolar disassembly is initiated

at the FC, whereas loss of both DFC and GC components occurs later (Leung et al., 2004).

When cells exit mitosis, nucleolar components reassemble around the respective nucleolar organiser regions (NORs) (Leung et al., 2004). During mitosis, the partially processed rRNA transcripts from the previous nucleoli form complexes with various processing factors, with the resulting structures called “prenucleolar bodies” (PNBs). Components are subsequently transferred from the PNBs into the reforming nucleoli at NORs (Leung et al., 2004). These PNBs contain a host of nucleolar components including U3 and U8 snoRNAs, fibrillarin, MPP10, nucleolin and B23. Initially, pre-rRNA processing factors are directly recruited to NORs, independently of rDNA transcription (Dousset et al., 2000; Jimenez-Garcia et al., 1994; Leung et al., 2004). Subsequently, partially processed pre-rRNAs and further processing components are recruited to NORs either directly or via the PNBs (Dousset et al., 2000). The NORs are able to coalesce to form either one or multiple functional nucleoli (Leung et al., 2004), with the supply of previously transcribed pre-rRNA thought to provide a ready supply of pre-ribosomes to the daughter cells (Dundr and Olson, 1998).

1.9.3 The Regulation of Ribosome Biogenesis

The regulation of ribosome biogenesis is crucial to any eukaryotic cell in order to maintain sufficient protein synthesis levels, required for cell growth and division. Conversely, during cellular stress the nucleolus is observed to be structurally altered, the nucleolar proteins linked to pre-rRNA processing become dispersed and ribosome biogenesis is downregulated. Furthermore, it is believed that the nucleolus may have a number of roles outside that of ribosome biogenesis (Boisvert et al., 2007).

1.9.3.1 The Regulation of Ribosome Biogenesis Under Stress

It has been demonstrated that the nucleolus is not only important in ribosome biogenesis but also as a stress sensor. It was previously observed that a vast array of factors result in activation of the p53 tumour suppressor and its downstream targets, although it was not known what links them. It has now been shown that a number of stresses including UV irradiation, heat shock, NTP depletion and hypoxia are all capable of causing nucleolar disruption which in turn, leads to p53 stabilisation (Rubbi and Milner, 2003). Furthermore, it was shown that it is nucleolar disruption, rather than DNA damage, which causes stabilisation of the tumour suppressor protein; thought to be important in allowing its activation and thus either cell cycle arrest and DNA repair, or apoptosis (Rubbi and Milner, 2003).

In normal, growing cells, p53 protein levels are kept low by the action of the MDM2 protein which targets p53 for proteasomal degradation (Ljungman, 2000). Co-compartmentalisation of the two proteins is therefore essential for p53's degradation, with disruption of their association usually required for p53 activation (Xirodimas et al., 2001). Moreover, it has been recently demonstrated that defects in pre-rRNA maturation also stabilise p53 (Holzel et al., 2010). Collectively, this suggests that rather than being actively induced, a p53 response must constantly be suppressed by the degradation of the protein, promoted by an active nucleolus and ribosome biogenesis. Therefore, this intimately ties ribosome biogenesis with the tumour suppressor p53, with ribosome biogenesis appearing incompatible with an efficient tumour repressor response mediated by the protein. Interestingly, a number of drugs have been shown to affect the localisation of proteins linked to processing of the small ribosomal subunit, and p53 stabilisation. These include Actinomycin D (Andera and Wasylyk, 1997; Leary et al., 2004; Turner et al., 2009), Leptinomycin B (Freedman and Levine, 1998; Rouquette et al., 2005; Zemp et al., 2009) and Rapamycin (Kao et al., 2009; Vanrobays et al., 2008).

1.9.3.2 Actinomycin D

Drug treatments have been widely used to examine nucleolar function and to sub-categorise complexes. They are useful in selectively blocking specific pathways when initially characterising proteins, as disruption of the protein's associations or localisations implicate it in the targeted pathway. Actinomycin D (ActD) is a chemotherapeutic frequently used at low concentrations to block RNA pol. I transcription of rRNA; blocking ribosome biogenesis. ActD selectively inhibits RNA pol. I activity as a result of the higher G/C content of the rDNA (Perry, 1963). As the SSU processome is said to assemble on the nascent pre-rRNA, potentially co-transcriptionally, blocking rRNA transcription blocks SSU processome recruitment; giving rise to a 50S pre-SSU processome complex (Turner et al., 2009). When treated with ActD, mammalian nucleoli have been shown to exhibit a segregated phenotype. Factors from the granular component (GC) remain in the center of the nucleolus, whereas the fibrillar (FC/DFC) components move to the periphery of the nucleoli and form nucleolar caps; suggested as a potential method of stopping ribosome biogenesis under stress conditions (Journey and Goldstein, 1961; Leary et al., 2004; Reynolds et al., 1964).

1.9.3.3 Leptinomycin B

The antibiotic and potential chemotherapeutic, leptomycin B (LMB) is an inhibitor of CRM1; a protein required for nuclear export of proteins containing a NES motif (Fukuda et al., 1997). CRM1 is also important in the nucleolar localisation of snoRNPs including U3 (Boulon et al., 2004) and potentially the cytoplasmic export of pre-U8 snoRNA (Watkins et al., 2004; Watkins et al., 2007). Importantly, late-stage pre-ribosomal particles are known to move from the granular component of nucleoli to the nucleoplasm and are then exported to the cytoplasm via a CRM1-mediated pathway (Moy and Silver, 2002), which can be specifically and effectively disrupted by LMB (Fukuda et al., 1997).

More recently, it has been demonstrated that LMB treatment of HeLa cells inhibits processing in the 5'ETS of pre-rRNA, at a site homologous to A_0 in yeast pre-rRNA, and at site 1; both thought to occur within the nucleolus (Rouquette et al., 2005). Treatment with LMB also prevents shuttling of late pre-40S processing components between the cytoplasm and nucleus, believed to require CRM1 and adapter proteins for translocation (Zemp et al., 2009). Accordingly, the block in shuttling has been shown to result in the loss of processing at site 3 at the 3' end of 18S rRNA; believed to occur cytoplasmically (Rouquette et al., 2005). Collectively, this suggests that active nuclear export of the pre-40S particle is required not only for downstream processing, but also for upstream pre-rRNA processing steps in human cells.

1.9.3.4 Rapamycin

Rapamycin is an immunosuppressant and antibiotic. It is known to bind to the cytosolic FK-binding protein 12 (FKBP12). This complex then inhibits the mammalian target of rapamycin (mTOR) pathway by directly binding the mTOR Complex 1 (mTORC1) (Hay and Sonenberg, 2004). mTOR kinase acts as a nutrient sensor and major effector of cell growth and proliferation via the regulation of protein synthesis. Some targets are phosphorylated directly by mTOR, but many are phosphorylated indirectly via the mTORC1 or mTORC2 complexes, although only mTORC1 is inhibited by rapamycin (Hay and Sonenberg, 2004; Kim et al., 2002). The activity of mTOR is said to be stimulated by a range of factors including insulin, growth factors, serum, amino acids and oxidative stress (Hay and Sonenberg, 2004; Kim et al., 2002). In yeast, TOR has been shown to regulate the subcellular distribution of PNO1; a KH domain protein required for co-transcriptional ribosome assembly and pre-40S ribosome export. This suggests a possible method for nutrient availability controlling ribosome biogenesis (Vanrobays et al., 2008) although if this is also the case in human cells is not known. It should be also be noted that mTOR is not thought to be a single, linear pathway, but potentially a central player; integrating cellular physiological and environmental cues to control growth (Mayer and Grummt, 2006). It has been

shown that rapamycin treatment or TOR depletion in yeast inhibits cell cycle progression; arresting cells at the G1/S boundary, reducing the size of the nucleolus, and limiting Pol I and Pol III transcription, thus inducing a G0-type stationary phase (Zaragoza et al., 1998).

1.9.4 Additional Roles of the nucleolus

Proteomic analysis of human nucleoli has revealed that it contains over 700 proteins, only a third of which are known to be involved in ribosome biogenesis (Andersen et al., 2005; Andersen et al., 2002; Boisvert et al., 2007; Scherl et al., 2002). This indicates that the nucleolus may be involved in a host of other processes outside of ribosome biogenesis. Potential roles are thought to include regulation of mitosis, cell-cycle progression and proliferation, many forms of stress response and biogenesis of multiple ribonucleoprotein particles, with dysregulation leading to a number of human diseases (Boisvert et al., 2007).

1.10 The Nucleolus, Ribosome Biogenesis in Cell Cycle Control and Differentiation

In both yeast and humans, ribosome biogenesis is a key control point for the regulation of cell growth and division (Belin et al., 2009; Bernstein and Baserga, 2004; Bernstein et al., 2007; Dai and Lu, 2008; Montanaro et al., 2008), with protein translational capacity influenced by the number of ribosomes in the cell (Burrin et al., 1997). Ribosome synthesis is down regulated in terminally differentiated cells (Bowman and Emerson, 1977; Poortinga et al., 2004) and up-regulated in many cancers, with several proto-oncogenes and tumour suppressors able to directly regulate ribosome biogenesis (Oskarsson and Trumpp, 2005; Ruggero and Pandolfi, 2003).

1.10.1 Ribosome Biogenesis and Cell Cycle Control

In eukaryotes, cell growth and the cell cycle are said to be separate processes. However, the doubling time in yeast is not limited by the time required to duplicate the genome, but rather by the time required to double the cell's mass (Jorgensen et al., 2004), with protein accounting for more than 70% of eukaryotic cells' dry mass (Oskarsson and Trumpp, 2005). Therefore, cell cycle progression is typically dependent upon cell growth (Jorgensen et al., 2004; Rudra and Warner, 2004) with the two processes coordinately controlled (Figure 1.13 (Neufeld et al., 1998; Neufeld and Edgar, 1998; Oskarsson and Trumpp, 2005)). Interestingly, signaling pathways such as Ras/PKA and mTOR (Guertin et al., 2006; Jorgensen et al., 2004; Soulard et al., 2009; Yang et al., 2008), c-Myc (Dai and Lu, 2008; Li et al., 2010; Shiue et al., 2009; Teleman et al., 2008), p53, pRB and ARF (Larminie et al., 1998; Oskarsson and Trumpp, 2005; Sugimoto et al., 2003; White, 1997; White, 2005) are all key regulators in both ribosome biogenesis and cell cycle control in higher eukaryotes. Furthermore, these pathways involve a significant amount of cross-talk between them, as illustrated by the activity of Myc; shown to be an important mediator of TOR-dependent ribosome biogenesis in *Drosophila* (Teleman et al., 2008). Moreover, defects in the nucleolus and pre-rRNA maturation have been shown to stabilise p53 (Holzel et al., 2010; Rubbi and Milner, 2003). In turn, p53-mediated transcriptional repression of c-Myc has been demonstrated to be required for both G_1 cell cycle arrest and cellular differentiation (Ho et al., 2005).

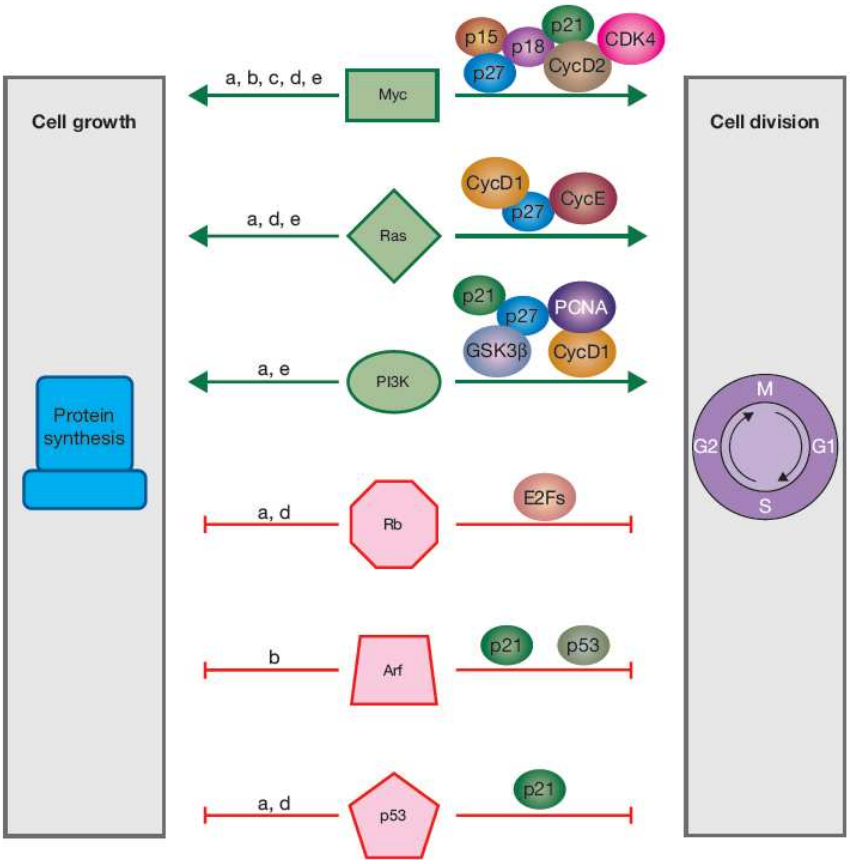


Figure 1.13 A Model for the Simultaneous Control of Cell Growth and Division
Each pathway displays a 'growth' (left) and a 'division' (right) branch, which is either activating (oncogenic; green) or repressive (tumour suppressor; red). The processes affecting cell growth are: (a) RNA Pol I transcription; (b) pre-rRNA processing; (c) RNA Pol II transcription; (d) RNA Pol III transcription; and (e) mRNA translation. The protein factors affecting cell division are indicated within colour circles, these include oncoproteins, tumour suppressors. Figure from (Oskarsson and Trumpp, 2005).

In humans it has been shown that rRNA is produced at increased levels in a range of tumours and it is suggested that overexpression of pre-rRNA may be a general feature of cancer (Williamson et al., 2006). Indeed, as early as the 1970's, changes to the nucleolus have been recognised as useful diagnostic markers for tumourogenesis, although the significance of this was not fully understood (Gani, 1976). Whilst the number of nucleoli in transformed cells is observed to increase, a crude measure such as argyrophilic nucleolar organizer regions (AgNOR; silver stained NORs) staining is not a very accurate tool for prognosis (Derenzini et al., 2004; Karakok et al., 2001; Lumachi et al., 2004) or only useful in a sub-set of cancers (Gunther et al., 2000; Rodrigues et al., 1997). It is therefore important to

understand the role that the nucleolus and ribosome biogenesis play in cell cycle control and cancer progression more fully.

Interestingly, casein kinase II (CKII) was the first kinase shown to regulate UBF activity with phosphorylation promoting transcription of rRNA (Lin et al., 2002; Tuan et al., 1999). Accordingly, CKII is found overexpressed in many cancers and is believed to phosphorylate components of cell cycle machinery including c-Myc (Luscher et al., 1989; Ruggero and Pandolfi, 2003). Of particular interest, the CKII subunits are key components of the UTP-C complex; crucial for efficient pre-rRNA processing in yeast (Krogan et al., 2004; Perez-Fernandez et al., 2007). This suggests that ribosome production and cell cycle progression may share key components.

In yeast it has been demonstrated that ribosome biogenesis directly promotes passage through the start of the cell cycle as, when ribosomes are no longer made, cells stall in the G1 phase (Bernstein and Baserga, 2004). This is thought to occur through inhibition of Whi5 (equivalent to the human tumour suppressor retinoblastoma; pRB), which when active, can sense blocks in ribosome biogenesis and thus inhibit passage through Start (when cells commit to cell division during late G1) (Bernstein et al., 2007).

A similar mechanism may occur in human cells. Normally, pRB inhibits S phase progression by associating with and blocking the action of E2F transcription factors. pRB becomes progressively phosphorylated through the cell cycle, allowing release and activation of the E2F transcription factors (Classon and Harlow, 2002). The pRB gene is frequently disrupted in human cancers, thus allowing the deregulation of E2F factors (Ruggero and Pandolfi, 2003). In untransformed cells, pRB is also capable of restricting production of rRNA. This is achieved through associating with UBF (Cavanaugh et al., 1995), believed to prevent recruitment of other RNA pol. I cofactors required for initiation of transcription, such as SL1 and the TBP-associated factors (TAFs) (Comai et al., 2000; Voit et al., 1997). Furthermore, pRB has been shown to accumulate in the

nucleoli of differentiated U937 cells, correlating with inhibition of rDNA transcription (Cavanaugh et al., 1995). It is also thought that pRB may be directly involved in differentiation through the activation of transcription factors involved in the process (Classon and Harlow, 2002), thus providing links between ribosome biogenesis, cellular differentiation and tumourigenesis.

This may be illustrated by the potentially dual role of the ESF2 protein, shown to be an SSU processome component in *S. cerevisiae* (Granneman et al., 2006b; Hoang et al., 2005). In mice, its role in ribosome biogenesis has not been studied but instead, it has been shown to associate with a component of the transcription factor TFIID complex; responsible for binding the TATA box and is known to associate with factors such as c-Fos, c-Myc and p53 (Oda et al., 2000). Furthermore, expression of the protein was observed to promote basal transcription levels, suggesting it may aid cellular growth, although this has not yet been demonstrated (Oda et al., 2000).

In Burkitt's lymphoma, a human B-cell tumour, the onocoprotein c-Myc is activated; triggering proliferation (Pajic et al., 2000). In P493-6 cells, derived from this lymphoma, only the processing of the 47S rRNA precursor to mature 18S and 28S rRNA, but not the synthesis of the 47S transcript, was dependent on the presence of c-Myc (Schlosser et al., 2005). Both N-Myc (neuronal Myc) and c-Myc have been shown to control the levels of mRNAs encoding pre-rRNA processing factors, with levels of mature rRNA increased upon Myc expression (Boon et al., 2001; Coller et al., 2000; Schlosser, 2003). This suggests that, in this instance, the level of pre-rRNA processing factors may be important in dictating the amount of mature rRNA produced.

It should however, be noted that rDNA transcription is also closely regulated throughout the cell cycle (Heath et al., 2000). c-Myc has been demonstrated to enhance RNA pol. I transcription of rRNA (Arabi et al., 2005; Grandori et al., 2005) and to induce expression of a host of translation initiation factors including eIF-4E, eIF2A, eIF5A and eIF4G (Coller et al., 2000); known to cause cellular

transformations (Schmidt, 1999). It would therefore appear that the factors used to drive cell cycle progression also drive the production of ribosomes at the levels of both transcription and processing, as well as promoting mRNA translation (Figure 1.13). This suggests that cellular growth and division are much more intimately associated than once thought.

1.10.2 Ribosome Biogenesis and Cellular Differentiation

During cellular differentiation, many cell types undergo arrest in the G0/G1 phase of the cell cycle. This is often accompanied by a reduction in cell growth and ribosome biogenesis (Poortinga et al., 2004). Upon differentiation, the adenocardinoma cell line CaCo-2 is said to lose its tumourigenic phenotype, so represents a useful tool for examining tumour progression (Stierum, 2003). Moreover, proliferation and differentiation are mutually exclusive events, therefore many malignancies present as their immature cell type (Heath et al., 2000).

It has been reported that upon myoblast differentiation, the ribosome half life is reduced and pre-rRNA turnover is increased, whereas there is no significant change in the rRNA transcription levels (Bowman and Emerson, 1977). In regenerating rat livers, ribosome biogenesis is also controlled post-transcriptionally, in part by accelerating the occurrence of pre-rRNA cleavages to produce more ribosomes, more quickly (Dudov and Dabeva, 1983). Moreover, ribosomal proteins are produced in excess in higher eukaryotes; with unused proteins rapidly degraded to enable swift changes in the rate of ribosome production (Bowman, 1987; Lam et al., 2007; Tsay et al., 1988). Collectively, this shows that processing and post-transcriptional regulation are crucial in controlling ribosome biogenesis, especially in tumourogenesis and cellular differentiation.

It has however, also been shown that rDNA transcription is closely regulated throughout differentiation. MAD1 (Mitotic spindle assembly checkpoint protein) negatively regulates cell growth and rDNA transcription by associating with UBF (upstream binding factor; essential for rRNA transcription) during granulocyte

differentiation. Conversely, exogenous c-Myc expression (down regulated during differentiation (Heath et al., 2000)) can increase both UBF expression and rRNA transcription (Arabi et al., 2005; Grandori et al., 2005; Poortinga et al., 2004), whilst ribosomal protein L11 has been shown to inhibit c-Myc-induced transcription and cellular proliferation (Dai et al., 2007), demonstrating a possible mechanism of regulating rRNA transcription during cellular differentiation.

The ribosomal proteins L11 and L5 have also been shown to bind and suppress the E3 ligase function of human MDM2 (HDM2), thus activating p53 (Bhat et al., 2004; Horn and Vousden, 2008). It has also been demonstrated that a decrease in L11 NEDDylation (association of the NEDD8 protein) during nucleolar stress causes relocalisation of the L11 protein from the nucleolus to the nucleoplasm, providing the signal for p53 activation (Sundqvist et al., 2009). This demonstrates the complexity of the stress-response pathways and their close association with ribosome biogenesis. However, whilst there is evidence that ribosome biogenesis is regulated at both the level of rRNA transcription and rRNA processing (Schlosser, 2003; Schlosser et al., 2005), the majority of the work to date has focused upon regulation of transcription.

Of particular interest, is the observation that mammalian U3 snoRNA expression is down-regulated in response to serum starvation and during myoblast differentiation; coinciding with a reduction in ribosome production (Glibetic et al., 1992; Sienna et al., 1996). Moreover, during *Xenopus* oocyte development, U3 snoRNA levels are regulated independently of fibrillarin (Caizergues-Ferrer et al., 1991).

Interestingly, peroxisome proliferator-activated receptors (PPAR) are a group of transcription factors able to regulate cellular differentiation and development. When malignant cancer cell lines such as colon, breast, prostate and liposarcoma, have been forced to differentiate by treatment with PPAR-γ agnoists, a reversal of the malignant phenotype has been observed, giving rise to the prospect of “differentiation therapy” (Mueller et al., 1998; Mueller et al., 2000; Sarraf et al.,

1998; Spira and Carducci, 2003; Tontonoz et al., 1997). Other forms of differentiation therapy have also shown promise in treating acute promyelocytic leukemia (Spira and Carducci, 2003; Waxman, 2000) and reducing metastases of breast cancer cells (Beug, 2009), demonstrating the importance of understanding cellular differentiation.

It is suspected that many of the factors that drive ribosome biogenesis and cellular growth similarly drive the cell cycle and tumourogenesis. Conversely, it would appear that many of the factors involved with the suppression of ribosome biogenesis may also drive cellular differentiation (Figure 1.13). It would therefore be tempting to speculate that a better understanding of these processes may lead to the discovery of novel therapeutic targets for some cancers.

1.11 Research Aims

The production of the small ribosomal subunit through pre-rRNA cleavages and the association of protein factors within the SSU processome have been widely studied in yeast. A large amount of this data has recently been provided by proteomic analysis of the SSU processome in *S. cerevisiae* yet, surprisingly little is known about the composition, assembly and activity of SSU processome human cells, with even less known about its regulation.

In both humans and yeast, the U3 snoRNP is essential for the cleavages surrounding the 18S pre-rRNA. Furthermore, in human cells, the motif responsible for association of the hU3-55k protein is indispensable for U3 snoRNP incorporation into the SSU processome, indicating that protein-protein interactions are key to its integration into the complex (Granneman and Baserga, 2004; Venema et al., 2000). In *Xenopus* oocytes, U3 snoRNA levels have been shown to be regulated independently of fibrillarin (Caizergues-Ferrer et al., 1991) although it is unclear how U3 snoRNP levels are regulated, specifically in vertebrates. We therefore, set out to investigate the role of hU3-55k in regulating the U3 snoRNP, and how it may regulate the production of ribosomes in human cells.

Once produced, the U3 snoRNP must associate with the pre-rRNA and the SSU processome complex must be assembled. It is known that protein-protein interactions are most likely key to forming this complex (Granneman et al., 2004). In yeast, and most likely in humans, it is thought that the SSU processome is assembled in a hierarchical manner with the loading of a series of pre-assembled protein sub-complexes (Merl et al., 2010; Perez-Fernandez et al., 2007; Turner et al., 2009). However, the primary rRNA binding protein(s) that may allow these complex(s) to assemble onto the pre-rRNA are completely unknown. Moreover, it is not clear, particularly in humans, how the potential sub-complexes may interact.

Interestingly, there are a host of SSU processome-associated proteins which have been observed in yeast proteomic screens, that contain putative RNA-binding and RNA-modifying domains, yet their functions are largely unknown (Bernstein et al., 2004; Dragon et al., 2002; Grandi et al., 2002; Krogan et al., 2004; Schafer et al., 2003). Furthermore, little is known about the temporal order of the components linked to processing events, especially with respect to the processing of the 3' end of human 18S rRNA. It has however, been demonstrated that preventing this cleavage step has repercussions for both downstream and upstream processing events (Rouquette et al., 2005; Zemp and Kutay, 2007).

Many of the putative RNA-binding / RNA-modifying proteins identified however are relatively small in size, making them attractive components to express and study. A key example is RRP7; one of only two non-CKII components of the UTP-C complex (Krogan et al., 2004) and the only UTP-C component to contain a putative RNA recognition motif (RRM). This suggests it may recruit the complex to the pre-rRNA although this has never been demonstrated. It should also be noted that when this work began, there was no UV cross-linking and cDNA analysis (CRAC) data available from yeast (Granneman et al., 2009; Granneman et al., 2010) meaning that the binding sites for almost all of the SSU processome proteins on the rRNA were unknown.

The objective of this study was therefore, to gain a better understand of the SSU processome in human cells, particularly with respect to the U3 snoRNP, hU3-55k and proteins involved with binding and modifying the pre-rRNA. We wished to know how U3 snoRNA levels were controlled and the importance of the U3-specific hU3-55k protein. We also wanted to find out if proteins present in the yeast SSU processome were also components of the human complex. Moreover, examine if any of these were able to directly bind pre-rRNA sequences, in particular, proteins that may bind within the 5' ETS or ITS1 to direct pre-rRNA processing. We also wished to examine the processing of the 3' end of 18S rRNA and investigate if it was the same as that observed in yeast, as an additional processing step is known to be required. This would be achieved in through the following aims:

1) **To investigate U3 snoRNA regulation in differentiated and undifferentiated cells**
2) **To examine the regulation of the SSU processome and the potential role played by hU3-55k and its conserved domains**
3) **To investigate potentially orthologous SSU processome proteins in human cells, believed to be involved in rRNA binding and modifications in yeast**
4) **To investigate 3' end processing of the pre-18S rRNA through the characterisation of the human proteins NOB1, PNO1 and DIM1; crucial to the process in yeast**

Chapter Two

Materials & Methods

2.1 Cloning & Mutagenesis

2.1.1 PCR & Cloning Genes of Interest

PCRs (polymerase chain reactions) were used to amplify DNAs (deoxyribonucleic acids) of interest. Reactions were set up using 100 ng of template DNA, 1 x Qiagen PCR Buffer (final concentration 1.5 mM $MgCl_2$), 0.4 mM dNTPs (deoxyribonucleotide triphosphates), 2 µM forward primer, 2 µM reverse primer and 2.5 U (units) of HotStarTaq DNA Polymerase (Qiagen), in a total volume of 50 µl. The reactions were incubated for 15 minutes at 95℃ to activate the HotStarTaq Polymerase, then cycled 35 x 1 minute at 95℃ to de nature the DNA, 1 minute at 57℃ to anneal primers to the template and 1 minute at 72℃ for every kb of DNA to be amplified. A final extension step of 10 minutes at 72℃ was used after the last cycle to ensure completion of the reaction.

All template cDNA clones were purchased from RZPD / imaGenes as I.M.A.G.E. Full Length cDNA clones with the exception of tUTP4 in pcDNA6.2/nLumio-DEST which was kindly provided by Brian McStay, NUI Galway (Prieto and McStay, 2007), hU3-55k and hU3-55k Del193-352 in pCI-neo, and U3-StreptoTag in pCR4 TOPO were kindly provided by Sander Granneman, University of Edinburgh (Granneman et al., 2002; Granneman et al., 2004; Lukowiak et al., 2000; Pluk et al., 1998), and StreptoTagged Wt. U3 and U3 C'Consensus snoRNAs in pGEM-T Easy were kindly provided by Hannah Richardson, University of Newcastle. The StreptoTag sequence was composed of the streptomycin binding aptamer motif as described previously (Bachler et al., 1999), and was derived from a plasmid kindly provided by Sander Granneman (Granneman et al., 2004).

2.1.2 Agarose Gel Electrophoresis

A final concentration of 1 x loading buffer (40 % glycerol (v/v), 60 % TE buffer (Tris-ethylene diamine tetra-acetic acid [EDTA]) (v/v), 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 1 x bromophenol blue was added to the DNA to be analysed. Samples were loaded onto an appropriate percentage agarose gel, containing 1x SYBR®Safe (Invitrogen), and run in 1 x TBE (Tris/Borate/EDTA,) buffer (90 mM Tris-HCl, 90 mM Boric acid, 2 mM EDTA, pH 8.0) at 100 volts for 1 hour. Gels were visualised using a Syngene ultraviolet transilluminator.

2.1.3 DNA Extraction and Purification

DNA bands were excised from the agarose gel and purified using a QIAEX II Gel Extraction Kit, according to manufacturer's guidelines and eluted in 50 µl H_2O.

2.1.4 DNA Quantitation

Where relevant, 1µl of sample DNA was quantified using a Thermo Scientific NanoDrop at absorbance at 260nm and purity determined by the ratio of absorbance at 260 / 280 nm.

2.1.5 Restriction Digestion of DNA

Restriction digests were typically performed using 0.5 – 2 µg DNA, 10 Units of the required enzyme(s) and buffer in accordance with manufacturer's guidelines (Promega) in a final volume of 20 µl. Digestions were incubated at 37 ℃ for 2 -4 hours.

2.1.6 Ligation of Isolated DNA into Plasmids

In the case of pGEM-T Easy cloning (Promega, ampicillin resistant), reactions were set up with 2x Rapid Ligation Buffer, 50ng pGEM-T Easy Vector, a 1:1 molar ratio of 1:1 insert:vector (typically 5-10ng DNA) and 0.3 Units of T4 DNA Ligase (Promega) in a final volume of 10 µl.

Ligation reactions utilising pET100 D/TOPO (Invitrogen, ampicillin resistant) were set up according to manufacturer's instructions in 6 µl using 1 µl (20 ng) TOPO Vector (Invitrogen), 1 µl salt solution and 4 ng of DNA from a PCR. Reactions were incubated at room temperature for 5 minutes and then placed on ice for transformation of TOP10 *E. coli* cells.

Ligation reactions using appropriately digested DNA insert and vector were set up using a 3:1 molar ratio of insert:vector (typically 10-30 ng insert DNA, 50ng vector), 0.3 Units of T4 DNA Ligase (Promega) and 1x T4 DNA Ligase Buffer in a final volume of 20 µl.

All ligations were subsequently incubated at 4 ℃ overnight and transformed into chemically competent TOP10 *E. coli* cells, with the cloned products sequence verified.

2.1.7 Transformation of Chemically Competent *E. coli* with DNA

Chemically competent TOP10 *E. coli* were used for propagation of plasmids and newly ligated constructs whereas, chemically competent BL21 Rosetta *E. coli* were transformed for protein expression experiments. 50 µl of competent cells were defrosted on ice, 50ng of the relevant plasmid, or 5 µl of a ligation reaction added and incubated on ice for 30 minutes. The cells were then heat shocked at 42 ℃ for 45 seconds and placed back on ice for 2 minutes. 1 ml of Luria Broth (LB) was added to the cells, incubated at 37 ℃ for 1 hour with constant agitation, and plated on LB agar plates with the appropriate antibiotic(s) for selection of successful

transformants. Plates were incubated overnight at 37 ℃, single colonies picked and grown overnight at 37 ℃, shaking at 220 rpm in 5 mls LB containing the appropriate antibiotic(s) for selection. DNA was subsequently extracted and purified from TOP10 cells using a GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) as directed by manufacturer, DNA eluted in 100 µl H_2O.

2.1.8 DNA Sequencing

To confirm the correct clones had been isolated, analytical restriction digests and separation by agarose gel electrophoresis was initially used. To ensure that the sequence and orientation of inserts were indeed correct, subsequent DNA sequencing was performed by GATC Biotech using the relevant upstream and downstream primers to sequences contained within the vector of interest.

2.1.9 pcDNA3 and pcDNA5 Vector Modifications

In order to generate a pcDNA5/FRT/TO vector (Invitrogen) containing 2xFLAG – PreScission Protease Site – 6xHis Tag sequence, a previously modified pcDNA3(-) vector (Invitrogen) containing 2xFLAG tags (kindly provided by Ze'ev Ronai, Sanford-Burnham Institute for Medical Research, USA) was used (figure 2.1). The pcDNA3(-) was digested at the 3' end of the 2xFLAG sequence with *Bam* HI to allow insert of the subsequent tag cassette sequence. The vector was subsequently alkaline phosphatase treated to prevent re-circularisation. This was performed using 10 pmol of vector, 1x Calf Intestinal Alkaline Phosphatase (CIAP) Buffer (Promega) in a total volume of 50 µl. The reaction was incubated at 37 ℃ for 30 minutes, 0.01 Units/µl of CIAP (Promega) was then added and incubated at 37 ℃ for a further 30 minutes. This was separated on a 0.8% agarose gel and recovered as previously described.

Primer set "PreScission Protease - 6xHIS" (Table 2.1) were 5' phosphorylated by T4 kinase treatment to facilitate efficient ligation and limit self-ligation / multimerisation of the tag cassette. This was performed using 1x T4 Polynucleotide

Kinase (PNK) Buffer (Promega), 10 Units of T4 PNK (Promega), 1 mM ATP, 5 pmol of forward primer and 5 pmol of reverse primer in a total volume of 10 µl. The reaction was incubated at 37 ℃ for 1 hour, made up to 50 µl with H_2O, incubated at 95 ℃ for 3 minutes and allowed to cool. Approxi mately 0.4 pmoles of insert was then ligated with the previously *Bam* HI digested pcDNA3(-) 2xFLAG vector, and was used to transform chemically competent TOP10 *E. coli* cells as previously described. Clones were analysed by 5'*Hind* III / 3'*Bam* HI (Promega) restriction digestion and were separated upon a 2% agarose gel by electrophoresis. Positive clones were subsequently analysed by DNA sequencing to ensure correct orientation, sequence and to verify that only one repeat of the cassette was present.

Table 2.1 Primers and Restriction Sites / Overhangs Used to Create the PreScission Protease – 6xHis Tag in pcDNA3(-)

Destination Vector	Gene / Insert	Primer Sequence 5'-3'	Restriction Site
pcDNA3-FLAG	PreScission Protease - 6xHIS Forward	GATCACTGGAAGTTCTGTTCCAGGGG CCCCTGCATCACCACCATCACCATG	5' *Bcl* I - Fused into *Bam* HI site
	PreScission Protease - 6xHIS Reverse	GATCCATGGTGATGGTGGTGATGCAG GGGCCCCTGGAACAGAACTTCCAGT	3' *Bam* HI

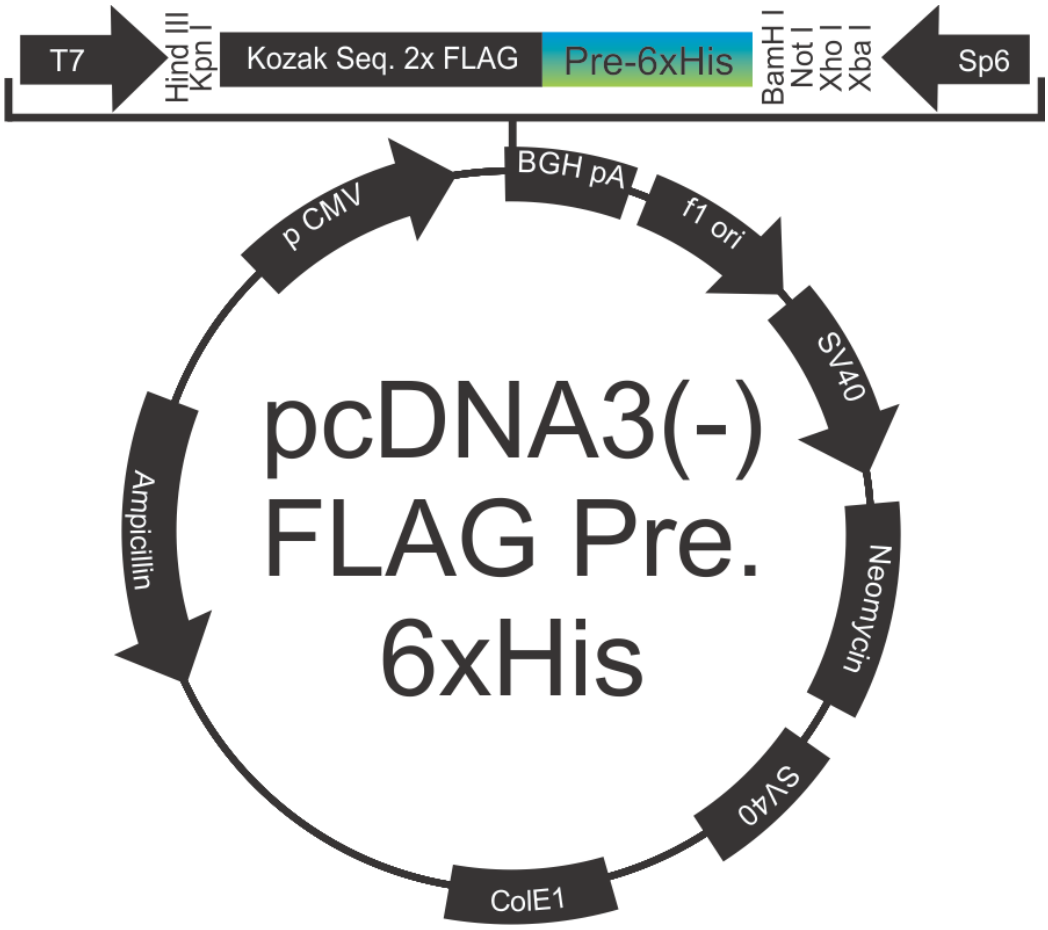


Figure 2.1 pcDNA3 2xFLAG Vector
pcDNA3(-) FLAG vector was linearised and the PreScission Protease 6x poly-histidine sequence (pre-6xHis; blue/green) was inserted to give the pcDNA3(-) FLAG Pre. 6xHis vector. Shown in the vector consecutively are; the CMV promoter; T7 promoter; multiple cloning site; SP6 promoter; BGH polyadenylation signal; SV40 promoter and origin of replication; Neomycin resistance gene; SV40 polyadenylation signal; ColE1 origin of replication and the Ampicillin resistance gene.

The novel pcDNA3(-) 2xFLAG – PreScission Protease – 6x His vector and pcDNA5/FRT/TO vector were subsequently digested 5' *Hind* III / 3' *Bam* HI (Promega) and separated by 2% and 0.8% agarose gel electrophoresis respectively. The relevant fragments were gel extracted and ligated together and selected on ampicillin (100 µg/ml) LB agar plates. As before, colonies were picked, grown overnight in LB with ampicillin (100 µg/ml) and DNA extracted, analysed by 5' *Hind* III / 3' *Bam* HI (Promega) restriction digestion and agarose gel electrophoresis. Positive colonies were DNA sequence verified to yield the pcDNA5-FLAG vector (Figure 2.2).

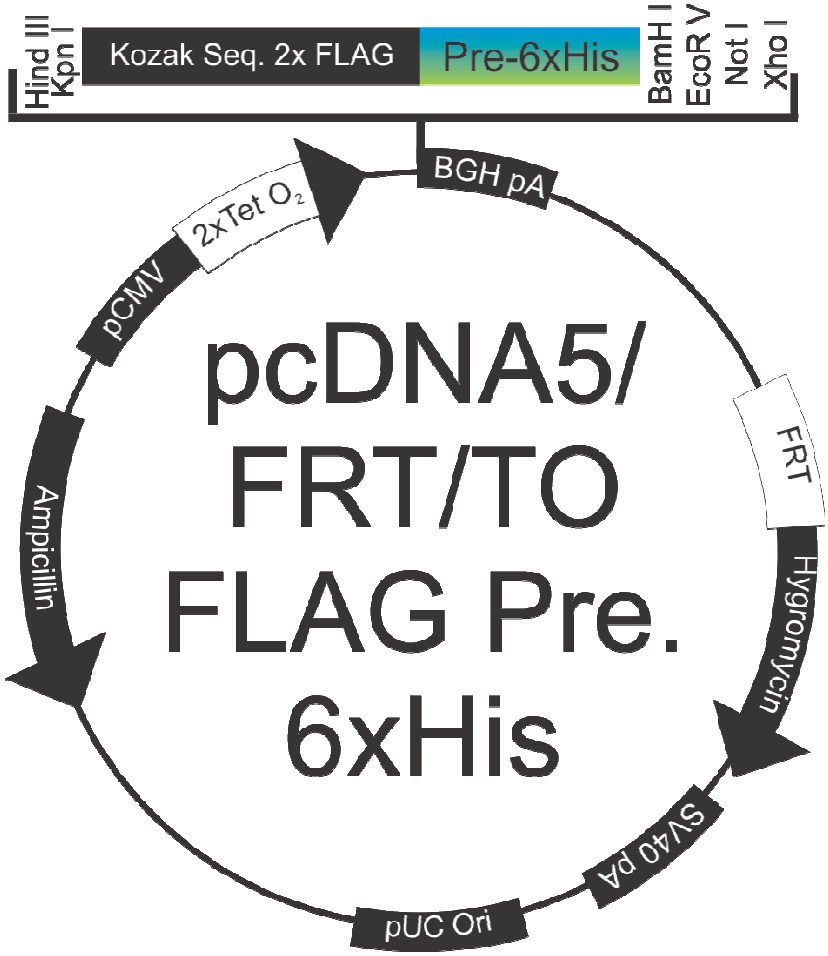


Figure 2.2 pcDNA5-FLAG Vector
The 2xFLAG – Precision Protease – 6x His tag sequence from pcDNA3(-) was inserted into pcDNA5/FRT/TO to create the novel pcDNA5/FRT/TO FLAG Pre. 6xHis vector (pcDNA5 FLAG). Shown in the pcDNA5 vector are consecutively; the CMV promoter; Tetracycline operator sequences; multiple cloning site; BGH reverse priming site and polyadenylation signal; Flp Recombinase Target site (FRT); Hygromycin resistance gene (lacking the start codon); SV40 early polyadenylation signal; pUC origin; and Ampicillin resistance gene.

2.1.10 hU3-55k Constructs

To create a FLAG-tagged hU3-55k (NM_004704) construct suitable for inducible, stable expression from HEK293 cells, hU3-55k DNA was amplified by PCR from hU3-55k pCI-neo, using primer set “Wt. hU3-55k” (Table 2.2). The resulting PCR product was initially cloned into pGEM-T Easy, and then sub-cloned by restriction digest into pcDNA5-FLAG (as in Table 2.2).

To create an hU3-55k construct lacking the initial 136 amino acids (hU3-55k Δ1-136; (Figure 2.3)), as previously described (Granneman et al., 2002), with the addition of a minimal nuclear localisation signal (NLS) of KKKRK (from the large tumour antigen of SV40 (Pap et al., 2001)), primer set “hU3-55k Del136+NLS” (Table 2.2) were used to amplify the desired region from hU3-55k pGEM-T Easy by PCR. The PCR product was initially cloned into pGEM-T Easy, and then sub-cloned by restriction digestion and ligation into pcDNA5-FLAG.

hU3-55k Del193-352 (ΔWD) in pCI-neo (Lukowiak et al., 2000) was used as a template for a PCR using primer set “hU3-55k Del193-352”, the product of which was cloned into pGEM-T Easy, and then sub-cloned by restriction digest into pcDNA5-FLAG, fusing the *Bcl* I restriction site of the insert into the *Bam* HI site of the vector.

hU3-55k mutants previously found in breast cancers (Sjöblom et al., 2006) Δ8R->G and Δ342A->E were created by QuikChange II Site-Directed Mutagenesis (Stratagene) using the Wt. hU3-55k pcDNA5-FLAG vector as template and primer set “hU3-55k Δ8R->G” and “hU3-55k Δ342A->E” respectively. Similarly, a non-phosphorylatable mutant, and a mutant mimicking constitutive phosphorylation at conserved N-terminal serines were created by QuikChange II Site-Directed Mutagenesis of the Wt. hU3-55k pcDNA5-FLAG vector using primers “hU3-55k –P” and “hU3-55k +P” respectively (Table 2.2 and section 2.1.12).

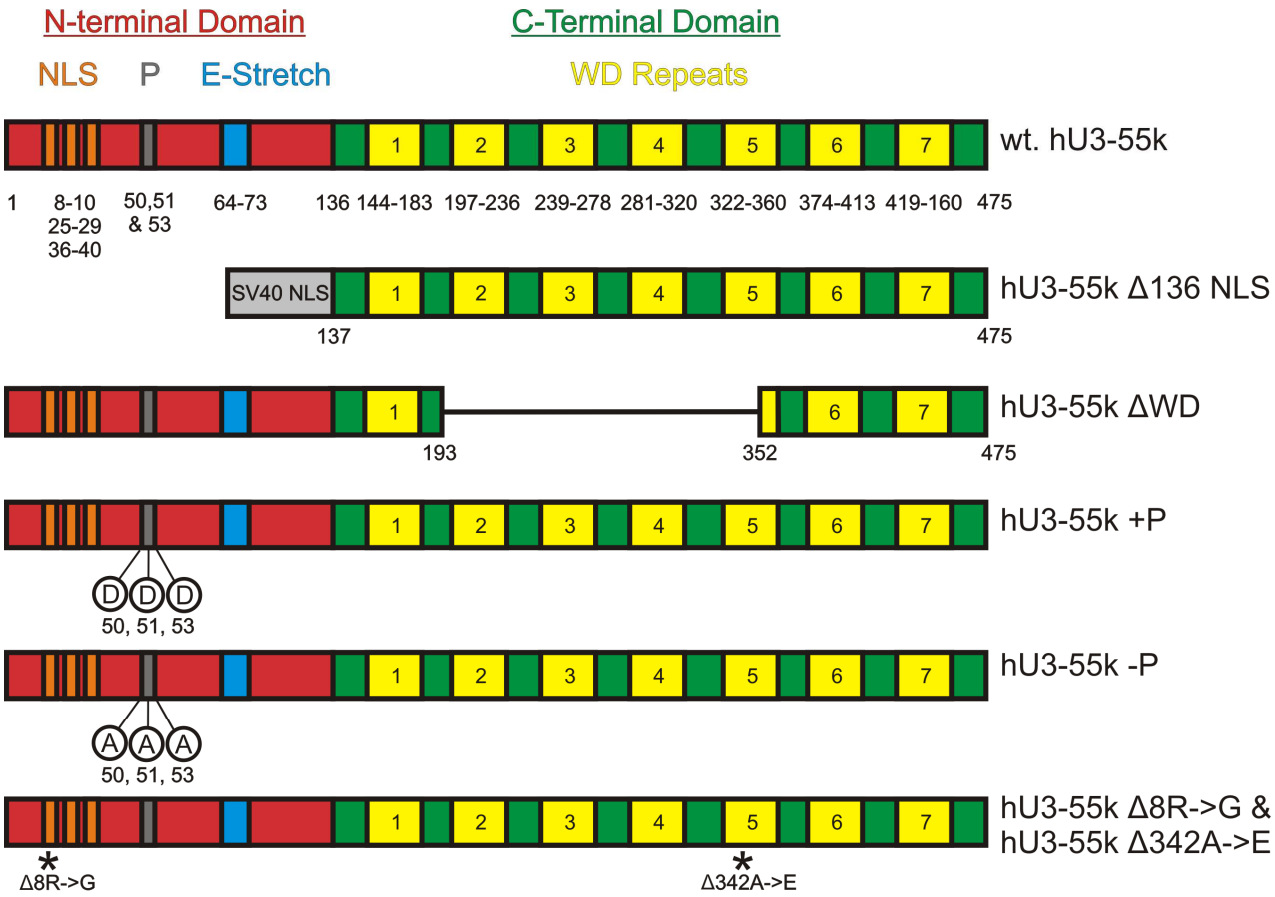


Figure 2.3 hU3-55k Mutants

hU3-55k is composed of several conserved domains, described previously (Granneman et al., 2002; Lukowiak et al., 2000; Pluk et al., 1998; Rouquette et al., 2005). Namely, a Nuclear Localisation Signal (NLS, orange); Phosphoserines (P 50, 51, 53, grey); Glutamic acid rich stretch (E-stretch, blue); Repeats of [GH]-(X23–41)-[WD] belonging to the WD repeat protein family (WD Repeats, yellow). hU3-55k mutants are as indicated; Δ136 NLS (lacking the initial 136 amino acids) with a minimal nuclear localisation signal (NLS); ΔWD lacking the core WD repeats (residues 193-352); constitutively phosphorylated (+P), or dephosphorylated (-P) mimics (serines 50,51 &53); point mutations at either residue 8 (R->G), or residue 342 (A-E; indicated by asterisks). Residue numbers are indicated under the relevant hU3-55k model, numbers within yellow boxes depict the WD repeat, and letters in circles depict the residue used to alter the original phosphoserine.

Table 2.2 Primers and Restriction Digests Used to Create hU3-55k pcDNA5-FLAG Expression Plasmids

Gene / Primer	Primer Sequence 5'-3'	Restriction Site
Wt. hU3-55k Forward	CACCGGATCCATGTCGGCAACAGCGGCTGCTCG	5' *Bam* HI
Wt. hU3-55k Reverse	GCGCGCGGCCGCTCAGGAACCAGCAGCTGGGGG	3' *Not* I
hU3-55k Δ8R->G Forward	CACCGGATCCATGTCGGCAACAGCGGCTGCTGGTAAGCGGGGAAAGCCG	5' *Bam* HI
hU3-55k Δ8R->G Reverse	GCGCGCGGCCGCTCAGGAACCAGCAGCTGGGGG	3' *Not* I
hU3-55k Δ342A->E Forward	CATGGTGTCCGGCGAGGACGATGGCTCTGTGG	5' *Bam* HI
hU3-55k Δ342A->E Reverse	CCACAGAGCCATCGTCCTCGCCGGACACCATG	3' *Not* I
hU3-55k -P Forward	GGCAAGATGAATGAGGAGATCGCCGCCGACGCCGAGAGCGAGAGCCTAGCTCCA	5' *Bam* HI
hU3-55k -P Reverse	TGGAGCTAGGCTCTCGCTCTCGGCGTCGGCGGCGATCTCCTCATTCATCTTGCC	3' *Not* I
hU3-55k +P Forward	GGCAAGATGAATGAGGAGATCGACGACGACGACGAGAGCGAGAGCCTAGCTCCA	5' *Bam* HI
hU3-55k +P Reverse	TGGAGCTAGGCTCTCGCTCTCGTCGTCGTCGTCGATCTCCTCATTCATCTTGCC	3' *Not* I
hU3-55k Del193-352 Forward	CACCTGATCAATGTCGGCAACAGCGGCTGCTCG	*Bcl* I (Fused into *Bam* HI site)
hU3-55k Del193-352 Reverse	GCGCGCGGCCGCTCAGGAACCAGCAGCTGGGGG	*Not* I
hU3-55k Del136+NLS Forward	CACCGGATCCATGAAGAAGAAGAGAAAGTCAGCTGACATTCGCGTTTTACG	5' *Bam* HI
hU3-55k Del136+NLS Reverse	GCGCGCGGCCGCTCAGGAACCAGCAGCTGGGGG	3' *Not* I

2.1.11 SSU Processome Protein Constructs

cDNAs encoding a series of putative RNA-associating proteins were purchased from imaGenes as I.M.A.G.E. Full Length cDNA clones in either ampicillin (100 µg/ml) or chloramphenicol (12.5 µg/ml) resistant vectors (Table 2.3). The provided stab cultures of *E. coli* were grown in 5 mls of LB media containing the appropriate antibiotic and DNA extracted using a GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) as previously described.

Table 2.3 SSU Processome cDNAs form imaGenes

Gene / Insert	Accession Number	imaGenes #	Vector	Antibiotic Resistance
NOB1	BC064630	IRATp970E0284D	pCMV-SPORT6	Ampicillin
PNO1	BC008304	IRAUp969D0338D	pOTB7	Chloramphenicol
DIM1	BC010874	IRATp970E069D	pCMV-SPORT6	Ampicillin
tUTP4	BC009348	IRAUp969B1160D	pOTB7	Chloramphenicol
UTP6	BC035325	IRATp970D0445D	pCMV-SPORT6	Ampicillin
UTP23	BC022441	IRAUp969D1284D	pDNR-LIB	Chloramphenicol
UTP24	NM_015962	IRAUp969B1286D	pDNR-LIB	Chloramphenicol
ESF2	NM_013375	IRATp970B0274D	pCMV-SPORT6	Ampicillin
KRR1	BC026107	IRATp970B1030D	pBluescriptR	Ampicillin
RCL-1	BC001025	IRAUp969D089D	pOTB7	Chloramphenicol
RRP7	NM_015703	IRATp970G11135D	pCMV-SPORT6	Ampicillin
MRD1	BC006137	IRAUp969C1035D	pOTB7	Chloramphenicol

The appropriate purified vector DNA was then used as a template in PCR reactions with the suitable primer set (Table 2.4) to amplify the genes of interest. These were then cloned into pET100 D/TOPO and sequence verified as previously described, to allow expression from *E. coli* of the resultant N-terminally His-tagged NOB1, PNO1, UTP23, UTP24, ESF2 and KRR1 proteins. RCL-1 was cloned by PCR amplification, restriction digested using 5' *Bam* HI / 3' *Not* I and ligated accordingly into pET28a(-) and selected upon LB kanamycin (50 µg/ml) plates.

UTP24 however, contained a number of mutations when compared to both the NCBI sequence NM_015962, and that of orthologues. The initial 71 bp. were absent from the clone, base 213 was changed from T ->G and base 587 was absent. The initial 71 bp. were restored using sequential PCRs, first using primer set "UTP24 1" and subsequently primer set "UTP24 2", using the PCR product from the first round of PCR as the template for the second. The reverse primer "UTP24 Reverse 1 & 2" contained the additional C nucleotide required to repair the 3' end of the gene. Fortunately, the internal base 213 was a silent mutation, therefore did not result in an amino acid change (remaining a valine) and so was not altered.

Table 2.4 Primers Used to Create pET100 D/TOPO Expression Plasmids for Expression and Purification of His-tagged Proteins

Gene / Primer	Primer Sequence 5'-3'
NOB1 Forward	CACCGGATCCATGGCTCCAGTGGAGCACGTTGTGG
NOB1 Reverse	TCTAGATCACCTTTTCTTCACAAACTTCTTTCTGGAAGCG
PNO1 Forward	CACCGGATCCATGGAATCCGAAATGGAAACG
PNO1 Reverse	TCTAGATCAGAATCGATCTGCTGATCTGCTAGC
UTP23 Forward	CACCGGATCCATGAAGATCACAAGGCAGAAACATGCC
UTP23 Reverse	TCTAGATCATTCTCCTTCTGCATTCTGCTTCTC
UTP24 Forward 1	GCGACCATGAAGCGAATGCTTAGTCTCAGAGATCAGAGGCTTAA AGAAAAGGATAGATTAAAACC
UTP24 Forward 2	CACCAGATCTATGGGGAAGCAAAAGAAAACAAGGAAGTATGCGA CCATGAAGCGAATGCTTAG
UTP24 Reverse 1 & 2	CTCGAGAATCGAGGGGCTCCATAATCATCTGGC
ESF2 Forward	CACCGGATCCATGGAGGCAGAGGAATCGGAGGAGG
ESF2 Reverse	TCTAGATCAGGAGTCCCTGACAAGGGAAGG
KRR1 Forward	CACCGGATCCATGGCGTCTCCCTCGCTGGAG
KRR1 Reverse	TCTAGATTACTTTTTTTTCTTCTTTTTCTTTTCATCTGCC
RCL-1 Forward	CACCGGATCCATGGCGACTCAGGCGCACTCCCTC
RCL-1 Reverse	TCTAGATCACTTGAGGGTCTTGCTAAGG

To create FLAG-tagged constructs suitable for inducible, stable expression from HEK293 cells, the genes corresponding to a range of putative SSU Processome components were cloned into the pcDNA5-FLAG vector. The genes previously cloned into pET100 D/TOPO were unable to be sub-cloned as an alternative 3' restriction site was required, due to the presence of an *Xba* I site within the locus of recombination in the pcDNA5-FLAG plasmid. For this reason, all genes were amplified by PCR from either pET100 D/TOPO or, in the case of additional genes, from their corresponding cDNAs (Table 2.3) and ligated into pGEM-T Easy. These were then sub-cloned by restriction digestion (Table 2.5) and ligated into pcDNA5-FLAG, digested with 5' *Bam* HI / 3' *Xho* I or *Not* I (as appropriate) to yield N-terminally tagged fusion constructs.

Table 2.5 Primers and Restriction Digests Used to Create pcDNA5-FLAG Expression Plasmids Containing cDNAs of SSU Processome Proteins

Gene / Primer	Primer Sequence 5'-3'	Restriction Site
NOB1 Forward	CACCGGATCCATGGCTCCAGTGGAGCACGTTGTGG	5' *Bam* HI
NOB1 Reverse	CGCGCTCGAGTCACCTTTTCTTCACAAACTTCTTTCTGGAAGC	3' *Xho* I
PNO1 Forward	CACCGGATCCATGGAATCCGAAATGGAAACG	5' *Bam* HI
PNO1 Reverse	CGCGCTCGAGTCAGAATCGATCTGCTGATCTGCTAGC	3' *Xho* I
DIM1 Forward	CACCGGATCCATGCCGAAGGTCAAGTCGGGGGC	5' *Bam* HI
DIM1 Reverse	CGCGCTCGAGCTAGGAAAAATGAATACCTTCTGC	3' *Xho* I
tUTP4 Forward	CACCAGATCTATGGGTGAATTTAAGGTCCATCG	5' *Bgl* II
tUTP4 Reverse	CGCGCTCGAGTTAGGTTCCAAATTTCTTCTT	3' *Xho* I
UTP6 Forward	CACCTGATCAATGGCAGAGATAATTCAGGAACG	5' *Bgl* II
UTP6 Reverse	CGCGCTCGAGTCATAAATGGCCAGTCTGATGC	3' *Xho* I
UTP23 Forward	CACCGGATCCATGAAGATCACAAGGCAGAAACATGCC	5' *Bam* HI
UTP23 Reverse	CGCGCTCGAGTCATTCTCCTTCTGCATTCTGCTTCTC	3' *Xho* I
UTP24 Forward	CACCAGATCTATGGGGAAGCAAAAGAAAACAAGGAAGTATGCGACCATGAAGCGAATGCTTAG	5' *Bgl* II
UTP24 Reverse	CGCGCTCGAGTTAGAATCGAGGGGCTCCATAATCATCTGG	3' *Xho* I
ESF2 Forward	CACCGGATCCATGGAGGCAGAGGAATCGGAGGAGG	5' *Bam* HI
ESF2 Reverse	CGCGCTCGAGTCAGGAGTCCCTGACAAGGGAAGG	3' *Xho* I
KRR1 Forward	CACCGGATCCATGGCGTCTCCCTCGCTGGAG	5' *Bam* HI
KRR1 Reverse	CGCGCTCGAGTTACTTTTTTTTCTTCTTTTTCTTTTCATCTGCC	3' *Xho* I
RCL-1 Forward	GCGCGGATCCATGGCGACTCAGGCGCACTCCC	5' *Bam* HI
RCL-1 Reverse	GCGCGCGGCCGCTCACTTGAGGGTCTTGCTAAGG	3' *Not* I
RRP7 Forward	CACCGGATCCATGGTGGCGCGCAGGAGGAAGTGCGC	5' *Bam* HI
RRP7 Reverse	CGCGCTCGAGTCAGTACGGTCGGAATTTGCGC	3' *Xho* I
MRD1 Forward	CACCGGATCCATGTCGCGACTGATCGTGAAGAATCTCC	5' *Bam* HI
MRD1 Reverse	CGCGCTCGAGTCACAGCTGAAGGGTCTGCTCCTCGC	3' *Xho* I

2.1.12 Site-Directed Mutagenesis

The D Box of StreptoTagged U3 snoRNA was modified to a mutant sequence, similar to that previously examined in the U4 snRNA (Nottrott et al., 1999) and U14 snoRNA (Watkins et al., 2002; Watkins et al., 2000), mutating the site from UCU GAG to UCU CCG (Figure 2.4). This was done using a StreptoTagged (msl2) wt. U3 snoRNA in pGEM-T Easy (donated by Hannah Richardson, originally cloned by Sander Granneman (Granneman et al., 2004)) as a template for PCR. The StreptoTagged U3 snoRNA construct contained the entire U3 snoRNA sequence under its endogenous promoter to enable expression from the plasmid in human cell lines. It also contained a streptomycin binding aptamer sequence (StreptoTag (Bachler et al., 1999)) between nucleotides U135 and C138 of the U3 snoRNA coding sequence (Figure 2.4), as previously described (Granneman et al., 2004). However, as the D Box had previously been demonstrated to be required for snoRNA accumulation in HeLa cells (Granneman et al., 2004), we only wanted to use this construct for *in vitro* snoRNA assembly assays. For *in vitro* assays, only transcription from upstream of the C' Box to the D Box was required. Therefore, we were able to create a Box D mutant using a standard PCR and cloning approach. The primer set "U3 Box D Mut with 5' T7" (Table 2.6) was used in a PCR to amplify the U3 snoRNA, mutate the site of interest and introduce a T7 promoter sequence. The subsequent product, ranging from the C' Box to the D Box with a 5' T7 promoter sequence, was then cloned into pGEM-T Easy as previously described.

Table 2.6 Primers Used to Generate U3 snoRNA Mutants

Template	Gene / Primer	Primer Sequence 5'-3'
StreptoTagged U3 pGEM-T Easy	U3 Box D Mut with 5' T7 Forward	GCTAATACGACTCACTATAGGGAGAGTGTAGA GCACCGAAAACCACGA
	U3 Box D Mut with 5' T7 Reverse	ACCACGGAGACCGCGTTCTC
StreptoTagged U3 pGEM-T Easy	U3 MutC Forward	CCTGCAACTGCCGTCAGCCATACTGCTAGCTT CTTCTCTCCGTATTGGGGAG
	U3 MutC Reverse	CTCCCCAATACGGAGAGAAGAAGCTAGCAGT ATGGCTGACGGCAGTTGCAGG
StreptoTagged C'Con U3 pGEM-T Easy	U3 MutC Forward	CCTGCAACTGCCGTCAGCCATACTGCTAGCTT CTTCTCTCCGTATTGGGGAG
	U3 MutC Reverse	CTCCCCAATACGGAGAGAAGAAGCTAGCAGT ATGGCTGACGGCAGTTGCAGG

To introduce a mutant C box (previously described (Granneman et al., 2004)) into both the wt. U3 snoRNA and the C'Consensus U3 snoRNA pGEM-T Easy constructs (donated by Hannah Richardson, Figure 2.4), a QuikChange II Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer's protocol, using primers "U3 MutC" (Table 2.6). This enabled use of the constructs for *in vivo* expression analysis as they maintained the entire U3 snoRNA sequence and endogenous promoter. Steps to enable *in vitro* analysis of the constructs are described in section 2.5.6.

Similarly, hU3-55k mutants were generated in the pcDNA5-FLAG vector using the relevant primers (described in section 2.1.10 and Table 2.2) and QuikChange II Site-Directed Mutagenesis. Briefly, three 50 µl reactions were set up with either 5 ng, 20 ng or 50 ng of plasmid template to be mutated, with 125 ng of each relevant primer, 0.4 mM dNTPs, 1x reaction buffer and 1.25 Units of *PfuUltra* HF DNA Polymerase (Stratagene). The reactions were heated in a PCR block for 30 seconds at 95 ℃, then cycled 18 times between 95 ° C for 30 seconds, 55 ℃ for 1 minute and 68 ℃ for 7.5 minutes. Reactions were th en cooled on ice, 10 Units of *Dpn* I were added (to cleave methylated, template DNA) and incubated for 2 hours at 37 ℃. 1 µl of each reaction was then used to tr ansform 50µl of XL1-Blue super competent cells. Subsequently, colonies were picked, DNA extracted and sequence verified.

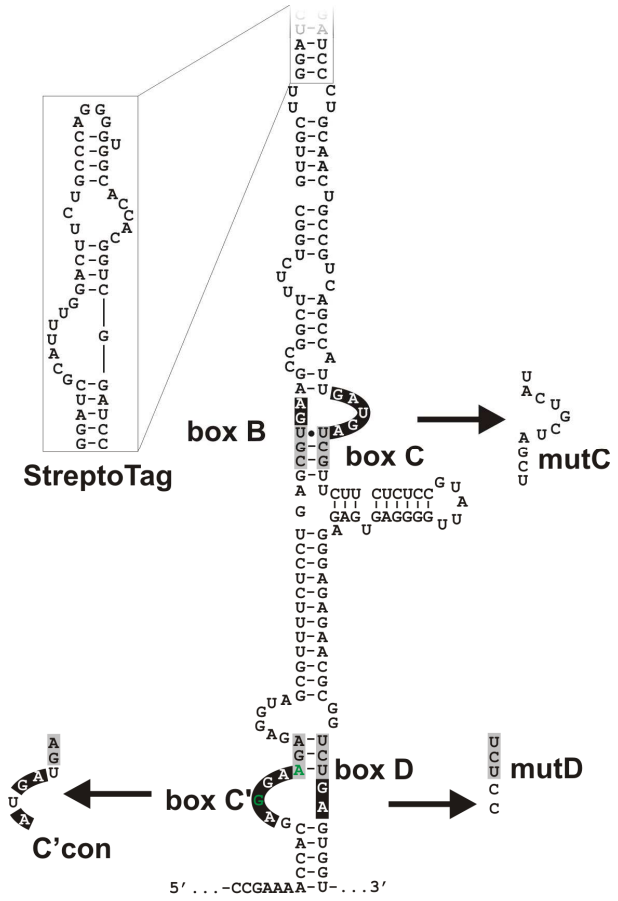


Figure 2.4 U3 snoRNA Mutants

The sequence of the human U3 snoRNA from box C' to box D is shown. Conserved box stems are shown with a grey backdrop, and loops with a black backdrop. In humans the consensus UGAUGA of box C' is changed to GGAAGA with the non-conserved nucleotides shown in green. Mutations are indicated, with the StreptoTag sequence also shown.

2.2 Recombinant Protein Expression & Purification

2.2.1 His-tagged Protein Expression and Purification

pET100 D/TOPO vectors containing either NOB1, PNO1, ESF2, KRR1, UTP23 or UTP24, or RCL-1 pET28a(-), all with 5' proximal 6x His-tags, were used to transform BL21 Rosetta *E. coli* (section 2.1.7) which were selected on LB agar plates containing Ampicillin (100 µg/ml, pET100 D/TOPO) or Kanamycin (50 µg/ml, pET28a(-)). Single colonies were picked and used to inoculate 10 mls LB media containing the relevant antibiotic. The following day, this was used to inoculate 1 litre of LB media (also containing the relevant antibiotic) which was incubated at 37 ℃, shaking at 225 rpm until an optical density (OD) of 0.4 was reached, as measured using 1 ml a cuvette in a spectrophotometer set at A_{600} nm. To induce expression of the proteins, cultures were cooled to 4℃ before IPTG (isopropyl-beta-D-thiogalactopyranoside) was added to a final concentration of 1 mM and

cultures incubated at 18 ℃, shaking at 225rpm over night. Cells were pelleted in a Beckman swing bucket centrifuge at 4200 rpm for 30 min., 4 ℃ and resuspended in 50 mls of protein buffer (20 mM Tris-HCL [pH 8], 5 mM $MgCl_2$, 300 mM KCl, 0.1% Tween-20 (v/v), 10% glycerol (v/v), 0.5 mM THP (tris(hydroxypropyl)phosphine)) on ice. For each of the proteins in pET100 D/TOPO, 8 litres of cells were grown overnight, pelleted and resuspended together in a total volume of 50 mls of protein buffer. To this, 1 Complete Protease Inhibitor Cocktail Tablet (Roche Applied Science) was added along with Ribonuclease A (20 µg/ml, 70 units/mg, Sigma-Aldrich, R6513-50MG), Benzonase Nuclease (5 µl, 1250 Units, Novagen, 71205-3) and lysozyme (200 µg/ml, 40,000 Units/mg, Sigma-Aldrich, L6876). The cell suspension was sonicated on ice, for 2 min., at 0.5 cycle pulsing and 70% power. The debris was pelleted by centrifugation at 20,000 rpm in a Beckman JA-20 rotor, for 30 min., at 4 ℃. Tagged proteins were purified by immobilised metal (Ni^{2+}) affinity chromatography (IMAC) using 1 ml HiTrap Ni Sepharose columns (GE Healthcare) and the ÄKTA purification system (GE Healthcare). Firstly, protein was loaded onto the Ni^{2+} beads by passing the lysate through the HiTrap Column, this was then attached to the ÄKTA system and washed with 12 mls protein buffer with 50mM imidazole, then with 5 mls protein buffer with 75mM imidazole. Elution was performed by passing 10 mls of protein buffer with 500 mM imidazole over the column, the eluate collected in 1 ml fractions (at approx. 1 mg/ml), and then snap-frozen in liquid nitrogen. 20 µl of each fraction was mixed with 20 µl of 2x protein sample buffer (75 mM Tris-HCl [pH 6.8], 1.25 mM EDTA, 20 % glycerol (v/v), 2.5 % SDS (w/v), 0.125 mM bromophenol blue), supplemented with 200 mM DTT), denatured at 95 ℃ for 5 minutes, analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). Coomassie staining was used to assess which fractions contained the protein of interest (section 2.2.7).

2.2.2 Thioredoxin-tagged Protein Expression and Purification

N-terminally thioredoxin-tagged, C-terminally His-tagged constructs pBAD/Thio-TOPO NOP56, NOP58 and thioredoxin alone were kindly provided by Kenny

McKeegan (University of Newcastle), described previously (McKeegan et al., 2007). NOP56 (residues 1-458) and NOP58 (residues 1-435) lacked the C-termini; this was required for efficient expression. These domains are thought not to be required for Box C/D snoRNP formation in *S. cerevisiae* (Gautier et al., 1997; Lafontaine and Tollervey, 2000). Recombinant proteins were expressed from the pBAD/Thio constructs when at 0.4 OD A_{600} nm with 0.02% arabinose, pelleted, sonicated and then purified using HiTrap Ni Sepharose columns as described for His-tagged proteins.

2.2.3 Glutathione S-transferase (GST) tagged Protein Expression and Purification

pGEX-6P-1 constructs containing GST-tagged Fibrillarin ΔRGG (containing residues 82-321), TIP48, TIP49, NOP17, BCD1 (residues 1–360) and GST alone were provided by Kenny McKeegan (University of Newcastle), as previously described (McKeegan et al., 2007). pGEX-4T-2 plasmids encoding GST-tagged 15.5K were provided by Reinhard Lührmann (Schultz et al., 2006). pGEX-2T NUFIP was provided by P. Cabart (Sir William Dunn School of Pathology, University of Oxford) as previously described (Cabart et al., 2004). Recombinant proteins were expressed from the pGEX constructs when at 0.4 OD A_{600} nm with 1mM IPTG, pelleted and sonicated, as described for His-tagged proteins.

For each litre of culture, 100 µl of glutathione sepharose beads (GE Healthcare) were washed 3x in 1 ml of protein buffer and pelleted at 3,000 rpm for 30 seconds in a microfuge. The beads were then resuspended in 1 ml protein buffer and added to cell lysate supernatant, then mixed on a rotary incubator (8 rpm) at 4 ℃ for 2 hours. The beads were then pelleted at 3,000 rpm, 4 ℃ for 30 seconds and washed 3x with 50 mls protein buffer. GST-tagged proteins were eluted from the beads by resuspending the pelleted beads in 3 mls of protein buffer supplemented with 50 mM reduced glutathione (pH 8) and again agitated at 4 ℃, on a rotary incubator for 1 hour. The beads were pelleted at 3,000 rpm, 3 mins, 4℃ and the supernatant retained. The reduced glutathione was removed from the sample by

passing proteins over a HiTrap Desalting column (GE Healthcare) as described below and snap frozen.

2.2.4 Ion Exchange

After initial purifications, proteins were further purified and concentrated by ion exchange. PNO1 and RCL-1 did not require ion exchange. However, NOB1, ESF2, KRR-1, UTP23 and UTP24 were required to be further purified and concentrated. To do this, the theoretical pI for each protein was calculated using the ExPASy Proteomics Server “Compute pI/Mw Tool” so the correctly charged column (Cation Exchange Resource S; KRR1, UTP23 or Anion Exchange Resource Q; NOB1, ESF2, UTP24 (GE Healthcare)) could be used.

Samples from the relevant fractions (as described in section 2.2.1) were pooled and diluted 1:1 with protein buffer containing no KCl or imidazole, pH 8 for Resource Q samples, or pH 6 for Resource S samples. The relevant column was primed with the appropriate buffer. Samples were loaded onto the columns and washed with 5 mls of protein buffer of the appropriate pH, then eluted over a 10 ml gradient of protein buffer increasing from 50 mM to 500 mM KCl with a 5 ml final elution in protein buffer with 500 mM KCl.

1ml fractions were collected and 20 µl of each fraction was mixed with 20 µl of 2x protein sample buffer, denatured at 95 ℃ for 5 min utes, analysed by SDS PAGE and Coomassie stained to assess which fractions contained the protein of interest.

2.2.5 Desalting of Proteins

So as not to allow salts, in particular imidazole, to interfere with downstream reactions, proteins were desalted using HiTrap Desalting columns (GE Healthcare). Columns were equilibrated with 5 ml of protein buffer (pH8) and then 2 mls of sample injected onto 2 HiTrap columns attached in series. Samples were eluted over 10 mls of protein buffer, with salts such as imidazole coming off after

the protein of interest. 1 ml fractions were collected and 20 µl of each fraction was mixed with 20 µl of 2x protein sample buffer, denatured at 95 ℃ for 5 minutes, analysed by SDS PAGE and Coomassie stained. Proteins were verified by anti-His Western blots (section 2.5.2).

2.2.6 Bradford Assay

The concentration of purified proteins was determined by Bradford assay, dependent on an absorbance shift from red to blue when the Coomassie dye binds to protein in the sample as measured by absorbance at 600 nm in a spectrophotometer. A standard curve was configured using known concentrations of BSA in protein buffer ranging from 5 µg/ml to 1 mg/ml, in a total volume of 1ml with 500 µl of Bradford assay (BIO-RAD) in 1 ml cuvettes, OD measured at 600 nm. 10 µl of each protein sample was then measured in a similar way and the concentration derived from the standard curve using the slope intercept line equation “y = mx + c” whereby “m” is the slope and “c” gives the slope intercept.

2.2.7 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) and Coomassie Blue Staining

Protein samples were mixed with 1x protein loading buffer (75 mM Tris-HCl [pH 6.8], 1.25 mM EDTA, 2% glycerol (v/v), 2.5% SDS (w/v), 0.125 mM bromophenol blue), supplemented with 200 mM DTT and denatured at 95 ℃ for 5 min. Samples were loaded upon gels along side 10 µl of pre-stained protein ladder (SeeBlue Plus 2, Invitrogen) to assess the size of the proteins. SDS polyacrylamide gels consisted of a 4% acrylamide stacking gel (4% acrylamide (37.5:1 acrylamide: bisacrylamide), 125 mM Tris-HCl [pH 6. 8], 0.1% SDS (w/v)) and a 12% acrylamide resolving gel (12% acrylamide (37.5:1 acrylamide: bisacrylamide), 375 mM Tris-HCl [pH 8.8], 0.1 % SDS (w/v)). Proteins were resolved on the denaturing gels at 200 volts in 1x running buffer (25 mM Tris [pH 8.3] / 250 mM glycine, 0.1 % SDS (w/v)).

Proteins separated on gels which were not to be transferred by Western blotting, were visualised by Coomassie blue staining. This was performed by incubation in staining solution (0.1 % Coomassie blue (w/v), 40 % methanol (v/v), 10 % acetic acid (v/v)) with agitation. Subsequently, gels were destained several times in 40 % methanol (v/v), 10 % acetic acid (v/v) until the proteins were clearly visible.

2.2.8 *In vitro* Protein Translation

mRNA transcriptions and translations were set up using a ProteinScript II Kit (Ambion) according to manufacturer's instructions. Briefly, 1 x Transcription Mix, 0.5 µg of template DNA (hU3-55k in pCI-neo) and 2 µl of Enzyme Mix (containing T7 RNA Polymerase, as supplied by Ambion), were made up to 10 µl with H_2O and incubated at 30 ℃ for 1 hour to produce mRNA for t ranslation.

Subsequently, a 50 µl reaction was set up containing 1x Translation Mix, 4 µl ^{35}S-Methionine (final concentration 0.8 nM, 1.5 MBq, PerkinElmer), 35 µl Rabbit Reticulocyte Lysate and 2 µl of the aforementioned transcription reaction, this was then incubated at 30 ℃ for 60 minutes, snap-frozen in liquid nitrogen and stored at -80 ℃. Protein samples were verified by separation upon a 12% SDS polyacrylamide gel which was fixed and exposed to a PhosphorImager screen. Labelled protein was detected using a Typhoon imager and ImageQuant Software analysis (GE HealthCare).

2.3 RNA, DNA and Protein Handling

2.3.1 RNA Extraction and Ethanol Precipitation

200 µl, 250 µl and 400 µl of RNA extraction buffer (1% SDS (w/v), 50 mM Tris [pH 7.5], 50 mM NaCl, 0.5 mM EDTA) was added to glycerol gradient fractions, immunoprecipitation reactions and cell pellets respectively. An equivalent volume of phenol: chloroform: isoamyl alcohol (25:24:1) [pH 8.0] was added, samples vortexed at full speed for 30 sec. and then centrifuged in a benchtop microfuge for

3 minutes at 13,000 rpm. The upper aqueous phase (containing RNA) was removed and RNA precipitated by the addition of 1/20 (v/v) of 3M NaOAc, 1 µl tRNA (10 mg / ml, not required for whole cell lysates) and 1ml of 100% ethanol. The samples were vortexed, stored at –80 ℃ for 30 minutes, or -20 ℃ overnight, centrifuged in a microfuge for 15 minutes at 13,000 rpm, 4 ℃. The supernatant was removed and any remaining liquid evaporated using a speed vac.

Pellets were resuspended in 10 µl H_2O and their concentration measured using a Nanodrop (Thermo Scientific, for RNA from whole cells). The appropriate amount of RNA (usually 4 µg of whole cell RNA, or 2.5µl from other reactions) was then mixed 1:1 (v/v) with RNA loading buffer (40% formamide, 0.5 mM EDTA, 50 µg/ml bromophenol blue, 50 µg/ml xylene cyanol). Samples were denatured at 95 ℃ for 2 minutes and cooled on ice, before separation by electrophoresis.

2.3.2 Protein Precipitation with Acetone

To extract the proteins from the phenol phase (from section 2.3.1), 1 ml of acetone was added to the samples and incubated at -20 ℃ overnight, then centrifuged at 13,000 rpm in a benchtop microfuge (4 ℃) for 15 minutes to produce a pellet. This was washed in 70% ethanol, centrifuged at 13,000 rpm (4 ℃) for 5 minutes, the ethanol removed and the pellet dried using a speed vac. Pellets were resuspended in 50 µl 1x protein sample buffer (75 mM Tris-HCl [pH 6.8], 1.25 mM EDTA, 20 % glycerol (v/v), 2.5 % SDS (w/v), 0.125 mM bromophenol blue), supplemented with 200 mM DTT), denatured at 95 ℃ for 5 minutes and analysed by SDS PAGE.

2.3.3 RNA Analysis by Gel Electrophoresis and Northern Blotting

RNA extracted (as previously described) from glycerol gradient fractions, immunoprecipitation reactions and cell pellets, was mixed 1:1 (v/v) with RNA loading buffer (as in 2.3.1), separated upon an 8% acrylamide gel containing 7 M urea and run in 1 x TBE buffer at 400 volts until the bromophenol blue reached the bottom of the gel. RNA was transferred onto Hybond N membrane (Amersham

Biosciences) using a Trans-Blot ® Cell (BIO-RAD) in 0.5x TBE buffer at 70 volts for 1 ½ hours, then crosslinked to the membrane using a Stratalinker® 2400 UV Crosslinker (Stratagene).

2.3.4 Gel Electrophoresis for Northern Blotting of pre-rRNA

To analyse pre-rRNAs within the cell, RNA was separated upon glyoxal agarose gels. Pellets of 10 million cells were resuspended in 500 µl of RNA extraction buffer as described previously, and incubated at 95℃ for 5 min. 500 µl of Phenol: chloroform: isoamyl alcohol (25:24:1) [pH 6.6/8.0] was added, samples vortexed for 30 sec. and centrifuged in a microfuge for 3 min at 13,000 rpm, 4℃. The aqueous top layer was removed and the phenol: choloroform extraction repeated on the supernatant. Next, 500 µl chloroform: isoamyl alcohol (24:1) was added to the supernatant to remove traces of Phenol and was subsequently centrifuged as before. The supernatant was removed and RNA precipitated by the addition of 20 µl of 3M NaOAc and 1 ml of 100% ethanol. The mixture was vortexed for 30 sec. and incubated at -80 ℃ for 30 min. before being centrifuged for 15 min at 13,000 rpm, 4℃. The supernatant was removed and the pellets dried briefly in a speed vac, resuspended in 10 µl H_2O and their concentration measured as described previously. 10 µg of total RNA (approximately 2 million cells) were mixed with 1:5 RNA: glyoxal buffer (v/v) (61.2% DMSO (v/v), 20.4% glyoxal (v/v), 12.2% 1x BPTE buffer [28.7 mM Bis-Tris, 9.9 mM PIPES, 1 mM EDTA] (v/v), 4.8% glycerol (v/v), and 0.02 mg/ml EtBr). Samples were then incubated at 55℃ for 1 hour.

RNAse Zap® (Ambion) was used to remove RNases from all electrophoresis equipment before use. RNA samples were separated by electrophoresis upon a 1.2% agarose / 1x BPTE gel in 1x BPTE buffer at 45 V for 16 hours. The RNA in the gel was visualised under UV light to ensure sufficient migration had occurred before blotting.

The glyoxal gel was washed in 75 mM NaOH for 20 min, 2x in 0.5 M Tris [pH 7.4], 1.5 M NaCl for 15 min and once in 6x SSC (0.9M sodium chloride, 90mM trisodium

citrate, pH 7) for 20 min. RNA was transferred onto a Hybond N membrane (Amersham Biosciences) by capillary blotting in 6x SSC overnight. RNA was then crosslinked to the membrane using a Stratalinker® 2400 UV Crosslinker (Stratagene).

2.3.5 Northern Blot Hybridisation

Radiolabeled probes to the relevant RNA sequences were produced by either; T7 RNA polymerase driven transcription (polymerase kindly provided by Nikolay Zenkin, University of Newcastle); extension of random hexamer primers using the large fragement of Klenow polymerase (Promega); or polynucleotide kinase labelling of an oligonucleotide (Table 2.7).

Table 2.7 Templates and Enzymes Used to Produce Radiolabeled Probes

Template	Enzyme used to Radiolabel	Reference
U8 pBS+SP6	T7 RNA Polymerase	(Watkins et al., 1996)
U13 pGem9Zf-	T7 RNA Polymerase	(Boulon et al., 2004; Rouquette et al., 2005)
U15a pBS+	T7 RNA Polymerase	(Watkins et al., 1996)
U3 msl2 pGEMTeasy	T7 RNA Polymerase	(Granneman et al., 2004)
U3 pBS+SP6	Klenow Polymerase	(Watkins et al., 1996)
U1 pUC18	Klenow Polymerase	(Watkins et al., 2004)
18S-E Oligonucleotide Sequence	Polynucleotide Kinase	(Rouquette et al., 2005)

Probes to human U8, U13 and U15a snoRNAs, and the StreptoTag sequence (msl2) were transcribed from PCR templates containing a T7 promoter sequence proximal to the RNA of interest (Table 2.8), such that the T7 RNA polymerase transcribed the antisense strand. 50 ng of gel recovered PCR product was mixed with 1x transcription buffer (40mM Tris-HCl [pH7.9], 6mM $MgCl_2$, 10mM NaCl, 10mM DTT, 2mM Spermidine), 1mM ATP, 1 mM GTP, 1mM CTP and 0.1mM UTP, 0.5 µl RNasin (10 units, Promega), 1 µl T7 RNA Polymerase and 4 µl α-^{32}P UTP (1.32 µM, 1.48 MBq, PerkinElmer) in a total volume of 10 µl (made up with H_2O) which was incubated for 2 hours at 37 ℃. 1 µl Turbo DNase (2 units, Ambion) was added to remove template DNA and incubated for 30 min. at 37 ℃. The volume

was made up to 50 µl with H_2O and spun twice through G-50 columns (GE Healthcare) to remove unincorporated nucleotides.

Table 2.8 PCR Primers used to Amplify T7 RNA Polymerase Transcription Templates

Template	Primer	Primer Sequence 5'-3'
U8 pBS+SP6	T7 Forward	TAATACGACTCACTATAGGG
	T3 Reverse	ATTAACCCTCACTAAAGGGA
U13 pGem9Zf-	T7 Forward	TAATACGACTCACTATAGGG
	SP6 Reverse	CATTTAGGTGACACTATAG
U15a pGEMTeasy	T7 Forward	TAATACGACTCACTATAGGG
	SP6 Reverse	CATTTAGGTGACACTATAG
U3 msl2 pGEMTeasy	Strepto Forward	ATCGCATTTGGACTTCTGCCC
	Strepto Reverse	GGCTTAATACGACTCACTATAGGGCAGTTG CAGGGATCCGAC

U3 snoRNA and U1 snRNA probes were made against PCR products (Table 2.9) corresponding to the full length coding sequences by random primed labelling. 50 ng of vector DNA in 9 µl H_2O was denatured by incubation at 95-100℃ for 5 min . and then cooled on ice. To this was added; 3 µl of random hexamer mix (250 mM Tris [pH 7.5], 50 mM $MgCL_2$, 5 mM DTT, 500 µM dATP, 500 µM dGTP, 500 µM dTTP and150 µg/ml random deoxribonucleotides, Amersham); 2 µl of α-^{32}P labelled dCTP (PerkinElmer); and 1 µl (5 units) of Klenow polymerase (Promega) which was then incubated at 37℃ for 30 min. 35 µl H_2O was added to this and the reaction spun through a G-50 column (GE Healthcare) to remove unincorporated nucleotides.

Table 2.9 Primers Used to Produce Templates for Random-Primed Labelling Reactions

Template	Primer	Sequence 5'-3'
pBS+SP6 U3 vector	U3 Forward (T7)	TAATACGACTCACTATAGGG
	U3 Reverse (T3)	ATTAACCCTCACTAAAGGGA
U1 pUC18 vector	U1 Forward	GGGGAAAGCGCGAACGCAG
	U1 Reverse	TACTTACCTGGCAGGGGAG

A probe to the 18S-E 3' termini of 18S pre-rRNA was made by the addition of a radioactive phosphate group to the 5' end of an oligonucleotide. 1x T4 PNK buffer, 10 units T4 PNK (Promega), 200 pmoles of the sequence (5'-CCTCGCCCTCCGGGCTCCGTTAATGATC-3') and 5 µl of γ-^{32}P labelled ATP were incubated in a total volume of 20 µl at 37 ℃ for 1 hour. 35 µl H_2O was added to this and the reaction spun through a G-50 column (GE Healthcare) to remove unincorporated nucleotides, as before.

Hybond N membranes were pre-hybridised in hybridisation buffer (25mM NaH_2PO_4 / Na_2HPO_4 [pH 6.5], 6x SSC (0.9M sodium chloride, 90mM trisodium citrate, pH 7), 5x Denhardts, 0.5% SDS (w/v), 50% deionised formamide, 100 µg/ml denatured salmon sperm DNA) for 2 hours at 42 ℃. Radiolabelled probes were denatured at 95 ℃ for 5 minutes, then added directly to the hybridisation buffer and incubated overnight at 42 ℃. Membranes were washed at 42 ℃; 3x 5 min. in 2x SSC, 0.5 % SDS (w/v); 2x 5 min. in 2x SSC, 0.1% SDS (w/v). Those probed for U3 snoRNA or U1 snRNA were then washed at 50℃ for 30 min in 2x SSC, 0.1 % SDS (w/v). Membranes probed for U8, U13, U15 snoRNAs or StreptoTagged U3, using antisense RNA probes, were washed at 65 ℃ in 1x SSC, 0.1% SDS (w/v) for 1 hour.

Membranes containing pre-rRNA were pre-hybridised in SES-1 buffer (0.25 M NaH_2PO_4 / Na_2HPO_4, 7% SDS (w/v), 1 mM EDTA) at 37 ℃ for 20 min. The 18S-E probe was then denatured at 95 ℃ for 5 minutes, then added directly to the buffer and hybridised overnight at 37 ℃. Membranes were washed 2x 20 min. in 1x SSC 0.1% SDS (w/v).

Excess liquid was removed from the membranes and the signal was recorded by exposure to a PhosphorImager screen. The signal was detected by using a Typhoon imager and ImageQuant Software analysis (GE HealthCare).

2.3.6 RT-PCR and Southern Blot Analysis (U3 transfections)

RT-PCR was used to quantify the amount of StreptoTagged U3 snoRNA pGEM-T Easy vector transfected into HeLa cells. The linear range of amplification was determined by varying the number of cycles and amount of template used, as described previously (McKeegan et al., 2009; Will et al., 2004). 1µl of a 1/200 dilution of each DNA sample was used as template in each RT-PCR reaction. 20 cycles required to give suitable product within the linear range of amplification.

Forward Primer (T7): 5'-TAATACGACTCACTATAGGG-3'
Reverse Primer (SP6): 5'-CATTTAGGTGACACTATAG-3'

PCR products were then separated on a 1% agarose gel in 1x TBE as previously described. This was then incubated for 30 min. in 0.5M NaOH, 1.5M NaCl to denature the DNA, followed by neutralisation in 1.5M NaCl, 0.5M Tris-HCl, 1mM EDTA (pH 7.2) for 30 min. The DNA was then transferred onto a Hybond N membrane (Amersham Biosciences) by capillary blotting in 10x SSC. DNA was crosslinked to the membrane using a Stratalinker® 2400 UV Crosslinker (Stratagene) and then probed using a U3 snoRNA specific probe, made by random primed labelling as described previously.

2.3.7 S1 Nuclease Protection Assay for pre-rRNA

As in section 2.3.5, an oligonucleotide probe (below) was kinase labelled at the 5' terminus with 5 µl of γ-^{32}P labelled ATP.

Probe:
5'-GCGAGGGCCCCCCGAGAAGCTAGCTACACCACTGCAGCACGAGAGGGCC
CGGCCCAGGCT-3'

The probe was complimentary to bases 401-460 of the pre-rRNA, overlapping the A' cleavage sites in the 5'ETS (Figure 2.5), as previously published (Prieto and McStay, 2007).

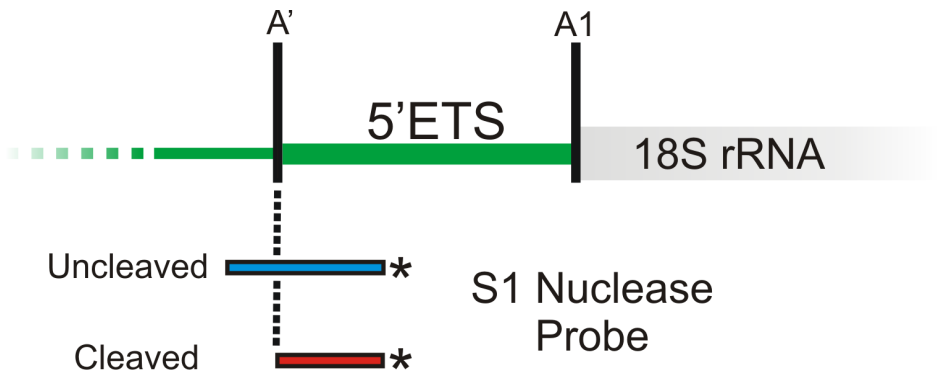


Figure 2.5 Schematic Representation of the 5' Region of Human pre-rRNA, Showing the Location of the S1 Probe Relative to the Primary Processing Site (A')
The 5' ETS is shown in green, 18S pre-rRNA is shown in grey, cleavage sites are shown as vertical black lines (A' and A1). The location of the S1 Nuclease Protection Probe is show as uncleaved in blue, cleaved in red, with the radiolabel indicated by an asterisk. The probe is shown spanning the primary (A') cleavage site.

A 30 µl reaction was set up in H_2O with 2.5 µl / 10 µl of sample RNA (isolated from immunoprecipitation experiments, as described in 2.3.1), 3 µl of 10x S1 hybridisation buffer (3M NaCl, 100 mM Tris [pH 7.9], 10 mM EDTA) and 1ul of radiolabeled probe. The reaction was incubated at 65 ℃ for 3 hours to allow the probe to anneal, then placed on ice and 270 µl of ice cold S1 nuclease buffer (1 mM $ZnS0_4$, 20 mM NaOAc [pH 5.4], 50 mM NaCl) with 50 units of S1 nuclease (Amersham) was added. Digestion was performed by incubation at 37 ℃ for 30 min. The reaction was subsequently stopped by the addition of 10 µl of 10% SDS (w/v) and 5 µl of 5 M EDTA.

RNA was precipitated by adding 15 µl NaOAc (3M), 1 µl tRNA (10 mg/ml) and 1 ml of 100% ethanol. The samples were treated as before (section 2.3.1), pellets were resuspended in 10 µl of H_2O and 10 µl of RNA loading dye (also described previously). Products were separated by electrophoresis on a 10% acrylamide, 7M urea gel in 1 x TBE buffer at 400 V until the bromophenol blue reached the bottom of the gel. The gel was then fixed in 10% methanol (v/v), 10% acetic acid (v/v) for 30 min. and dried using a gel drier at 70 ℃ for 1 ½ hours. Products were visualised by exposure to a PhosphorImager screen and detected using a Typhoon imager and ImageQuant Software for analysis (GE HealthCare).

2.4 Human Cell Culture

2.4.1 Cell Culture

Human HeLa SS6 (cervical carcinoma) and HEK293 (human embryonic kidney) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4500 mg/l glucose, L-glutamine, and sodium bicarbonate, without sodium pyruvate (Invitrogen) supplemented with 10% (v/v) foetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were incubated in a 5% CO_2 humidified incubator at 37℃ and passaged when at 80% confluency, using 1x trypsin EDTA (Sigma-Aldrich) in sterile phosphate buffer saline (PBS, Sigma-Aldrich).

The strain of HEK293 cells used were "Flp-In™ T-REx™-293" (Invitrogen). Flp-In T-Rex HEK293 cells contain a stably integrated pFRT/*lac*Zeo vector to provide a specific Flp Recombinase site, derived from *S. cerevisiae*. They also contain an integrated pcDNA6/TR vector allowing constitutive expression of the Tet-repressor under the control of the human CMV promoter and blasticidin S resistance (Figure 2.6). These enabled us to generate tetracycline-regulated stable cell lines. To maintain selection, 10 µg/ml of blasticidin was added to these every third passage.

CaCo-2 (human epithelial colorectal adenocarcinoma) and CaLu-3 (human lung epithelial non-small cell adenocarcinoma) cells were kindly provided by Sabine Quitard (Kenny Lab, University of Newcastle) and James Garnett (Gray Lab, University of Newcastle) respectively. The cells provided in an undifferentiated state were cultured as described for HeLa cells. Cells provided in a differentiated state had been plated onto Corning® Transwell® polycarbonate membrane inserts with 0.4 µm pore, 12mm diameter with approximately 250,000 cells per well and cultured for 15 days (CaLu-3) or 21 days (CaCo-2) at 5% CO_2 in a humidified incubator at 37℃. The differentiation of the cells had been verified by measurement of their transepithelial electrical resistance (TEER) as an indication of tight junction formation in the epithelial monolayers (between 300 – 500 Ω / cm^2) using an EVOM Voltohmmeter (Lee et al., 2009; Sun et al., 2008).

Cells were harvested by washing with PBS, incubated with 1x trypsin EDTA at 37 ℃ for either 5 minutes (HeLa and HEK293 cells) or 30 minutes (CaCo-2 and CaLu-3 cells), then resuspended in a suitable volume of media (*i.e.* 10 mls for a 10ml flask, 0.5 mls for a 0.5ml well) and cells pelleted using either a swing bucket centrifuge or benchtop microfuge at 800 rpm for 5 minutes.

HeLa and HEK293 cells were resuspended in PBS, 100 µl of trypsinised cells added to 10 mls of CASY®ton solution and counted in a CASY® cell counter (Innovatis AG) to give the number of cells / ml. The relevant number of cells were then re-pelleted as before and either used in electroporations, or snap-frozen in liquid nitrogen and stored at -80 ℃ for downstream analysis. CaCo-2 and CaLu-3 monolayers could not be counted after differentiation, therefore pellets were snap-frozen in liquid nitrogen and stored at -80 ℃.

2.4.2 Cell Chemical Treatments

Human cells were chemically treated as detailed in Table 2.10 before being harvested or fixed for immunofluorescence, or fluorescence *in situ* hybridisation analysis (FISH).

Table 2.10 Human Cell Chemical Treatments

Chemical	Concentration	Duration	Reference(s)
Actinomycin D (ActD.)	0.1 ug/ul	2 hours	(Turner et al., 2009)
Leptomycin B (LMB)	30 nM	2 hours	(Muro et al., 2008; Rouquette et al., 2005)
Rapamycin (Rap.)	100 nM	4 hours	(Pradelli et al., 2009; Vanrobays et al., 2008)

2.4.3 RNA Interference (RNAi) by Electroporation of HeLa Cells

Specific target proteins in human cells were selectively depleted using siRNA targeting specific mRNA sequences (Table 2.11). As a control, siRNA targeting firefly luciferase mRNA (GL2) was used. This gene is not present in HeLa cells and has previously been demonstrated to have no effect on cell growth or RNA levels in HeLa SS6 cells (Elbashir et al., 2002). NOP58 was targeted using duplexes previously described (Watkins et al., 2004). hU3-55k siRNA duplexes were purchased as a mixed pool, specific to the desired open reading frame (ORF), from Dharmacon. These were previously demonstrated to be specific and effective at reducing hU3-55k protein levels by the McStay lab (Prieto and McStay, 2007).

Table 2.11 siRNA duplexes used to deplete specific mRNAs in human cells

Target / Strand	Accession	siRNA sense sequence	Reference
GL2 (Firefly luciferase) Sense	X65324	5'-CGUACGCGGAAUACUUCGAUU-3'	(Elbashir et al., 2001; Elbashir et al., 2002; Rouquette et al., 2005)
GL2 (Firefly luciferase) Antisense		5'-UCGAAGUAUUCCGCGUACGUU-3'	
NOP58 Sense	NM_015934	5'-CAAGCAUGCAGCUUCUACCGUUCUU-3'	(Watkins et al., 2004)
NOP58 Antisense		5'-GAACGGTAGAAGCTGCATGCTTGUU-3'	
hU3-55k ON-TARGETplus SMARTpool	NM_004704	5'-GCGGCAAGAUGAAUGAGGA-3'	(Prieto and McStay, 2007)
		5'-GAAUCAAAGAGGCUCGGAA-3'	
		5'-GAGCAGGCCCUUCUGGAUAU-3'	
		5'-GGACUGUACGUGUGUGGAA-3'	

The sense and antisense strands were suspended at a concentration of 100 µM in H_2O whereas SMARTPool siRNAs were resuspended at a concentration of 20 µM in 1x siRNA annealing buffer (100 mM mM KOAc, 2 mM MgOAc, 30 mM HEPES-KOH (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) [pH 7.4]). Sense and antisense strands were mixed with one another to a final concentration of 20µM in 1x siRNA annealing buffer. All duplexes were then incubated at 90 ℃ for 1 min and annealed at 37℃ for 1 hour (Elbashir et al., 2 002). Duplexes were then stored at -20 ℃ until use.

2.4.4 Transient Transfection of HeLa Cells by Electroporation

Transfection of siRNA duplexes and StreptoTagged U3 snoRNA constructs into HeLa cells was performed by electroporation using the Amaxa II Electroporator and Nucleofector Kit R, using programme I-013, according to manufacturer's protocols. For each electroporation, 2 million cells were used with either 11 µl of 20 µM siRNA (220 pmoles) or 2 µg plasmid DNA. After electroporation, cells were either transferred to 10 ml flasks, or 200,000 cells / well of a 24-well plate and incubated for 60 hours. Cells were then harvested by trypsinisation, counted and pelleted.

2.4.5 Chemical Transfections and Creation of HEK293 Stable Cell Lines

HEK293 cells containing stably integrated genes of interest under a tetracycline inducible element were created using the "Flp-In™ T-REx™-293" / "pcDNA5/FRT/TO" system from Invitrogen (described in 2.4.1 and Figure 2.6).

Genes of interest were cloned into the modified pcDNA5/FRT/TO vector containing 2x FLAG tags (pcDNA5-FLAG, section 2.1.9), a tetracycline-regulated hybrid human cytomegalovirus (CMV)/TetO2 promoter, and a hygromycin B resistance gene with a FRT site in the 5' coding region (but lacking a promoter). Subsequently, Flp-In T-Rex HEK293 cells were chemically co-transfected with the pcDNA5-FLAG vector, containing the gene of interest, and pOG44, constitutively expressing the Flp recombinase. This facilitated homologous recombination between the FRT sites and thus, integration of the tagged gene of interest and the hygromycin resistance gene, both in frame of their relevant promoters (Figure 2.6). Cells were cultured in DMEM (as described previously) supplemented with 10 µg/ml of blasticidin and 100 µg/ml of hygromycin B to select and maintain the Tet-repressor and pcDNA5-FLAG inserts, respectively.

Chemical transfections were performed using cells grown in 2 ml tissue culture wells (6-well plates) to a confluency of ~60 %. 9 µl/well of FuGene HD (Roche

Applied Science) was mixed with 91 µl of Opti-MEM I (Invitrogen) and incubated at room temperature for 5 minutes. 1.8 µg pOG44 and 0.6 µg pcDNA5 were then added to the reaction, incubated at room temperature for 15 minutes and added back to the cells in a drop wise manner. Cells were incubated in a 5% CO_2 humidified incubator at 37℃ for 2 days before 10 µ g/ml of blasticidin and 100 µg/ml of hygromycin B were added for selection of successful integration.

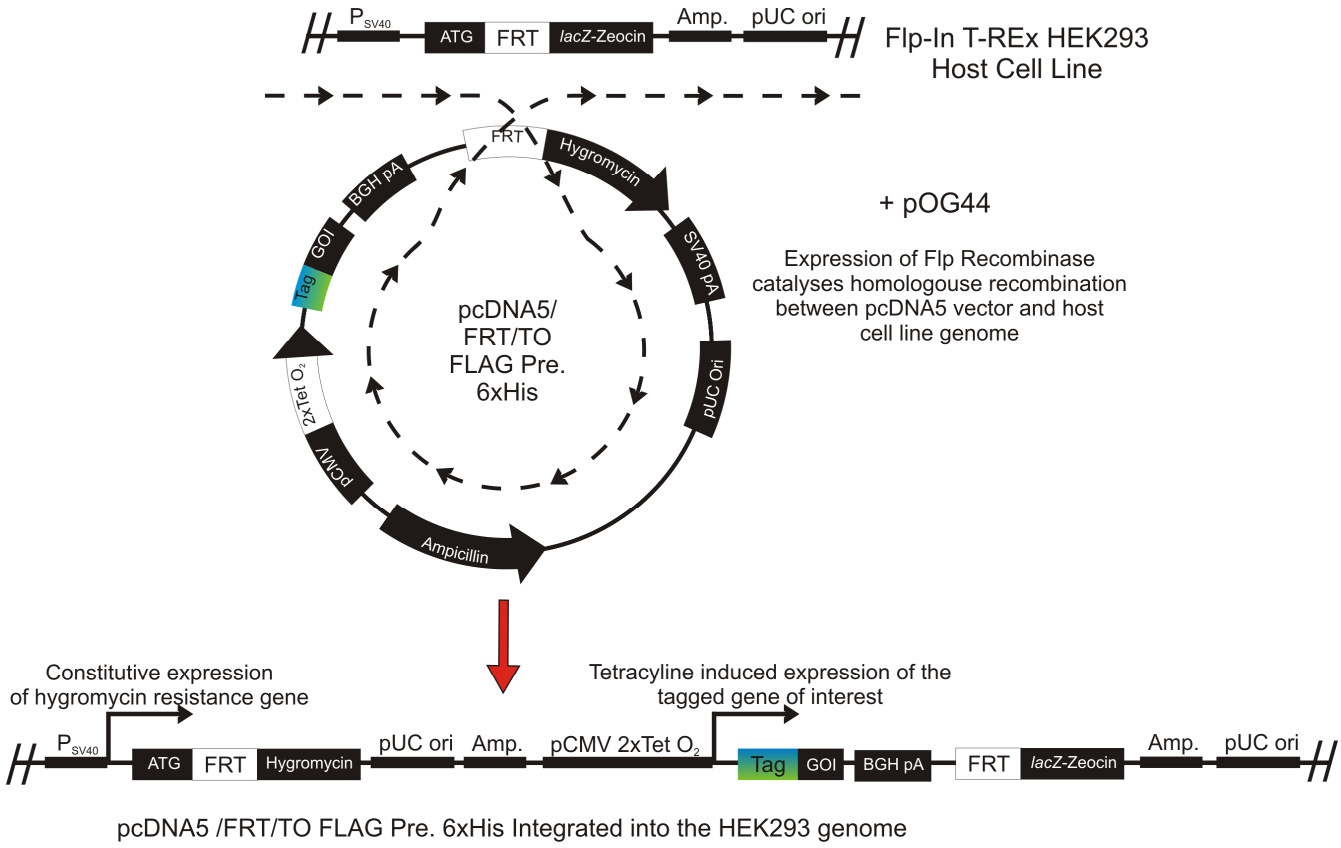


Figure 2.6 Schematic Representation pcDNA5/FRT/TO FLAG Pre. 6xHis Homologous Recombination into Flp-In T-REx HEK293 Host Cell Line
Homologous recombination (dotted line) is shown between the pcDNA5-FLAG vector and the Flp Recombination sites (FRT) in the modified HEK293 cell genome. This is aided by the Flp Recombinase encoded within pOG44. Shown are; SV40 promoter (P_{SV40}); start codon (ATG); Flp Recombination sites (FRT); hygromycin resistance gene (hygromycin); pUC origin (for propagation in *E. coli*); Ampicillin resistance gene (Amp.); CMV promoter (pCMV); Tetracycline operator sequences (2xTet O_2); 2xFLAG Precission Protease 6xHis Tag (Tag); Gene of interest (GOI); BGH polyadenylation signal (BGH pA, required for efficient termination and polyadenylation of mRNA); and *lacZ* fusion with the zeocin resistence gene (*lacZ*-Zeocin). Based on a figure from Invitrogen.

Cells were induced for 2 days with the suitable amount of tetracycline to give 1:1 expression of FLAG-tagged protein: endogenous hU3-55k (Table 2.12), unless otherwise stated.

Table 2.12 Tetracycline Concentrations Used to Induce Stably Transfected HEK293 Cells

Expressed Gene	Final Tet. Concentration
Wt. hU3-55k	0.5 ng/ml
hU3-55k Δ8R->G	0.5 ng/ml
hU3-55k Δ342A->E	1 ug/ml
hU3-55k -P	0.5 ng/ml
hU3-55k +P	0.5 ng/ml
hU3-55k Del193-352	100 ng/ml
hU3-55k Del136+NLS	1 ug/ml
NOB1	1 ng/ml
PNO1	2 ng/ml
DIM1	5 ng/ml
tUTP4	1 ug/ml
UTP6	5 ng/ml
UTP23	5 ng/ml
UTP24	100 ng/ml
ESF2	10 ng/ml
KRR1	5 ng/ml
RCL-1	1 ug/ml
RRP7	5 ng/ml
MRD1	10 ng/ml

2.4.6 Fluorescence *In Situ* Hybridisation (FISH)

Human cells were grown on 10 mm round glass coverslips in 24-well plates, each in 5 mls of media, as described in section 2.4.1. Cells were washed in PBS and fixed using 4 % paraformaldehyde / PBS (w/v) [pH 7.4] for 20 minutes after which they were washed 3x in 70% ethanol, rehydrated for 10 minutes in PBS / 5 mM $MgCl_2$, and pre-hybridised in 15% formamide, 2 x SSC, 10 mM NaH_2PO_4 / Na_2HPO_4 [pH 7.0] for 10 min. The RNA on each coverslip was hybridised with 1 µl of each FISH probe (10 µg/µl; Table 2.13) in 5 µl of 2 x formamide buffer (60 % formamide, 40 mM NaH_2PO_4 / Na_2HPO_4 [pH 7.0]), 10 µl FISH hybridisation buffer (20% dextrane sulphate, 4x SSC, 0.4% BSA (w/v)), 1 µl tRNA (10 µg/µl), 1 µl of

sonicated salmon sperm DNA (10 µg/µl), and H_2O to make the reaction to 20 µl, incubated for 4 hours in a humidified incubator at 37 ℃.

Table 2.13 Sequence of Probes Used in FISH Analysis of Human Cells

Probe	Sequence 5'-3'	Fluorescent Label	Reference
U3 snoRNA	C*GCTCTACACGTTCAGAGAAACTTCTCTA GTAACACACTATAGAAATGATCCC	Cy5	(Granneman et al., 2004)
U8 snoRNA	C*GTTCTAATCTGCCCTCCGGAAGGAGGAA ACAGGAAACAGGTAAGGATTATCCCACCC*	Cy3	(Granneman et al., 2002; Granneman et al., 2004; Watkins et al., 2002)
U2 snRNA	Alexa488.GAACAGATACTACACTTGATCTTA GCCAAAAGGCCGAGAAGC.Alexa488	Alexa 488nm	(Schaffert et al., 2004)
StreoTagged U3 msl2	Cy3.GCAGGGATCCGACCGTGGTGCCACCC TGGGCAGAAGTCAAATGCGATCCAAGC	Cy3	(Granneman et al., 2004; Lukowiak et al., 2000)

Oligonucleotides probes were either pre-labelled (U2 snRNA; Molecular Probes, Invitrogen, StreptoTagged U3 msl2; Amersham Pharmacia Biotech) or contained 5-allyl-cytidine groups (depicted by *, Table 2.13) at the 5' and 3' ends that were coupled to fluorophores containing N-hydroxysuccinimide, as described by the manufacturer (Molecular Probes, Invitrogen) (Granneman et al., 2004).

After hybridisation, cells were washed 2x for 30 mins at 37 ℃ in pre-hybridisation solution, 2x in 2x SSC, 0.1 % Triton-X100 (v/v) at room temperature for 15 minutes, then 2x in 1 x SSC, 0.1 % Triton-X100 (v/v) at room temperature for 15 minutes. To visualise the nucleus, DAPI (0.1 µg/ml, Sigma-Aldrich) was added to the penultimate wash. Coverslips were briefly immersed in H_20 and then ethanol, before mounting onto glass microscope slides, using 3.5 µl Moviol mounting solution (30% glycerol (w/v), 12% Mowiol (w/v), 0.12 M Tris pH 8.5).

2.4.7 Sonication and Extract Preparation from Human Cells

Human cells were cultured using the conditions previous described to a confluency of 80%, harvested and pelleted. To produce whole cell extract for analysis by glycerol gradient analysis or immunoprecipitation, 1 x 10^7 cells were resuspended (on ice) in 550 µl of gradient buffer (20 mM HEPES-NaOH [pH 7.9], 150 mM KCl, 0.5 mM EDTA, 1 mM DTT)) and disrupted by sonication using a Bandelin Sonopuls HD2070 ultrasonic homogeniser with a 2 mm MS72 titanium microtip (2 x 20 s continuous sonication at 20% power, with 30 sec. intervals). Triton X-100 (0.2% (v/v)), Glycerol (10% (v/v)) and $MgCl_2$ (1.5mM) were then added to the extract preparation before insoluble material was removed by centrifugation at 13,000 rpm in a benchtop microfuge (4 ℃) for 10 minutes and the supernatant assayed (Granneman et al., 2004).

2.4.8 Glycerol Gradient Analysis of U3 snoRNP Complexes and Related Proteins

Glycerol gradients were used to separate elements of the SSU Processome, analyse the incorporation of proteins into the complex and their effect on the U3 snoRNP. 10-40% glycerol gradients were made in 4ml, 11x60 mm Ultra-Clear tubes (Beckman). 2 mls of 10% glycerol solution (10% glycerol (v/v), 0.2% Triton-X100 (v/v), 1.5 mM $MgCl_2$) in gradient buffer (20 mM HEPES-NaOH [pH 7.9], 150 mM KCl, 0.5 mM EDTA, 1 mM DTT)) was added to the bottom of each tube, under which 2 mls of a 40% glycerol solution (40% glycerol (v/v), 0.2% Triton-X100 (v/v), 1.5 mM $MgCl_2$) in gradient buffer was inserted, ensuring no mixing of the solutions at the interface. The gradient was created by spinning at 22 rpm, 83° for 1 min. 10 sec. using a BioComp Gradient Master 107ip. The gradients were then chilled at 4 ℃ for 1 hour before removing 400 µl from the top and replacing this with sonicated whole cell extract. Samples were centrifuged in a Beckman optima L-100 ultra centrifuge using a Sw60ti rotor, for 1.5 hours at 52,000 rpm, 4℃, acceleration and deceleration set at 5. The gradient was then fractioned from the top, into 200µl aliquots and snap-frozen in liquid nitrogen.

2.5 Antibodies

2.5.1 Creation of Custom Antibodies

1 mg of each His-tagged purified protein (as described in section 2.2; NOB1, PNO1, ESF2, KRR1, RCL-1 and UTP24) were lyophilised and sent to Eurogentec for production of polyclonal antibodies from rabbits. For each protein, two rabbits were immunised and a series of bleeds taken (Pre-immune, PPI; Small bleed, PP; Large bleed, GP; Final bleed, SAB). These were then tested on western blots of whole cell lysate from HEK293 cells expressing the FLAG-tagged protein of interest.

hU3-55k antibody (produced in rabbit, immunised against the peptide sequence EEELEETAQEKKLRLAK) was kindly provided by Sander Granneman (Granneman et al., 2002). NOP56 and NOP58 antibodies were provided by Kenneth McKeegan and Nick Watkins (University of Newcastle) as previously described (Watkins et al., 2002; Watkins et al., 2000)). NOP56 antidbodies were affinity purified on Sulpho-link columns (Pierce) by Kenneth McKeegan using the peptide sequence CTVNDPEEAGHRSRSK. Murine hybridomas expressing fibrillarin (72B9) antibody were kindly provided by Michael Pollard and Ger Pruijn, antibodies were collected by Kenneth McKeegan as described previously (Reimer et al., 1987). Purified UTP12 and MPP10 antibodies (using their respective peptide sequences) were kindly provided by Amy Turner (University of Newcastle) as described previously (Turner et al., 2009). U5-116K was kindly provided by Claudia Schneider (University of Edinburgh), created as previously described (Fabrizio et al., 1997). For other antibodies, see Table 2.14.

2.5.2 Western Blot Analysis

Proteins separated by SDS PAGE were transferred onto a nitrocellulose membrane (Protran; Whatman / GE Healthcare) by western blotting in transfer buffer (25 mM Tris base, 150 mM glycine, 10% methanol, [pH 8.3]) at 65 volts for 2

hours. Membranes were incubated in blocking buffer (PBS, 3% Marvel skimmed milk powder (w/v), 0.05 % Triton X-100 (v/v)) for 1 hour at room temperature. Antibodies were diluted in blocking buffer at the desired concentration (Table 2.14) and incubated with the membrane for 1 hour at room temperature. Membranes were then washed 3x 5 minutes, 2x 15 minutes in PBS, 0.05 % Triton X-100 (v/v) then incubated with blocking buffer containing the appropriately diluted secondary antibody – HRP (horseradish peroxidase) conjugate, for 1 hour at room temperature and the previous washes repeated. Results were visualised by ECL (enhanced chemiluminescence) detection (BioRad) and hyperfilm (Amersham) as described by the manufacturers.

Table 2.14 Antibodies Used in Western Blot Analysis

Target / Antibody	Raised In	Manufacturer	Dilution in W.B.
FLAG (M2)	Mouse	Sigma-Aldrich F1804-1MG	1/2000
FLAG (Polyclonal)	Rabbit	Sigma-Aldrich F7425-.2MG	1/2000
Fibrillarin	Rabbit	Santa Cruz SC-25397	1/2000
hU3-55k	Rabbit	Sander Granneman (Eurogentec)	1/2000
NOP58	Rabbit	Nick Watkins (Eurogentec)	1/2000
NOP56	Rabbit	Nick Watkins (Eurogentec)	1/2000
MPP10	Rabbit	Amy Turner (Eurogentec)	1/2000
Nucleolin	Rabbit	Abcam Ab22758	1/2000
UTP12	Rabbit	Amy Turner (Eurogentec)	1/2000
DHX15 / PRP43	Rabbit	Bethyl A300-390A	1/2000
U5-116k	Rabbit	Claudia Schneider	1/2000
NOB1	Rabbit	Eurogentec (custom)	1/250
PNO1	Rabbit	Eurogentec (custom)	1/250
ESF2	Rabbit	Eurogentec (custom)	1/250
KRR1	Rabbit	Eurogentec (custom)	1/500
RCL-1	Rabbit	Eurogentec (custom)	1/250
UTP24	Rabbit	Eurogentec (custom)	1/250
Poly-His	Mouse	Sigma H1029	1/2000
Mouse IgG (HRP conjugate)	Rabbit	Dako P0260	1/2000
Rabbit IgG (HRP conjugate)	Donkey	Santa Cruz sc-25397	1/25,000

2.5.3 Immunofluorescence

Human cells grown on 10 mm round coverslips (described previously) were washed 1x in PBS and fixed in 4% paraformaldehyde (w/v) in PBS [pH 7.4]. Cells were then washed 3x in PBS [pH 7.4], incubated in PBS 0.1 % Triton-X100 for 15

minutes at room temperature, washed 3x in PBS and blocked for 1 hour at room temperature in PBS, 10% FBS (v/v), 0.1% Triton-X100 (v/v). Cells were then probed using the appropriate amount of antibody (Table 2.15) in 50µl of 10% FBS (v/v) / PBS [pH 7.4] for 1h at room temperature. Coverslips were washed 3x briefly and 3x 10 min. in PBS before incubation in 50µl of 10% FBS (v/v) / PBS [pH 7.4] containing the appropriate secondary antibody, for 1h at room temperature. Again, cells were washed 3x briefly and 3x 10 min. in PBS, with the addition of DAPI (0.1 µg/ml; Sigma-Aldrich) to the penultimate wash to visualise the nucleus. Finally, coverslips were briefly immersed in H_20 and then ethanol, before mounting onto glass microscope slides, using 3.5 µl Moviol and analysed by wide-field microscopy.

Table 2.15 Antibodies Used in Immunofluorescence

Target / Antibody	Raised In	Manufacturer	Dilution for I.F.
FLAG (M2)	Mouse	Sigma-Aldrich F1804-1MG	1/500
FLAG (Polyclonal)	Rabbit	Sigma-Aldrich F7425-.2MG	1/500
Fibrillarin (72B9)	Mouse	Michael Pollard and Ger Pruijn	1/500
BMS1	Rabbit	Amy Turner / Nick Watkins (Turner et al., 2009)	1/100
Mouse IgG (Alexa Fluor 555 conjugate)	Donkey	Invitrogen A31570	1/500
Rabbit IgG (Alexa Fluor 647 conjugate)	Donkey	Invitrogen A-31573	1/500

2.5.4 Wide-field Microscopy

Cells subjected to FISH and immunofluorescence were visualised using a Zeiss Axiovert 200M inverted microscope with a Plan-Apochromat, 100 x / 1.40 oil DIC, ∞ / 0.17 objective (Zeiss). The Zeiss filter sets used were: 02 (DAPI); 20 (Cy3); 26 (Cy5). Use of the microscope was provided by J. Brown, University of Newcastle, UK.

2.5.5 Immunoprecipitation (IP) Reactions

10µl of Protein G Sepharose beads (GE Healthcare) per immunoprecipitation (IP) were washed together 3x in 1 ml of IP buffer (20 mM HEPES [pH 8], 150 mM NaCl,

3 mM $MgCl_2$, 0.5 mM DTT, 10 % glycerol (v/v), 0.1 % Triton-X100 (v/v)), pelleted between each wash at 3,000 rpm for 30 sec. at 4 ℃ in a microfuge, and the supernatant removed. After the last wash step, the pellet was resuspended in 500 µl of IP buffer with 5 µl of mouse anti-FLAG M2 antibody (Sigma-Aldrich) per IP reaction, or no antibody for a negative control, and incubated on a rotary incubator (8 rpm; Rotator SB3, Stuart) overnight at 4 ℃. Sepharose beads were then washed 3x in IP buffer as described previously. The beads were resuspended in an appropriate volume and aliquoted into one tube per reaction. To each of these, 5 million sonicated HEK293 cells (250 µl of previously described supernatant, section 2.4.7) were added and the volume made up to 500 µl with IP buffer and incubated at 4 ℃ for 3 hours on a rotary incubator (8 rpm). 10% of sonicated cells (25 µl) were retained for downstream RNA and protein extraction to give a measure of inputs. IP reactions were washed 3x in IP buffer as described previously, resuspended in 1 ml IP buffer and transferred to a new 1.5 ml eppendorf tube (to reduce background signal) and pelleted at 3,000 rpm for 30 sec. at 4 ℃. RNA and protein was subsequently extracted from the samples as described in section 2.3.1 and analysed by Western and Northern Blotting.

2.5.6 *In vitro* U3 snoRNA Transcription & U3 snoRNP Assembly in Mouse Nuclear Extract

StreptoTagged U3 snoRNA constructs wt. U3; C' Consensus; Mutant C Box; C' Consensus / Mutant C; and Mutant D Box in pGEM-T Easy (described in section 2.1.12) were PCR amplified using "U3+T7 Forward" and either" U3 wt. Reverse" or "U3mutD Reverse" primers (as appropriate).

U3+T7 Forward: 5'-GCTAATACGACTCACTATAGGGAGAGTGTAGAGCACCGA AAACCACGA-3'
U3 wt. Reverse: 5'-ACCACGGAGACCGCGTTCTC-3'
U3mutD Reverse: 5'-ACCACGGAGACCGCGTTCTC-3'

Products were transcribed using T7 RNA polymerase and α-^{32}P UTP to give a radiolabeled product (as described for U15a in section 2.3.5, although in this instance, PCR products were designed such that sense strand products were made). 1 µl of the product was used to assess counts per minute (CPM) using a scintillation counter.

Mouse nuclear extract (kindly provided by Stuart Maxwell, North Carolina State University, USA) was centrifuged at 13,000 rpm in a benchtop microfuge at 4 ℃ for 10 min. to remove insoluble material. The assembly reaction was then set up in 20 µl with 10 µl nuclear extract, 1x assembly buffer (10 mM HEPES [pH7], 200 mM KCl, 3 mM MgCl2, 0.5 mM THP (tris(hydroxypropyl)phosphine)), 50 µg/ml tRNA and 100,000 CPM of radiolabeled StreptoTagged U3 snoRNA of interest. Reactions were incubated at 30 ℃ for 30 min. then centrifuged at 13,000 rpm for 10 min. in a microfuge.

10µl of Protein A Sepharose CL-4B beads (GE Healthcare) per reaction were washed together 3x in wash buffer (20 mM Tris-HCL [pH 8], 3 mM $MgCl_2$, 150 mM KCl, 0.1% Tween-20 (v/v), 10% glycerol (v/v), 0.5 mM THP) pelleted between each wash at 3,000 rpm for 30 sec. at 4 ℃ in a microfuge, and the supernatant removed. Either 20 µl of rabbit anti-hU3-55k; 100 µl rabbit anti-NOP58; or 200 µl mouse anti-fibrillarin (72B9) antibodies were added per reaction and made up to a total volume of 1 ml in wash buffer. Reactions were incubated on a rotary incubator (8 rpm; Rotator SB3, Stuart®) overnight at 4 ℃. Beads were then washed 3x in 1 ml of wash buffer, resuspended in 1 ml and pelleted once more in a fresh tube before being aliquoted into the appropriate number of reactions.

Antibody conjugated Protein A Sepharose beads were incubated with the relevant radiolabeled U3 snoRNA in a total of 250 µl made up in wash buffer and incubated on a rotary incubator (8 rpm) for 2 hours at 4 ℃. RNA was then extracted with 100 µl RNA extraction buffer (described in section 2.3.1), pellets resuspended in 20 µl of H_2O and 20 µl RNA dye, heated to 95 ℃ for 5 min. and 20 µl separated upon a 6% polyacrylamide / 7M urea gel by electrophoresis in 1x TBE at 400 volts. The gel

was then fixed in 10% methanol (v/v), 10% acetic acid (v/v) for 30 min. and dried using a gel drier at 70 ℃ for 1 ½ hours. Products were visualised by exposure to a PhosphorImager screen and detected using a Typhoon imager and ImageQuant Software for analysis (GE HealthCare).

2.5.7 *In vitro* Protein-Protein Interaction Assay with ^{35}S-Methionine-labelled hU3-55k and Thioredoxin or GST-tagged Recombinant Proteins

10µl of Protein A Sepharose CL-4B beads (GE Healthcare) per reaction were washed together 3x in 1 ml of protein buffer (20 mM Tris-HCL [pH 8], 5 mM $MgCl_2$, 300 mM KCl, 0.1% Tween-20 (v/v), 10% glycerol (v/v), 0.5 mM THP), pelleted between each wash at 3,000 rpm for 30 sec. at 4 ℃ in a microfuge, and the supernatant removed. After the last wash step, the pellet was resuspended in 1 ml of protein buffer with 5 µl of mouse anti-thioredoxin antibodies (Sigma-Aldrich T0803) per reaction, or no antibody for a negative control, and incubated on a rotary incubator (8 rpm; Rotator SB3, Stuart®) overnight at 4 ℃. Beads were then washed 3x in 1 ml of protein buffer, pelleted and aliquoted into the appropriate number of reactions.

300 ng of purified thioredoxin-tagged NOP56 or NOP58, or thioredoxin alone (section 2.2.2) were incubated with 5 µl of [^{35}S]methionine-labelled hU3-55k (section 2.2.8) in protein buffer (total volume 200 µl) at 30 ℃ for 1 hour on a shaking platform and centrifuged at 13,000 rpm for 5 min. in a microfuge. The supernatant of each reaction was then added to the anti-thioredoxin coupled protein A sepharose beads and incubated for 1 hour on a rotary incubator at 8 rpm, 4 ℃. Reactions were washed 3x in 1 ml of protein buffer as previously, resuspended in 1 ml protein buffer and transferred to a new 1.5 ml eppendorf tube (to reduce background signal) and pelleted at 3,000 rpm for 30 sec. at 4 ℃. Pellets were resuspended in 50 µl 1x protein sample buffer (75 mM Tris-HCl [pH 6.8], 1.25 mM EDTA, 20 % glycerol (v/v), 2.5 % SDS (w/v), 0.125 mM bromophenol blue), supplemented with 200 mM DTT), denatured at 95 ℃ for 5 minutes and 25 µl separated on a 13% SDS polyacrylamide gel. The gel was then fixed in 10%

methanol (v/v), 10% acetic acid (v/v) for 30 min. and dried using a gel drier at 70 ℃ for 1 ½ hours. Products were visualised by expos ure to a PhosphorImager screen and detected using a Typhoon imager and ImageQuant Software for analysis (GE HealthCare).

This reaction was repeated for analysis of hU3-55k interactions with GST-tagged proteins TIP48, TIP49, 15.5K, fibrillarin ΔRGG, BCD1, NUFIP, NOP17 or GST alone. In this instance, 10 µl of Glutathione Sepharose 4 Fast Flow (GE Healthcare) beads were used per reaction, without the addition of antibody. Otherwise, all steps remained the same. It is worth noting that the RGG motif of fibrillarin, and the KKE/KKD regions of NOP56 and NOP58 were deleted to facilitate expression of sufficient soluble protein from *E. coli* (section 2.2).

Chapter Three

Specific Regulation of U3 snoRNP Levels During Cellular Differentiation and Tumourogenesis

3.1 Introduction

In eukaryotes, biosynthesis of cytoplasmic ribosomes occurs within the sub-nuclear structure of the nucleolus and consumes a large amount of the cell's energy. In yeast, rRNA transcription accounts for 60% of total transcription, with 50% of RNA pol. II transcription and 90% of mRNA splicing devoted to generating ribosomal proteins (Warner, 1999). A proliferating HeLa cell is said to produce ~7500 ribosomes/min, requiring transcription of 150–200 rRNA genes and the synthesis of ~300,000 ribosomal proteins (Mayer and Grummt, 2006). As part of the maturation of the rRNA, various other components are also required, including assembly factors, ribonucleases, RNA helicases and small nucleolar ribonucleoprotein particles (snoRNPs) (Henras et al., 2008; Mayer and Grummt, 2006; Turner et al., 2009; Warner, 1999).

In both yeast and humans, ribosome biogenesis is a key control point for the regulation of cell growth and division (Belin et al., 2009; Bernstein and Baserga, 2004; Bernstein et al., 2007; Dai and Lu, 2008; Montanaro et al., 2008), with protein translational capacity influenced by the number of ribosomes in the cell (Burrin et al., 1997). This process is down regulated in terminally differentiated cells (Bowman and Emerson, 1977; Poortinga et al., 2004) and up-regulated in many cancers, with several proto-oncogenes and tumour suppressors able to directly regulate ribosome biogenesis (Oskarsson and Trumpp, 2005; Ruggero and Pandolfi, 2003). For instance, the production of ribosomes is directly linked to the stabilisation of the tumour suppressor p53 and the ability of the cell to detect stress (Dai and Lu, 2008; Rubbi and Milner, 2003).

The small subunit (SSU) processome is involved in the initial recognition of the pre-rRNA, and can be seen as the terminal knob structures in “Miller-spreads” (Dragon et al., 2002; Miller and Beatty, 1969; Mougey et al., 1993). This complex is essential for 18S rRNA processing and contains the U3 snoRNA along with many additional factors, such as RNA helicases (*e.g.* DBP4, HAS1 and DHR1) and RNA-binding proteins (*e.g.* RRP5 and MRD1) (Henras et al., 2008; Turner et al., 2009). The U3 snoRNA belongs to the box C/D snoRNA family, of which there are over 100 different members in humans (Lestrade and Weber, 2006). The majority of box C/D snoRNAs function as sequence-specific guides to direct 2’-*O*-methylation of rRNA. A subset of the box C/D snoRNAs however, such as U3, U8 and U14, are essential for rRNA processing and are believed to regulate rRNA folding through base-pairing with specific regions of the pre-rRNA (Hughes, 1996; Liang and Fournier, 1995; Peculis and Steitz, 1993; Sharma and Tollervey, 1999). Box C/D snoRNAs contain a conserved box C/D motif (termed C'/D in U3), which is initially bound by 15.5K (snu13p), followed by NOP56, NOP58 and the methyltransferase fibrillarin, collectively constituting the common “core proteins” of Box C/D snoRNPs (Watkins et al., 2002; Watkins et al., 2000). The U3 snoRNA however, contains a specific motif, termed Box B/C, believed to be important for snoRNP function but not biogenesis (Granneman et al., 2004; Mereau et al., 1997; Samarsky and Fournier, 1998; Venema et al., 2000; Watkins et al., 2000). To this, another 15.5K protein binds along with the U3-specific protein hU3-55k (Rrp9p in yeast; Figure 3.1) (Granneman et al., 2002). The myriad of SSU processome components are only then believed to associate with the U3 snoRNP as part of the processome complex.

The majority of box C/D snoRNAs are encoded within the introns of protein-coding genes and are processed by the lariat pathway in which, the spliceosome brings the two exon-intron junctions into proximity, removes the intron and ligates the two exons together (Terns and Terns, 2002). In contrast, a subset of box C/D snoRNAs, including U3, U8 and U13, are independently transcribed by RNA polymerase II and contain a co-transcriptionally added 5'- 7-methylguanosine (m^7G) cap which is hypermethylated to give a trimethylguanosine cap structure

($m^{2,2,7}G$ / m_3G cap), an important part of the nucleolar localisation signal, most likely added in the Cajal bodies by hTgs1 (Jacobson and Pederson, 1998; Terns and Terns, 2002; Verheggen et al., 2002). Furthermore, the C/D (C'/D) motif and the recruitment of the core box C/D proteins have also been shown to be essential for snoRNA accumulation and nucleolar localisation (Lafontaine and Tollervey, 1999; Lange et al., 1998a; Lange et al., 1998b; Narayanan et al., 1999; Samarsky and Fournier, 1998; Speckmann et al., 1999; Verheggen et al., 2002; Watkins et al., 2002).

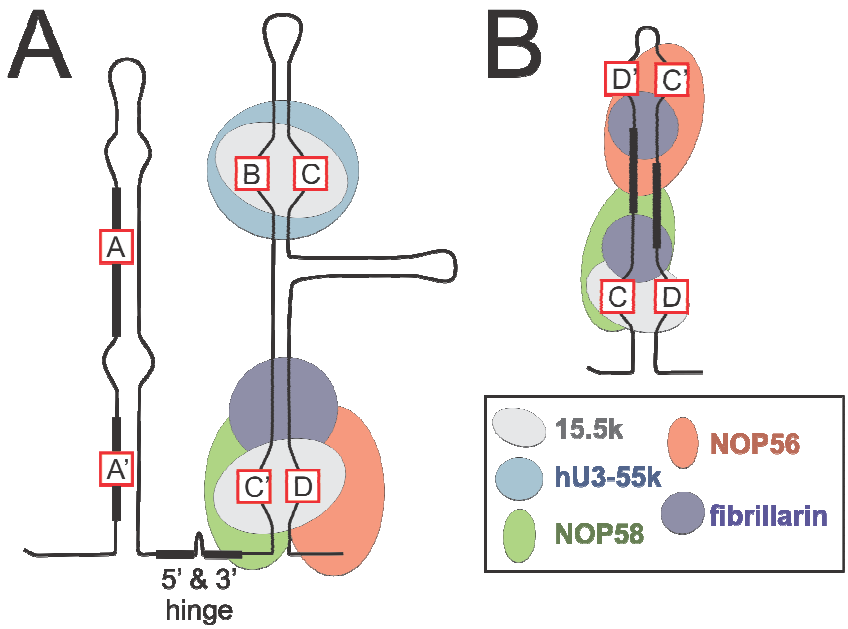


Figure 3.1 The U3 snoRNP and a Conventional Box C/D snoRNP
A) A model of the U3 box C/D snoRNP with core-proteins associated; essential for 18S pre-rRNA processing **B)** A conventional box C/D snoRNP with core proteins associated; required for methylation of pre-rRNA. Solid black line indicates RNA sequence; thick black lines indicate sections believed to base-pair with target pre-rRNA sequences (not shown). Conserved boxes are indicated by red boxes, with the name of the conserved motif shown inside. Proteins are as indicated within the key at the bottom right. Models based on previous data (Granneman et al., 2002; Granneman et al., 2004; Watkins et al., 2000).

SnoRNP biogenesis involves the dynamic association of numerous factors as part of a large multiprotein pre-snoRNP complex, believed to form at the site of transcription, with subsequent protein recruitment and processing within Cajal bodies (Verheggen et al., 2002). These factors include proteins linked to snoRNP assembly (TIP48, TIP49, NUFIP, TAF9, NOP17 and BCD1), molecular chaperones

(HSP90 and HSC70), nucleocytoplasmic transport factors (PHAX, CRM1, CBC, Nopp140, Ran and Snurportin1) and proteins involved in snoRNA maturation (TGS1, La, LSm proteins and the exosome) (Boulon et al., 2008; Boulon et al., 2004; McKeegan et al., 2007; Watkins et al., 2004; Watkins et al., 2007; Zhao et al., 2008). These pre-snoRNP factors are proposed to form a scaffold to facilitate snoRNP protein assembly, with several of the factors also predicted to function in the assembly of the U3-specific box B/C snoRNP complex (McKeegan et al., 2007; McKeegan et al., 2009).

Protein accounts for more than 70% of eukaryotic cells' dry mass (Oskarsson and Trumpp, 2005) therefore, cell cycle progression is typically dependent upon cell growth (Jorgensen et al., 2004; Rudra and Warner, 2004). Interestingly, the two processes coordinately controlled in eukaryotes (Neufeld et al., 1998; Neufeld and Edgar, 1998; Oskarsson and Trumpp, 2005).

During cellular differentiation, many cell types undergo arrest in the G0/G1 phase of the cell cycle. This is often accompanied by a reduction in cell growth and ribosome biogenesis (Poortinga et al., 2004). Upon differentiation, the adenocardinoma cell line CaCo-2 is said to lose its tumourigenic phenotype, so represents a useful tool for examining tumour progression (Stierum, 2003). Proliferation and differentiation are mutually exclusive events, therefore many malignancies present as their immature cell type (Heath et al., 2000). Interestingly, treatment of various malignant cancer cell lines with agonists of the transcription factor PPAR, have been shown to force them to differentiate, a reversal of the malignant phenotype, giving rise to the prospect of "differentiation therapy" (Mueller et al., 1998; Mueller et al., 2000; Sarraf et al., 1998; Spira and Carducci, 2003; Tontonoz et al., 1997). Other forms of differentiation therapy have also shown promise in treating acute promyelocytic leukemia (Spira and Carducci, 2003; Waxman, 2000) and reducing metastases of breast cancer cells (Beug, 2009), demonstrating the importance of understanding cellular differentiation.

In Burkitt's lymphoma, c-Myc is activated; triggering proliferation (Pajic et al., 2000). In P493-6 cells, derived from this lymphoma, only the processing of the 47S rRNA precursor, but not the synthesis of the transcript, was dependent on the presence of c-Myc (Schlosser et al., 2005). Interestingly, both N-Myc (neuronal Myc) and c-Myc have been shown to control the levels of mRNAs encoding pre-rRNA processing factors, with levels of mature rRNA increased upon Myc expression (Boon et al., 2001; Coller et al., 2000; Schlosser, 2003). It has also been reported that upon myoblast differentiation, the ribosome half life is reduced and pre-rRNA turnover is increased, whereas there is no significant change in the rRNA transcription levels (Bowman and Emerson, 1977). In regenerating rat livers, ribosome biogenesis is also controlled post-transcriptionally, in part by accelerating the occurrence of pre-rRNA cleavages to produce more ribosomes, more quickly (Dudov and Dabeva, 1983). Moreover, ribosomal proteins are produced in excess in higher eukaryotes; whereby unused proteins are rapidly degraded, to enable rapid changes in the rate of ribosome production (Bowman, 1987; Lam et al., 2007; Tsay et al., 1988). Collectively, this shows that processing and post-transcriptional regulation are crucial in controlling ribosome biogenesis, especially in tumourogenesis and differentiating cells.

It has however, also been shown that rDNA transcription is closely regulated throughout the cell cycle and differentiation. MAD1 (Mitotic spindle assembly checkpoint protein) negatively regulates cell growth and rDNA transcription by associating with UBF (essential for rRNA transcription) during granulocyte differentiation. Conversely, exogenous c-Myc expression (down regulated during differentiation (Heath et al., 2000)) can increase both UBF expression and rRNA transcription (Arabi et al., 2005; Grandori et al., 2005; Poortinga et al., 2004), whilst ribosomal protein L11 has been shown to inhibit c-Myc-induced transcription and cellular proliferation (Dai et al., 2007), demonstrating a possible method of regulating rRNA transcription during cellular differentiation. Whilst there is evidence that ribosome biogenesis is regulated at both the level of rRNA transcription and rRNA processing (Schlosser, 2003; Schlosser et al., 2005), the majority of the work to date has focused upon regulation of transcription.

Given the changes to the rate of ribosome biogenesis that occur during both cellular differentiation and tumourogenesis, the regulation of snoRNP homeostasis is of particular interest. The inherent stability of these complexes suggests that the best way to control snoRNP levels is at the point of production. With the exception of a few examples, however, surprisingly little is known about the regulation of snoRNP expression or indeed, that of other nuclear RNPs. Nonetheless, it is known that specific transcription signals drive the preferential expression of the mouse spliceosomal U1 snRNA variant U1b in stem cells (Caceres et al., 1992). Whereas, the levels of the human U6 snRNA and the *S. pombe* U3 snoRNA have been shown to be regulated post-transcriptionally (Nabavi et al., 2008; Noonberg et al., 1996). Of particular interest, is that mammalian U3 snoRNA expression is down-regulated in response to serum starvation and during myoblast differentiation, coinciding with a reduction in ribosome production (Glibetic et al., 1992; Sienna et al., 1996). Many eukaryotes also have paralogous U3 snoRNAs, and in mouse, the U3A variant displays tissue-specific expression (Mazan et al., 1990). During *Xenopus* oocyte development, U3 snoRNA levels are regulated independently of fibrillarin (Caizergues-Ferrer et al., 1991). It is, however, unclear how U3 snoRNP levels are regulated, specifically in vertebrates. Furthermore, it is not clear whether regulation is exclusive, or if it applies to all box C/D snoRNAs. As hU3-55K is the only U3-specific protein stably associated with the U3 snoRNP monoparticle and is important for incorporation of the U3 snoRNP into the SSU processome (Granneman and Baserga, 2004; Venema et al., 2000), we set out to investigate its role in regulating the U3 snoRNP, and how this may regulate the production of ribosomes in human cells.

3.2 Results

3.2.1 siRNA Targeting of hU3-55k and NOP58 Results in a Significant Reduction of U3snoRNA Levels

Firstly, we decided to test the requirement for hU3-55k in human cells, and in particular its relationship with the U3 snoRNP. We were interested if hU3-55k, the only U3-specific protein, could regulate U3 snoRNP levels. To examine this possibility, we used siRNAs to specifically deplete hU3-55k from HeLa cells. Duplexes targeting the firefly luciferase mRNA GL2 were used as a negative control, whereas, duplexes targeting NOP58, known to be crucial for human box C/D snoRNP accumulation, were used as a positive control (McKeegan et al., 2009; Watkins et al., 2004). HeLa cells were transfected with the relevant siRNA and harvested 60 hours later. The levels of individual proteins were determined by Western blot analysis which demonstrated that siRNAs targeting NOP58 and hU3-55k mRNAs specifically reduced the levels of the target proteins whereas the control siRNA had no effect; with fibrillarin levels remaining constant in all the knockdowns, demonstrating the specificity of the siRNA duplexes (Figure 3.2 A). RNA was also extracted from the siRNA-treated cells and analysed by Northern blotting, using probes specific to the U3 and U8 Box C/D snoRNAs, and the U1 snRNA. As previously described (Watkins et al., 2004), depletion of NOP58 resulted in the reduction of both U3 and U8 snoRNA levels without affecting those of the U1 snRNA, relative to the control knockdown (Figure 3.2 B). Of interest, reduced hU3-55k abundance resulted in a reduction of U3 snoRNA levels with U8 and U1 RNAs remaining unchanged. Previously, only factors essential to snoRNP biogenesis have been shown to affect U3 snoRNA levels in human cells, suggesting that hU3-55k is intricately involved in U3 snoRNP formation.

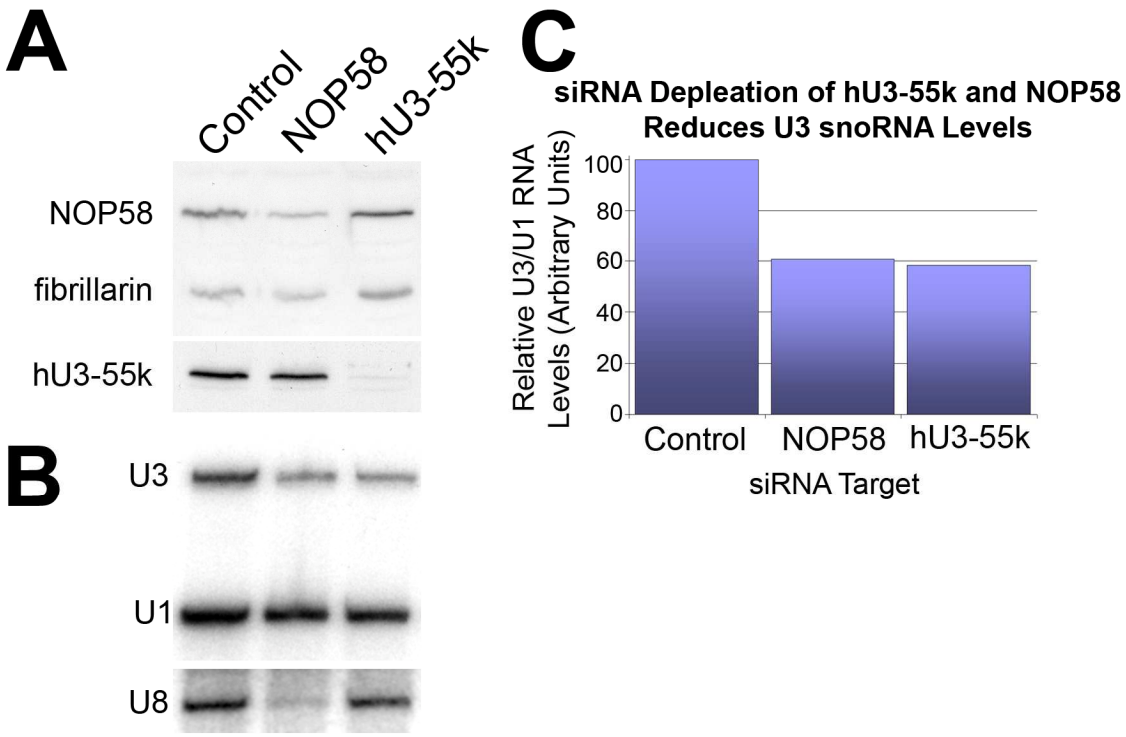


Figure 3.2 siRNA Knockdown of hU3-55k and NOP58 in HeLa Cells
A) HeLa cells were electroporated with siRNA duplexes specifically targeting firefly luciferase (control), NOP58, or hU3-55k mRNAs, as shown above each panel. Protein was extracted from siRNA targeted cells after 60 hours incubation post-electroporation, separated by SDS PAGE and Western blotted. Proteins of interest were detected by antibodies specific to each protein, indicated to the left of each panel. **B)** RNA was extracted from siRNA treated HeLa cells (as in A), separated by denaturing PAGE and Northern blot analysis performed using radiolabeled probes specific to U3, U1 and U8 sn / snoRNAs, as indicated to the left of each panel. **C)** A bar chart depicting the relative U3/U1 RNA levels in siRNA treated cells, with the protein targets of the duplexes shown under each bar. Quantitation of U3 snoRNA levels relative to that of U1 snRNA was performed on the same hU3-55k / NOP58 siRNA knockdown experiment / Northern blot. Quantitation was performed using ImageQuant TL (GE Healthcare) software with background substitution and relative RNA levels plotted. The results shown are representative of 3 repeated experiments.

3.2.2 Characterisation of Inducible Tagged Wild-type hU3-55k

We next wished to examine the effect of increasing hU3-55k levels in the cell, with respect to the U3 snoRNA. To do this, HEK293 stable cell lines were generated, capable of inducible expression of FLAG-tagged proteins of interest, such as hU3-55k; allowing titratable induction of the tagged protein. Firstly, a modified pcDNA5 vector (pcDNA5-FLAG) was created, containing a “2xFLAG - Precision Protease - 6xHis" tag sequence constituting 2x FLAG tag sequences, a precision protease

site for removal of the FLAG tags from the protein, and a poly-his tag sequence. This was designed to be added N-terminally to the coding sequence of any gene suitably cloned into the vector, allowing detection of tagged proteins by antibodies to either the FLAG sequence or 6x-His sequence (Figure 3.3). Genes of interest were cloned into the modified pcDNA5 vector containing, a tetracycline-regulated hybrid human cytomegalovirus (CMV)/TetO2 promoter, and a hygromycin B resistance gene with a FRT (Flp-In) recombination site in the 5' coding region (but lacking a promoter). Subsequently, Flp-In T-Rex HEK293 cells (containing an FRT recombination site and promoter; Invitrogen) were chemically co-transfected with the pcDNA5-FLAG vector, containing the gene of interest, and a vector constitutively expressing Flp recombinase. This facilitated homologous recombination between the FRT sites and thus, integration of the tagged gene of interest in frame of the relevant promoter, already within the genome; allowing titratable induction of protein expression (Figure 3.4). Thus, stable cell lines were generated in which either the hU3-55k protein, the core box C/D protein fibrillarin, or the FLAG-tag alone, were expressed under the control of a tetracycline-regulated promoter.

Expression of the hU3-55k-FLAG protein was tested by the addition of 1 µg/ml tetracycline to stably transfected HEK293 cells. The cells were harvested at set time intervals after the addition of tetracycline, analysed by Western blotting and probed for either hU3-55k or fibrillarin, with hU3-55k giving a double band due to the presence of the endogenous (smaller) protein and (larger) tagged form (Figure 3.4 A). This demonstrated that accumulation of the tagged protein continued for up to 48 hours with no effect on the levels of fibrillarin, used in this instance as a control. The concentration of tetracycline was also assessed over a titration of 0 – 1 µg/ml and Western blotting analysis employed, using an antibody that detected both the FLAG-tagged and endogenous proteins (Figure 3.4 B). This revealed that when cells were cultured in 0.5 ng/ml tetracycline for 48 hours, the level of hU3-55k-FLAG was 1:1 with the endogenous protein, and that tetracycline at 1 µg/ml resulted in over expression of the tagged protein with no effect on fibrillarin levels.

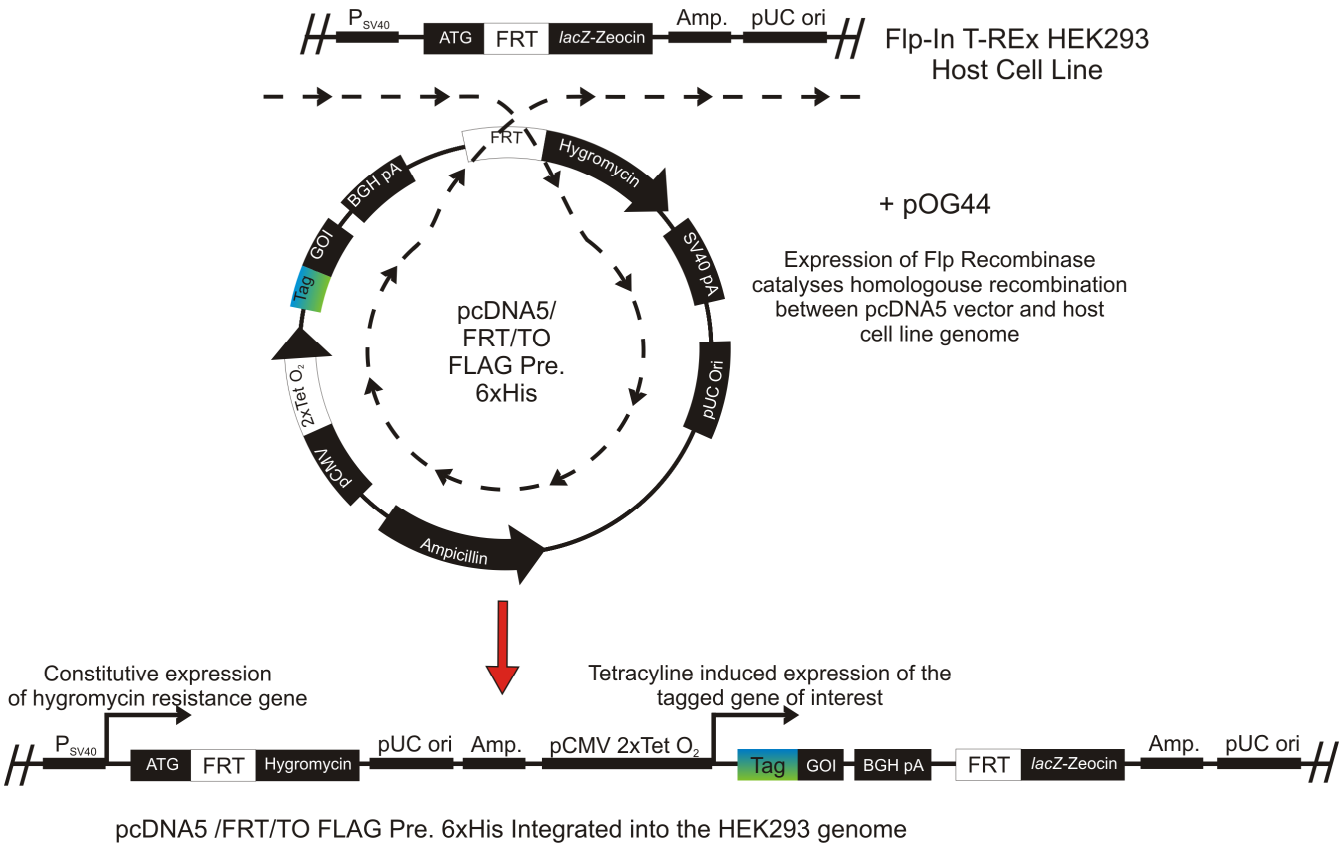


Figure 3.3 Schematic Diagram of pcDNA5/FRT/TO FLAG Pre. 6xHis Homologous Recombination into Flp-In T-REx HEK293 Host Cell Line

Homologous recombination is brought about by co-transfection of the pcDNA5 vector (shown) with pOG44 which constitutively expressed Flp Recombinase under the CMV promoter. This facilitates recombination between the Flp Recombination sites (FRT) in the vector and the pre-engineered HEK293 host cell line (shown by dotted line). Integration brings the SV40 promoter (P_{SV40}) and start codon (ATG) of the host cell line into proximity of the hygromycin resistance gene of pcDNA5, allowing selection of stable transfectants with hygromycin. Shown are; SV40 promoter (P_{SV40}); start codon (ATG); Flp Recombination sites (FRT); hygromycin resistance gene (hygromycin); pUC origin (for propagation in *E. coli*); Ampicillin resistance gene (Amp.); CMV promoter (pCMV); Tetracycline operator sequences (2xTet O_2); 2xFLAG Precission Protease 6xHis Tag (Tag); Gene of interest (GOI); BGH polyadenylation signal (BGH pA, required for efficient termination and polyadenylation of mRNA); and *lacZ* fusion with the zeocin resistence gene (*lacZ*-Zeocin). Based on a figure from Invitrogen.

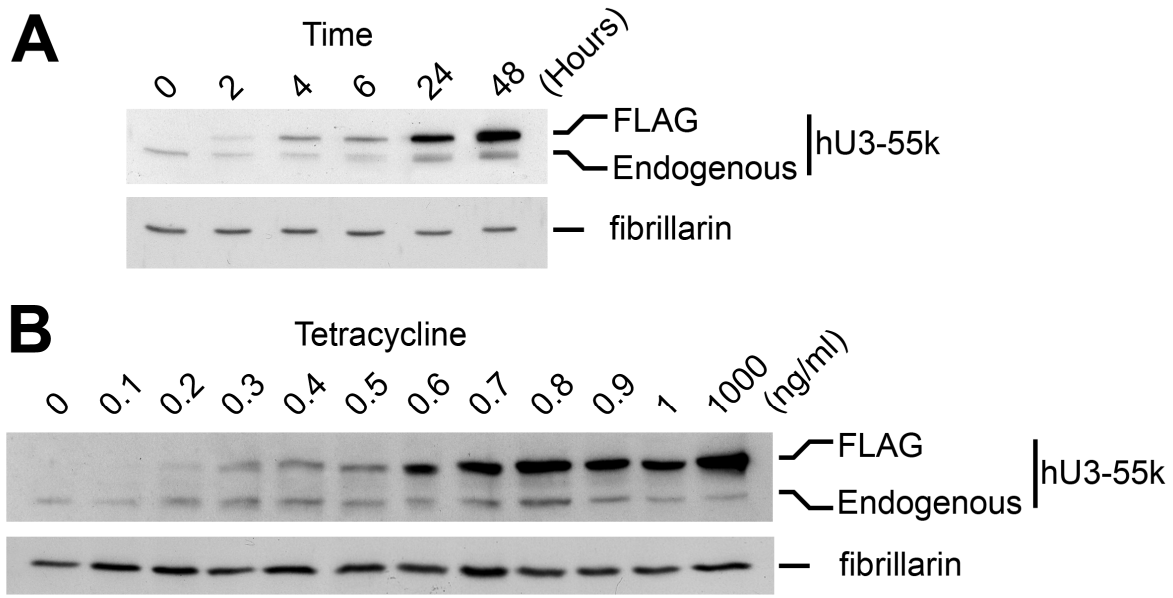


Figure 3.4 Induction of hU3-55k-FLAG Tagged Protein in HEK293 Cells
A) A HEK293 cell line, stably transfected with tagged hU3-55k-FLAG under a tetracycline inducible promoter, was cultured in the presence of 1 µg/ml of tetracycline for 0 – 48 hours as indicated above the panel. Proteins of interest were separated by SDS PAGE and analysed by Western blotting, by probing with primary antibodies to either hU3-55k (detecting both FLAG-tagged and endogenous hU3-55k), or fibrillarin, as indicated to the right of each panel. **B)** A range of tetracycline concentrations, as shown above the panel, were added to cultured hU3-55k-FLAG HEK293 cells for 48 hours before harvesting. Proteins of interest were separated by SDS PAGE and analysed by Western blotting as in (A).

To ensure the hU3-55k-FLAG protein was behaving as expected, we analysed its subcellular localisation, along with that of fibrillarin, by immunofluorescence. Similarly, fibrillarin-FLAG and the FLAG-tag alone inducible cell lines were examined as controls, kindly provided by Charlie Debieux, University of Newcastle. hU3-55k-FLAG cells were induced with either 0, 0.5 ng/ml or 1000 ng/ml tetracycline whereas fibrillarin-FLAG and FLAG-alone (vector) cells were incubated with either no tetracycline (data not shown) or 1000 ng/ml tetracycline, as controls (previously characterised by Charlie Debieux). Antibodies specific to either the FLAG-tagged protein or fibrillarin were used and the nucleus visualised by staining with DAPI; capable of strongly binding DNA in the nucleoplasm (Figure 3.5). This showed that, as widely known, DAPI stains the nucleoplasm much more strongly than the nucleolus, aiding in identification of the nucleolus in all of the conditions. When examining the localisation of the proteins, a low level of hU3-55k-FLAG expression was detectable in the absence of tetracycline. Both without and with 0.5

ng/ml tetracycline, hU3-55k-FLAG localised within the nucleolus, identified by the absence of DAPI staining and the presence of endogenous fibrillarin (Figure 3.5 A & B). Under these conditions, hU3-55k appeared throughout the nucleolus whilst the endogenous fibrillarin appeared slightly more restricted. In contrast, when hU3-55k-FLAG was overexpressed by the addition of 1 µg/ml tetracycline, mislocalisation of the protein was observed such that, whilst much of the protein remained nucleolar, some could also be detected in the nucleus, as shown in comparison to DAPI staining, although none appeared cytoplasmically. The fibrillarin-FLAG cell line showed that the tagged protein co-localised with the endogenous form upon the addition of 1 µg/ml tetracycline indicating expression at this level did not result in mis-localisation, whereas expression of the FLAG protein could not be detected from the FLAG-alone cells upon addition of the same concentration of tetracycline.

Fluorescence *In Situ* Hybridisation (FISH) was performed on similarly treated cells, using fluorescently conjugated probes specific to U3 and U8 snoRNAs (Figure 3.5 C). This enabled us to identify the subcellular localisation of these snoRNAs, with U8 appearing more restricted within the nucleoli; similar to the localisation of fibrillarin, whereas U3 snoRNA appeared more diffuse throughout the nucleoli; similar to that of hU3-55k-FLAG. The U3 snoRNP is usually observed throughout the nucleolus as, during ribosome biogenesis, it moves from the DFC to the GC as a key component of the SSU processome (Gerbi and Borovjagin, 1997; Granneman et al., 2004; Turner et al., 2009). In contrast, the majority of other box C/D snoRNPs, to which fibrillarin is also associated and which there are many more of in total compared to U3, function in the DFC; guiding 2'-*O*-methylation of pre-rRNA. Similarly, the U8 snoRNA is restricted to the FC and DFC where it is required for 28S and 5.8S processing. Accordingly, upon FISH analysis of cells expressing either hU3-55k-FLAG, fibrillarin-FLAG or FLAG-tag alone, U3 snoRNA was observed to have a DFC / GC localisation in comparison to the FC / DFC localisation of U8 snoRNA in all conditions (Figure 3.5 C).

Interestingly, the FISH data demonstrated a marked and specific increase in U3 snoRNA levels (at a set, timed exposure) upon the addition of 1 µg/ml tetracycline to the hU3-55k-FLAG HEK293 cell line, in comparison to U8 snoRNA levels which remained constant between the 0 and 1 µg/ml (+) tetracycline conditions. Moreover, no change in either U3 or U8 snoRNA levels, or localisation, was observed in control cell lines fibrillarin-FLAG and FLAG-tag (vector) in the presence of 1 µg/ml tetracycline, when compared to cells in the absence of tetracycline (Figure 3.5 C and data not shown). Therefore, the data suggest that hU3-55k is specifically and dramatically aiding U3 snoRNA accumulation within the cell. The hU3-55k-FLAG protein also appeared to be stable in the absence of U3 snoRNA as upon expression of the protein with 1 µg/ml tetracycline, some was observed to localise to the nucleoplasm whereas, U3 snoRNA was not observed in the nucleoplasm by FISH analysis of the same cells; suggesting the presence of free hU3-55k-FLAG protein.

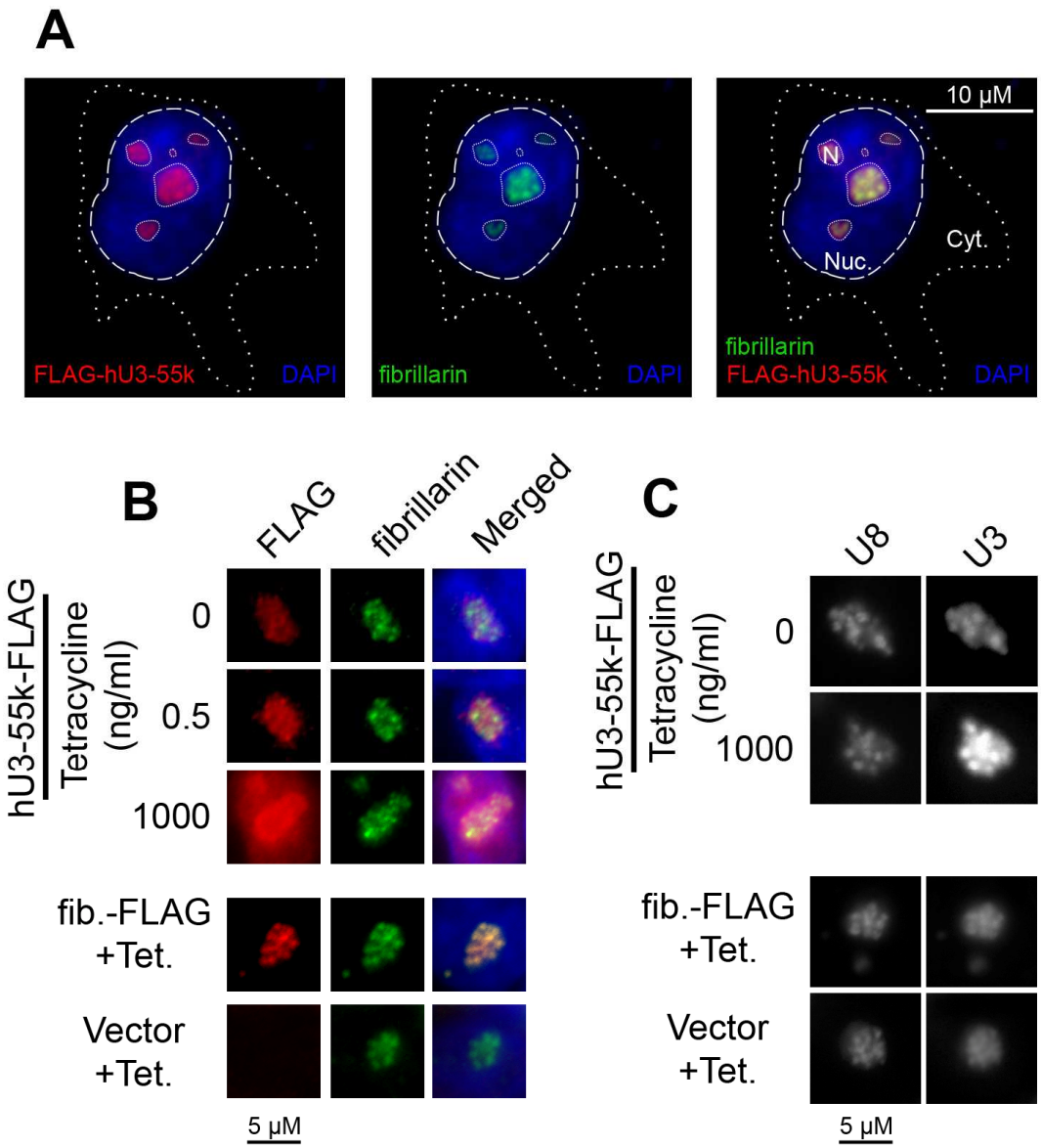


Figure 3.5 Localisation and Expression of hU3-55k and fibrillarin proteins
A) Expression of FLAG-hU3-55k (red) was induced with 0.5ng/ml tetracycline for 48 hours from stably transfected HEK293 cells, fixed and assayed by immunofluorescence using antibodies specific to either FLAG (red) or fibrillarin (green), with the nucleus visualised by staining with DAPI (blue). The cytoplasm (Cyt.), nucleoplasm (Nuc.) and nucleolus (N) are indicated, enclosed with dashed lines. A 10 µM scale bar is shown in the top right. **B)** Protein expression of FLAG-hU3-55k, or controls fibrillarin-FLAG (fib.-FLAG) or FLAG-tag alone (Vector) were induced from stably transfected HEK293 cells by the addition of tetracycline, as shown to the left of each panel, whereby +Tet. indicates 1 µg/ml tetracycline. Cells were grown with or without tetracycline for 48 hours, fixed and assayed as in (A) shown above each panel. **C)** Fluorescence *In Situ* Hybridisation (FISH) analysis was performed on HEK293 cells capable of hU3-55k-FLAG, fibrillarin-FLAG or vector-FLAG expression. Cells were incubated with or without tetracycline (indicated to the left of each panel, as in (A)). Subsequently, cells were fixed and probed using Cy3 and Cy5 conjugated nucleotide probes, complementary to U8 snoRNA and U3 snoRNA, respectively (indicated above each panel). A 5 µM scale bar is shown beneath. Cells and nucleoli shown are representative of 20 cells in each instance.

In order to confirm that the tagged hU3-55k and fibrillarin proteins were behaving as expected, interacting with the U3 snoRNA and forming part of the U3 snoRNP, immunoprecipitation experiments were carried out (Figure 3.6). HEK293 stable cells were induced with the suitable amount of tetracycline to give approximately a 1:1 stoichiometric ratio of tagged protein to endogenous. To achieve this, the cells were incubated with either 1 µg/ml (FLAG-tag alone and fibrillarin-FLAG expression), or 0.5 ng/ml tetracycline (hU3-55k-FLAG expression) and 48 hours later the cells were harvested and extracts prepared. Immunoprecipitation reactions were performed using anti-FLAG antibodies conjugated to Protein G Sepharose beads. Co-precipitated protein and RNA were separated by gel electrophoresis and then analysed by Western or Northern blotting respectively.
The input levels of endogenous hU3-55k and fibrillarin for the FLAG-tag (vector) alone, hU3-55k-FLAG and fibrillarin-FLAG cell lines (lower bands) were approximately equal, with expression of the tagged hU3-55k and fibrillarin (upper bands) comparable to the level of the endogenous proteins (Figure 3.6). Furthermore, each protein resolved at the expected size; hU3-55k at 55 kDa, with the addition of the tag at 64 kDa; fibrillarin at 35 kDa, with the addition of the tag at 45 kDa, approximately (markers not shown). The immunoprecipitations by anti-FLAG antibodies indicated that the FLAG-tag alone was unable to co-precipitate either hU3-55k or fibrillarin. The tagged hU3-55k but not the endogenous protein was efficiently precipitated from the hU3-55k-FLAG cell line, with endogenous fibrillarin also co-precipitated from these cells. Conversely, fibrillarin-FLAG but not the endogenous protein was efficiently precipitated from the fibrillarin-FLAG cell line, with endogenous hU3-55k also co-precipitated. This demonstrated that control cells did not co-precipitate either protein, tagged fibrillarin and hU3-55K proteins specifically co-precipitated the endogenous forms of one another, demonstrating association of the proteins, most likely through the U3 snoRNP.

U1 snRNA levels appeared equal between the inputs although U3 snoRNA levels were slightly increased in the hU3-55k-FLAG cell line, potentially due to the expression of the protein, as previously observed through FISH analysis. The increase of U3 snoRNA levels was however modest, although it should be noted

that this experiment used cells induced at a low level. The FLAG-tag alone did not co-precipitate either RNA whereas both hU3-55k-FLAG and fibrillarin-FLAG specifically co-precipitated U3 snoRNA, hU3-55k-FLAG more efficiently so than fibrillarin-FLAG. This confirmed that FLAG-tagged hU3-55K and fibrillarin were associated with the box C/D U3 snoRNP. It should be noted that this experiment used cells induced at a low level to ensure the proteins were not overexpressed, enabling efficient, yet unbiased incorporation of proteins into the U3 snoRNP.

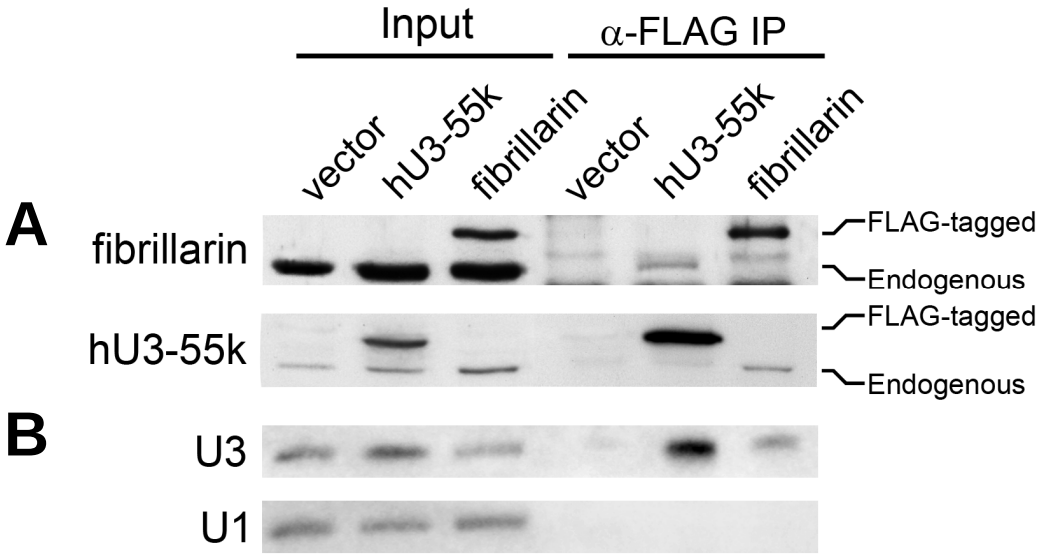


Figure 3.6 FLAG-tagged Proteins are Incorporated into the U3 snoRNP
FLAG-tagged proteins in HEK293 cells induced by the addition of either 1 µg/ml (vector and fibrillarin expression), or 0.5 ng/ml tetracycline (hU3-55k expression) for 48 hours. Cells were harvested and immunoprecipitation reactions performed using anti-FLAG antibodies, with co-precipitated protein and RNA separated by gel electrophoresis. The cell line used is shown above each panel, whereby "vector" contained only the FLAG tag sequence; not detectable by western blotting. **A)** Western blot analysis was performed using antibodies to either fibrillarin or hU3-55k (as shown to the left of each panel), detecting both FLAG-tagged and endogenous proteins (shown to the right of each panel). Note, a background band was present in immunoprecipitations probed for fibrillarin; above that of the endogenous protein. **B)** Northern blot analysis was performed on RNA samples derived from the same cells as used for Western blotting, using radiolabeled probes specific to U3 snoRNA or U1 snRNA.

3.2.3 hU3-55K levels Specifically Control the Cellular Abundance of the U3 snoRNA

We next wished to examine the effect of raising the levels of hU3-55K upon the amount of U3 snoRNA in the cell. To do this, protein expression of hU3-55k-FLAG, fibrillarin-FLAG or FLAG-tag alone from HEK293 cells was induced by incubation with 1 µg/ml tetracycline for 48 hours. Western blot analysis was performed on whole cell lysate from the HEK293 cells, with fibrillarin as a comparison and FLAG-tag alone as a control (Figure 3.7 A). Blots were probed with antibodies specifically detecting epitopes in either the FLAG-tag sequence or fibrillarin. This indicated that expression of endogenous fibrillarin was equal between the different cells. Similarly, expression of tagged fibrillarin and hU3-55k appeared comparable whereas, expression of the FLAG-tag protein alone was not detectable. RNA was extracted from the same cells expressing hU3-55k-FLAG, fibrillarin-FLAG or FLAG-tag alone and equally loaded relative to total RNA levels, separated by denaturing PAGE and analysed by Northern blotting using probes specific to U3 or U8 snoRNAs, or U1 snRNA (Figure 3.7 B). U1 snRNA levels were constant between the cell lines whereas U8 appeared marginally increased in the hU3-55k-FLAG expressing cells, comparative to the fibrillarin-FLAG and FLAG-tag alone cells (Figure 3.7 C). Interestingly, U3 snoRNA levels were significantly increased in the hU3-55k-FLAG expressing cells relative to levels in the other cell lines, and relative to the endogenous controls of U1 snRNA and U8 snoRNA within the same cell line.

Specifically, the over-expression of hU3-55K-FLAG facilitated a three-fold increase in the level of U3 snoRNA relative to U1 snRNA levels, and in comparison to the other cell lines (Figure 3.7 B&C). Taken together, the data demonstrate that raising or reducing the levels of hU3-55K results in a corresponding change in the amount of U3 snoRNA in the cell, thereby providing a novel means of regulating the production of this snoRNP independently of other box C/D snoRNPs. This would suggest that the levels of hU3-55K are limiting for the production of this complex, and that this protein is crucial to the accumulation and/or stability of the U3 snoRNP.

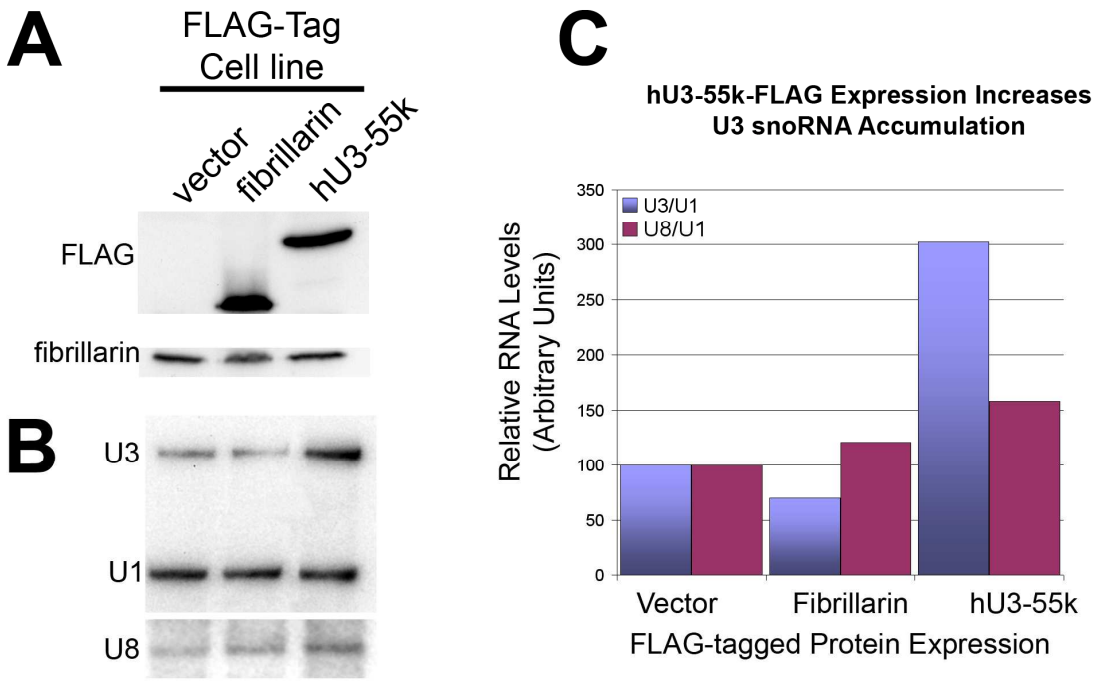


Figure 3.7 hU3-55k-FLAG Expression Specifically Increases U3 snoRNA Accumulation

Expression of FLAG-tagged proteins from HEK293 cells were induced by the addition of 1 µg/ml for 48 hours, cells were harvested and protein and RNA separated by gel electrophoresis. **A)** Western blot analysis of FLAG-tag alone (vector), fibrillarin, or hU3-55k FLAG-tagged proteins (shown above each panel) from the relevant HEK293 stable cell lines. Membranes were probed with antibodies to either FLAG or fibrillarin, as indicated to the left of each panel. **B)** Northern blot analysis of RNA extracted from stable cell lines expressing proteins of interest, indicated above the panels in (A). Equal amounts of total RNA were loaded in each lane. Membranes were probed with radiolabeled probes to either U3 or U8 snoRNA, or U1 snRNA. **C)** A bar chart showing quantitation of U3 (blue) and U8 (red) snoRNA levels relative to that of U1 snRNA, performed on a typical hU3-55k-FLAG induction experiment / Northern blot as seen in (B), whereby equal amounts of total RNA were separated. The three cell lines are shown along the x-axis. Quantitation was performed using ImageQuant TL (GE Healthcare) software with background substitution and relative U3/U1 RNA levels are plotted in arbitrary units along the y-axis.

3.2.4 Glycerol Gradient Analysis of hU3-55k Demonstrates Additional U3snoRNA is Restricted to the 12S U3 snoRNP Monoparticle

Next, we investigated incorporation of hU3-55k-FLAG into the SSU processome, and asked whether increasing the levels of the U3 snoRNP resulted in an increase in the amount of SSU processome. Extracts were prepared from cells expressing either hU3-55K-FLAG at equimolar amounts to the endogenous protein (0.5 ng/ml

tetracycline), overexpressing hU3-55k-FLAG (1 µg/ml tetracycline), or expression of the FLAG-tag alone (1 µg/ml tetracycline), sonicated and separated by glycerol gradient centrifugation. Protein and RNA was extracted from the individual fractions and analysed by Western and Northern blotting respectively. Western blots were probed using antibodies specific to hU3-55k to detect both endogenous and FLAG-tagged hU3-55k proteins. Northern blot hybridisation was performed using probes specific for the U3 snoRNA and U1 snRNA.

It has been previously demonstrated that the U3 snoRNA is present in either a 12S (Svedberg unit) peak; representing the U3 monoparticle containing only the core proteins and hU3-55k, or an 80S peak; representing the SSU processome complex containing the U3 snoRNP with accessory factors and the pre-rRNA (Dragon et al., 2002; Grandi et al., 2002; Granneman et al., 2004; Turner et al., 2009). In all instances, the U3 snoRNA was found distributed between the 12S and 80S complexes (Figure 3.8). Upon expression of either the FLAG-tag alone, or hU3-55k at an equimolar ratio to the endogenous protein, the U3 snoRNA and hU3-55k protein(s) were evenly distributed between the two complexes. In contrast, in cells overexpressing hU3-55K, with three times more U3 snoRNA, the snoRNA was biased towards the 12S complex. This, therefore, implies that the extra U3 snoRNA produced in this cell line was not incorporated into the larger processing complex of the SSU processome, suggesting other limiting factors to SSU processome formation.

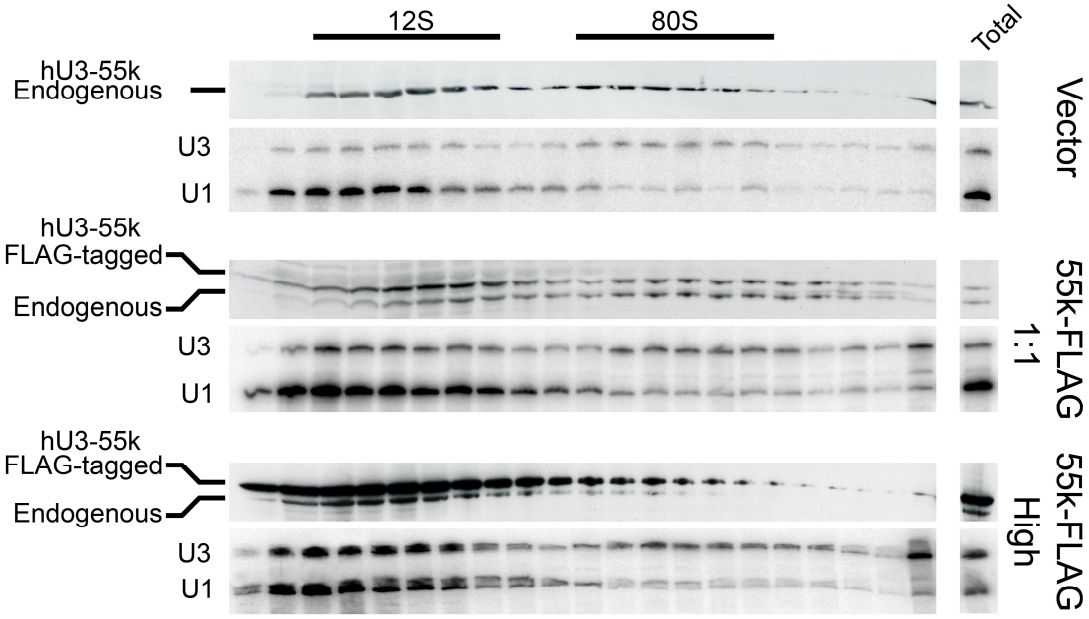


Figure 3.8 Incorporation of Exogenous hU3-55k-FLAG / U3 snoRNP into the SSU Processome

HEK293 cells were cultured for 48 hours in the presence of 0.5 ng/ml (hU3-55k-FLAG 1:1 expression), or 1 µg/ml tetracycline (Vector and hU3-55k-FLAG High expression; as indicated to the right of each panel). Cells were harvested and extracts made by sonication before separation upon 10-40% glycerol gradients. This separated the U3 snoRNP monoparticle (12S) from the SSU processome (80S) as indicated above the panels. Fractions were collected from each gradient, protein and RNA extracted, then separated by gel electrophoresis, with 10% of the original input (Total) run on the right of each gel. Proteins were transferred by Western blotting and probed with anti-hU3-55k antibodies, recognising both endogenous and FLAG-tagged hU3-55k, indicated to the left of each panel. RNA was Northern blotted and hybridised with radiolabeled probes to detect U3 snoRNA and U1 snRNA, indicated to the left of each panel.

3.2.5 The C' Box is Important in Regulating U3 snoRNA Expression

Our data indicate that hU3-55k is able to regulate U3 snoRNP accumulation. As previous evidence has only ever demonstrated necessity of the core box C/D domain and associated proteins (Granneman et al., 2004; Lafontaine and Tollervey, 1999; Samarsky and Fournier, 1998; Watkins et al., 2002), we postulate that hU3-55k influences recruitment of these core proteins to the box C'/D (C/D) motif of U3 snoRNA. Furthermore, as hU3-55K specifically affects U3 snoRNP accumulation, this would suggest that something in the U3 snoRNA, most likely in the C'/D (C/D) motif, renders assembly of this snoRNP dependent on the binding of hU3-55k.

Analysis of the human U3 C'/D (C/D) motif revealed that the C' Box sequence differs from that of the C Box consensus (Figure 3.9 A), whilst the D Box conforms to the consensus sequence. The two uracils of the C Box consensus, are replaced by guanine and adenine, respectively, in the human U3 C' Box sequence. Interestingly, it is known that changing the first U in the U14 snoRNA's C Box motif to a purine, decreases the affinity for 15.5K (Nick Watkins, personal communication; data not shown), with 15.5K said to act as a primary RNA-binding protein that nucleates the assembly of Box C/D snoRNP complexes (Schultz et al., 2006). Furthermore, mutation of the second U in the U14 snoRNA's C Box has been shown to result in a significant reduction in the binding of NOP56, NOP58 and fibrillarin (Watkins et al., 2002). This unusual C' Box was found in vertebrates (excluding fish), but not plant, insect or yeast U3 snoRNA sequences; all of which conform closely to the consensus Box C sequence (Figure 3.9 A). It is possible that this, in theory, "weaker" C' Box renders U3 snoRNA accumulation dependent on hU3-55K association. To test this, we analysed the effect of U3 snoRNA mutations on the snoRNA's ability to accumulate (Figure 3.9 B-D). U3 snoRNA mutants were generated whereby, the Box C motif was altered to block hU3-55K-binding (Granneman et al., 2002; Lukowiak et al., 2000) and C'/D motif optimised, by conversion of the C' Box to that of the consensus sequence (Figure 3.9 D). The mutations were generated in the StreptoTagged U3 expression construct, in which the streptomycin tag had been inserted into the snoRNA sequence (Figure 3.9 B), previously used to analyse the role of conserved sequences in U3 snoRNP formation and function (Bachler et al., 1999; Granneman et al., 2004), with the constructs under the control of the endogenous promoter. The wild-type and mutant U3 snoRNA-containing plasmids were transfected into HeLa cells, and 12 hours later nucleic acid extracted and analysed by Northern blotting. Membranes were analysed using specific probes to the StreptoTag sequence (Figure 3.9C; upper panel). To control for transfection efficiency, we also performed semi-quantitative PCR, using primers specific for the plasmid encoding the StreptoTagged U3 snoRNA (Figure 3.9 C; lower panel). The amount of StreptoTagged U3 snoRNA expressed was then normalised to the levels of the

transfected plasmid for each sample and plotted as a percentage of the expression seen for the wild-type snoRNA (Figure 3.9D).

A clear signal was detected for wild-type tagged U3 snoRNA levels from transfected cells, whilst no accumulation was observed in the untransfected control sample, as expected (Figure 3.9C). After normalisation, we observed that mutation of Box C (mutC), thus presumably preventing hU3-55k association, resulted in significantly reduced U3 snoRNA levels (Figure 3.9D). This is consistent with previous observations (Granneman et al., 2004) although the significance of this at the time was unclear. Conversely, alteration of the C' Box to the optimal consensus sequence (C'con), increased U3 snoRNA levels ~60%. Importantly, mutation of Box C' to the consensus sequence, in the presence of the Box C mutation (mutC/C'con), resulted in restoration of tagged U3 snoRNA accumulation. One would assume this occurred through by-passing the requirement for hU3-55k association. This data, therefore, supports the hypothesis that the presence of a "weak" C' Box in U3 snoRNAs makes accumulation dependent upon the B/C motif in higher eukaryotes.

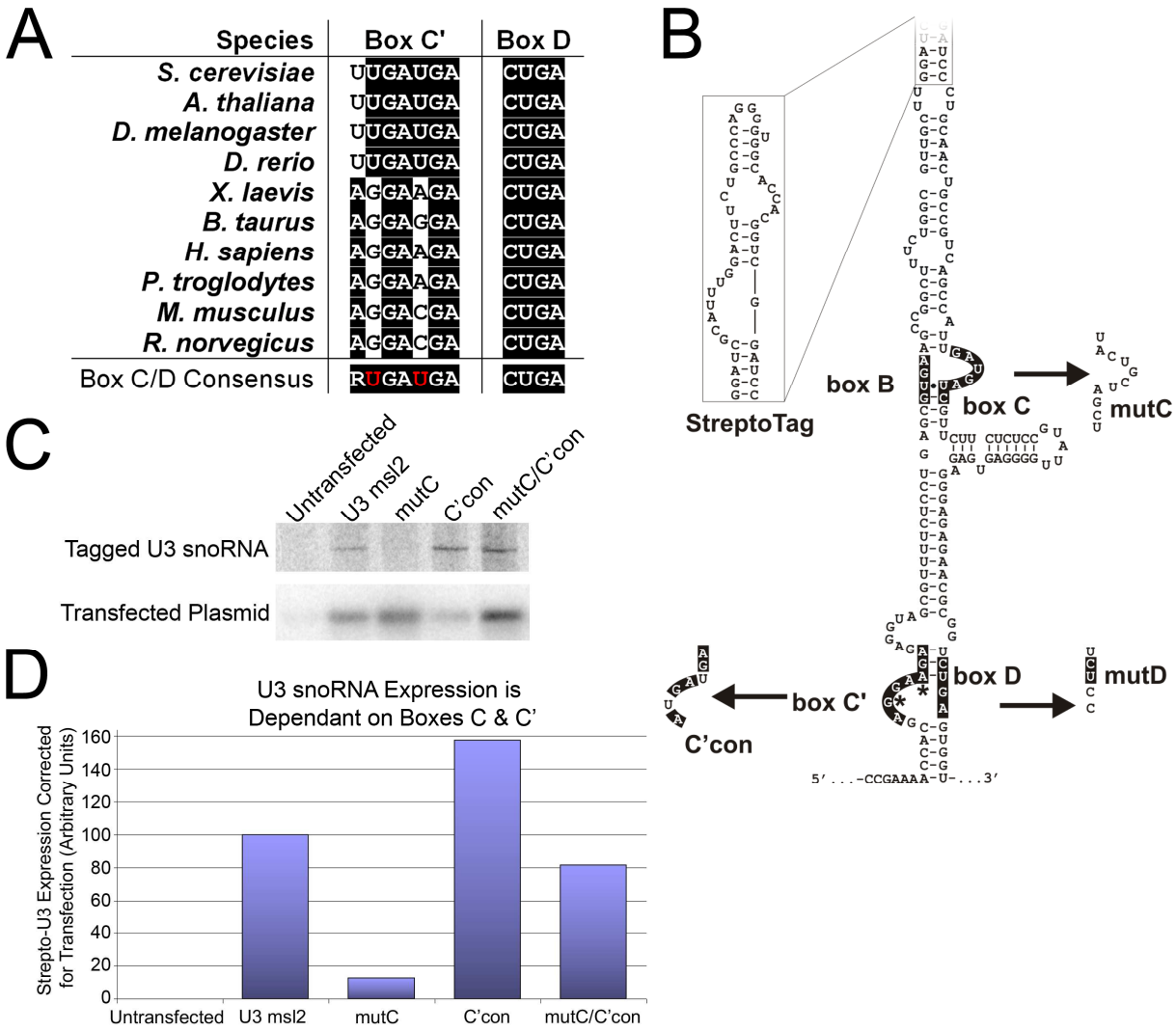


Figure 3.9 The C Box is Not Required in the Presence of a C' Consensus Box

A) Alignment analysis of U3 snoRNA box C'/D sequences from a range of species (shown to the left). The conserved box C/D consensus, whereby R is a purine, is shown at the bottom. Nucleotides not conserved in the higher eukaryote U3 snoRNA C' box are drawn in red. Conserved nucleotides in either the box C'/D motif are depicted as white on a black background. Alignments performed using ClustalX. **B)** A series of StreptoTagged (drawn as a boxed sequence) U3 snoRNA mutants were generated by site-directed mutagenesis in expression vectors, with mutations shown by arrows and the novel sequences depicted. The non-conserved G and A of Box C' are indicated by askterisks, conserved boxes with a black background, drawn with the same secondary structure as previously described (Granneman et al., 2004). **C)** U3 snoRNA mutants were transfected into HeLa cells and expressed for 12 hours. Cells were harvested, RNA extracted, separated by gel electrophoresis, Northern blotted and probed with a radiolabeled probe specific to the StreptoTag sequence; top panel. DNA was also extracted and subject to semi-quantitative PCR, using specific primers to the U3 snoRNA-containing plasmids to detect transfection efficiencies. Products were separated by agarose gel electrophoresis, Southern blotted and probed for a sequence specific to the plasmid; bottom pabel. **D)** A bar chart showing the relative expression of each tagged U3 construct. The experiment described in (C) was repeated 3 times and quantified using ImageQuant TL (GE Healthcare). The average expression of the tagged-U3 constructs relative to transfection efficiencies was plotted in arbitrary units (y-axis). The tagged U3 constructs transfected in each case are shown along the x-axis, as depicted in (B).

To examine the nucleolar, and sub-nucleolar localisation of the various U3 snoRNA mutants, FISH analysis of transfected cells was carried out. The StreptoTagged U3 snoRNA constructs described previously were transfected into HeLa cells and incubated for 12 hours. Cells were then fixed and analysed by FISH, using probes specific to the StreptoTag sequence or U3 snoRNA, with exposure times adjusted for signals to appear equal (Figure 3.10 A). Similarly, cells were probed separately for U8 snoRNA (to identify the DFC of the nucleolus), U2 snRNA (to identify Cajal bodies) and the StreptoTag sequence (Figure 3.10 B). In both cases, DAPI staining was used to identify the nucleus, and nucleoli (not shown). In untransfected cells, U3 snoRNA was observed throughout the nucleolus (DFC/GC), U8 snoRNA only in the DFC region of the nucleolus and U2 in Cajal bodies (as previously described (Carmo-Fonseca et al., 1992; Granneman et al., 2004)) with no signal detectable for the StreptoTag. Upon transfection with the wild-type U3 snoRNA, the signal from the StreptoTag and endogenous U3 snoRNA both appeared throughout the nucleolus. Endogenous U3 snoRNA appeared largely unaffected by transfection of the U3 snoRNA constructs, localising throughout the nucleolus in all cases. It should be noted that although the U3 snoRNA specific probe detected both endogenous and tagged U3 snoRNA, expression of the endogenous U3 was much higher than the tagged form (due to the difference in microscopy exposure times and Northern blots; data not shown), so is expected to have provided the majority of the signal. Similarly, the localisation profile of U8 snoRNA, and U2 snRNA appeared unchanged, compared to the control, upon the transfection of all the constructs.

The data illustrated that whilst wild-type and Box C' consensus (C'con) tagged U3 snoRNAs co-localised with the endogenous U3 snoRNA, neither U3 snoRNA construct containing the mutC motif (mutC or C'con/mutC), were able to do so; appearing restricted to the DFC region of the nucleolus, co-localising with U8 snoRNA, in a similar fashion to previous data (Granneman et al., 2004). Furthermore, the DFC/GC localisation of U3 snoRNA compared to the DFC restricted StreptoTagged mutC U3 snoRNA, illustrated that whilst the U3 snoRNA specific probe detected both endogenous and tagged U3 snoRNA, the majority of

signal detected from the U3 snoRNA probe was due to the endogenous snoRNA, allowing dissemination of the two U3 snoRNAs present (Figure 3.10 A).

Mutation of box C (mutC), and the mutation of box C' to the consensus sequence, in the presence of the box C mutation (mutC/C'con), resulted in tagged U3 snoRNA co-localisation not only with U8 snoRNA in the FC/DFC, but also appearing enriched in Cajal bodies, co-localising with U2 snRNA; believed to be a site of snoRNA maturation (Jacobson and Pederson, 1998; Terns and Terns, 2002; Verheggen et al., 2002) (Figure 3.10 B; indicated by arrows). This suggests that, similar to hU3-55k depletion by siRNAs, mutation of the C box and therefore, presumably loss of hU3-55k association, prevents efficient biogenesis of functional U3 snoRNP. Furthermore, it would appear that the small amount of mutC U3 snoRNP which was produced, was unable to translocate with the pre-rRNA into the granular component of the nucleolus as a component of the SSU processome (as previously described (Granneman et al., 2004)), where it is believed to play a role in processing, emphasising the importance of hU3-55k not only in accumulation, but also in function. It should be noted that mutC U3 snoRNA localisation to the DFC is expected to be due to a very low level of NOP58 association in the absence of hU3-55k, as indicated in the next section (Figure 3.11).

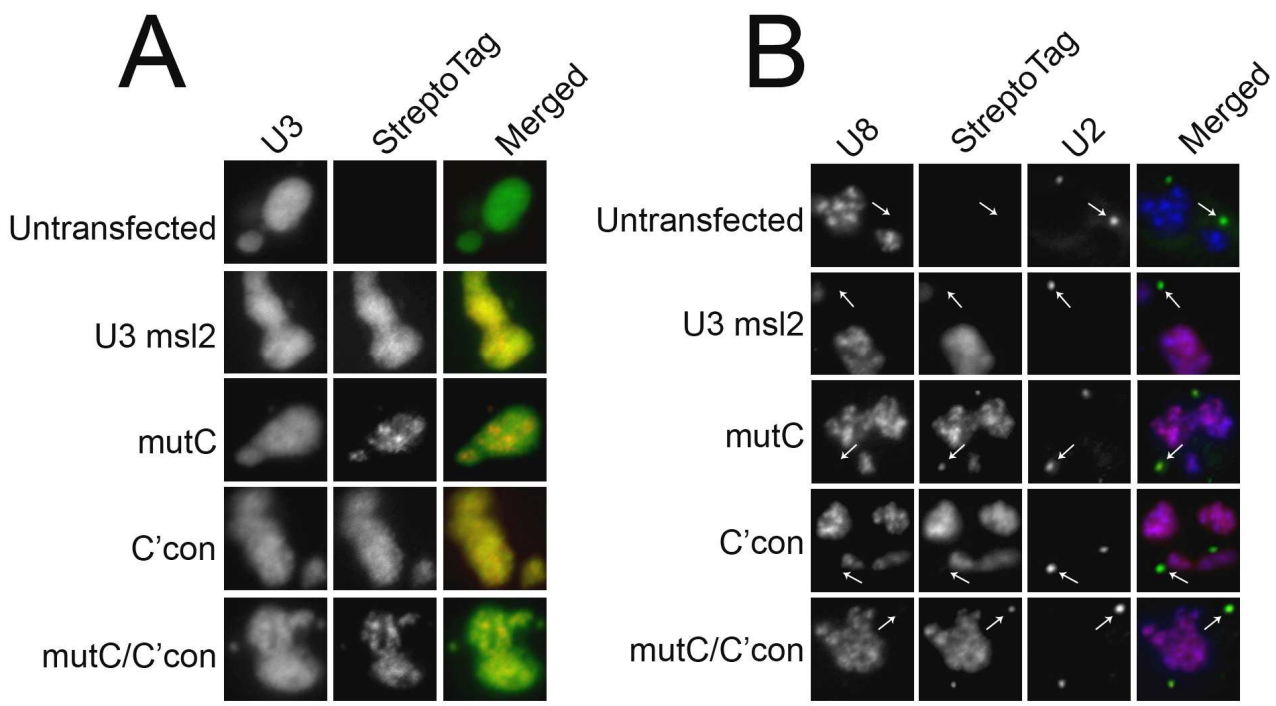


Figure 3.10 An Intact C Box Motif is Required for Correct Nucleolar Localisation of U3 snoRNA

StreptoTagged U3 snoRNA mutants (shown to the left of each panel) were transfected into HeLa cells and expressed for 12 hours. Cells were fixed and analysed by FISH using probes to specific RNAs. **A)** Cells were probed with fluorescently labelled oligonucleotides to either U3 snoRNA (U3; green) or to the StreptoTag sequence (StreptoTag; red) as indicated above each panel with the composite of the two channels shown as merged. It should be noted that whilst the U3 snoRNA specific probe detected both endogenous and tagged U3 snoRNA, expression of the endogenous U3 was much higher than the tagged form, so is the source of the majority of the signal in this channel. **B)** FISH analysis was performed on transfected HeLa cells as in (A) using probes to U8 snoRNA (U8; blue), the StreptoTag sequence (StreptoTag; red), U2 snRNA (U2; green) as shown above each, with the final panel displaying the merged channels. U2 snRNA localised in Cajal bodies; white arrows. It should be noted that exposure times were adjusted for signals to appear equal. DAPI staining was used to identify the nucleus but has not been shown for simplicity. Transfection efficiency ~ 30% of cells, images representative of 20 cells examined in each instance.

3.2.6 *In vitro* Characterisation of hU3-55k Demonstrates that hU3-55k Binding to the U3 snoRNA is Required for snoRNP Assembly

In light of our findings, that due to the "weak" C' box in U3 snoRNAs of higher eukaryotes, efficient assembly of the U3 box C/D snoRNP is dependent upon hU3-55K-association. We therefore, postulated that hU3-55k is involved in the assembly of the core box C/D complex. To test this, we analysed the role of hU3-55k in an *in vitro* assembly of the U3 snoRNP. Wild-type U3 snoRNA, as well as constructs

containing mutations in the C box, C' box and D box (Figure 9 B), were all produced and ^{32}P-labeled with UTP^{32} through *in vitro* transcription. Radiolabeled U3 snoRNAs were then incubated in mouse nuclear extract and the complexes formed were immunoprecipitated using antibodies that specifically recognised hU3-55K or NOP58 proteins, as described previously (Granneman et al., 2002; Watkins et al., 2002). Co-precipitated RNAs were separated on a denaturing polyacrylamide gel, analysed by PhosphorImager and quantified using ImageQuant software (GE Healthcare).

The wild-type, C'con and mutD U3 snoRNAs were all efficiently co-immunoprecipitated by anti-hU3-55K antibodies after incubation in nuclear extract (Figure 3.11). In contrast, anti-hU3-55K antibodies failed to co-purify the mutC and mutC/C'con RNAs; consistent with previous data that identified the B/C motif as the binding site for hU3-55K (Granneman et al., 2002; Lukowiak et al., 2000). The wild-type U3 snoRNA and the C'con mutant were co-precipitated by anti-NOP58 antibodies whereas, the mutC and mutD RNAs were poorly co-precipitated. Interestingly, the mutC/C'con double mutation was efficiently co-precipitated by anti-NOP58, but not by anti-hU3-55K antibodies. Therefore, mutating the U3 snoRNA C' box to that of the consensus sequence removed the requirement of an intact B/C box, and presumably hU3-55k association, for core U3 snoRNP assembly *in vitro*. This data strongly suggests that hU3-55K-binding to the B/C motif is directly involved in the assembly of the core box C/D proteins onto the C'/D motif of the U3 snoRNA. Furthermore, the mutD mutant displayed only reduced NOP58 association whereas, it has previously been demonstrated in U14 snoRNA that this abolishes association of NOP58, and all other core box C/D proteins (Watkins et al., 2002). In contrast, the mutD U3 snoRNA mutant did not noticeably reduce hU3-55k association, suggesting that in the U3 snoRNP, hU3-55k's binding of U3 snoRNA may facilitate association of NOP58 independently of the box C/D motif, allowing hU3-55k to regulate U3 snoRNP accumulation.

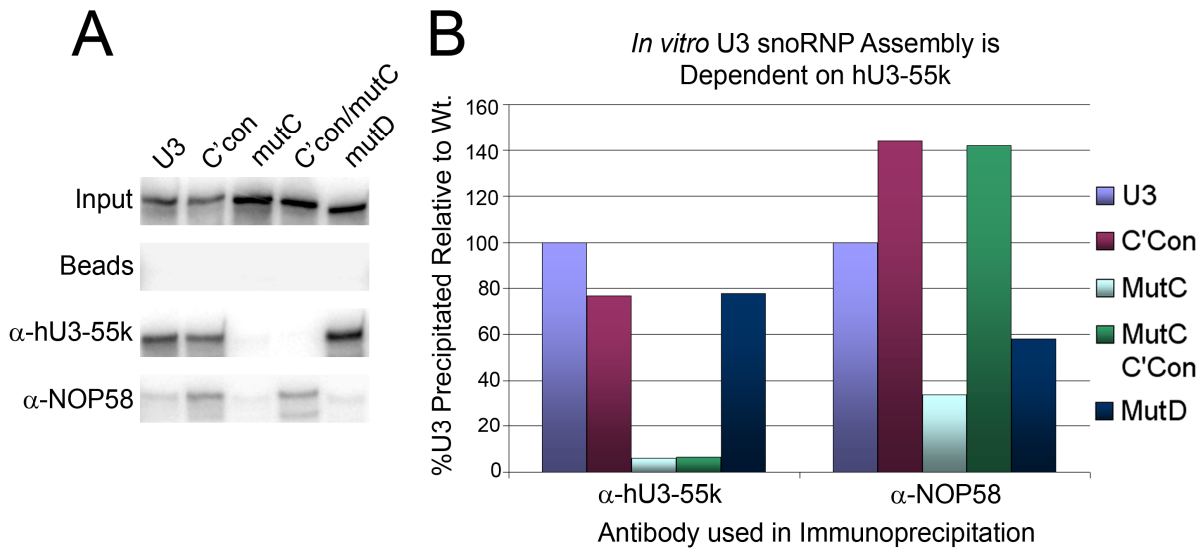


Figure 3.11 Assembly of U3 snoRNP is Dependent upon hU3-55k Association
A) Northern blot analysis of U3 snoRNP assembly. A wild-type U3 snoRNA construct (U3) and constructs with either a C' consensus box (C'con), mutant C box (mutC), or both (C'con/mutC) or a mutant D box (mutD; as shown above each panel) were transcribed and thus ^{32}P-labeled *in vitro*. Radiolabeled U3 snoRNA were incubated in mouse nuclear extract and the complexes formed were immunoprecipitated using antibodies specific to hU3-55K or NOP58 proteins, no antibodies were used as a negative control (beads; shown to the left of the panel). 10% of the transcription reaction was used as an input control (Input). Co-precipitated RNAs were separated on a denaturing polyacrylamide gel, analysed by PhosphorImager and quantified using ImageQuant software (GE Healthcare). **B)** Bar chart of the efficiency of U3 snoRNA co-precipitation by anti-hU3-55k and NOP58 antibodies. A representative *in vitro* assembly experiment, shown in (A) was quantified using ImageQuant software (GE Healthcare). The signal from beads alone was subtracted from the specific signal in each case. The percentage of each U3 snoRNA construct co-precipitated by antibodies recognising either hU3-55k or NOP58 (X axis), was plotted relative to co-precipitation of the wild-type U3 snoRNP by each antibody (Y axis). The U3 snoRNA constructs are as labelled in the figure legend to the right of the graph, named as in (A).

3.2.7 hU3-55k Associates with NOP56 and NOP58 *in vitro*

The hU3-55K protein is not predicted to bind the C'/D motif of the U3 snoRNA, where other core proteins are known to associate (Granneman et al., 2009; Granneman et al., 2002). Thus, it is likely that hU3-55k interacts with either the core box C/D proteins and/or the snoRNP biogenesis factors, to directly facilitate snoRNP formation. We therefore tested the interaction of *in vitro* translated, [^{35}S]methionine-labeled hU3-55K with either GST-tagged 15.5K and fibrillarin, or thioredoxin-tagged NOP56 and NOP58; core box C/D proteins (Figure 3.12 A and B). Similarly, [^{35}S]methionine-labeled hU3-55K interactions were examined with GST-tagged biogenesis factors (Figure 3.12). The tagged proteins were

overexpressed and purified from *E. coli* as described in materials and methods, and previously demonstrated (McKeegan et al., 2007; Schultz et al., 2006). In order to obtain suitable amounts of soluble protein, constructs were used in which the sequences coding for the RGG motif of fibrillarin, and the KKE/KKD regions of NOP56 and NOP58 were deleted. *In vitro* translated radiolabeled hU3-55K, translated in rabbit retic lysate, was used in these experiments as, when overexpressed with either thioredoxin or GST-tags in *E. coli*, hU3-55k was insoluble (data not shown). The recombinant proteins were incubated with [^{35}S]methionine-labeled hU3-55K and the complexes purified using either glutathione sepharose or anti-thioredoxin antibodies bound to protein A sepharose, as appropriate. The bound protein was then resolved by SDS PAGE and analysed by autoradiography.

The [^{35}S]methionine-labeled hU3-55K protein efficiently co-purified with thioredoxin-tagged NOP56 and NOP58 (Figure 3.12 B). The protein did not co-purify when either thioredoxin or antibody bound beads alone were used. hU3-55K did not co-purify, to any significant extent, with either GST-15.5K, GST-fibrillarin or GST alone (Figure 3.12 C). It is important to note that a weak interaction between 15.5K and hU3-55K was previously reported (Schultz et al., 2006). This interaction, however, only occurred at much higher concentrations of GST-15.5K than those used here (see Materials & Methods). Moreover, the interaction was not observed when less protein was used, in agreement with our observations (Schultz et al., 2006). [^{35}S]methionine-labeled hU3-55K did not co-purify above background levels with GST-tagged box C/D snoRNP biogenesis factors TIP48 or TIP49 whereas very weak signals were seen with GST-tagged fibrillarin, BCD1 and NUFIP. A weak interaction was reproducibly seen with GST-NOP17, shown to be crucial for maintaining box C/D snoRNP levels in human cells (McKeegan et al., 2007). Taken together, these data indicate that hU3-55K influences snoRNP assembly by directly interacting with the core box C/D proteins NOP56 and NOP58 and, to a lesser extent, the snoRNP biogenesis factor NOP17.

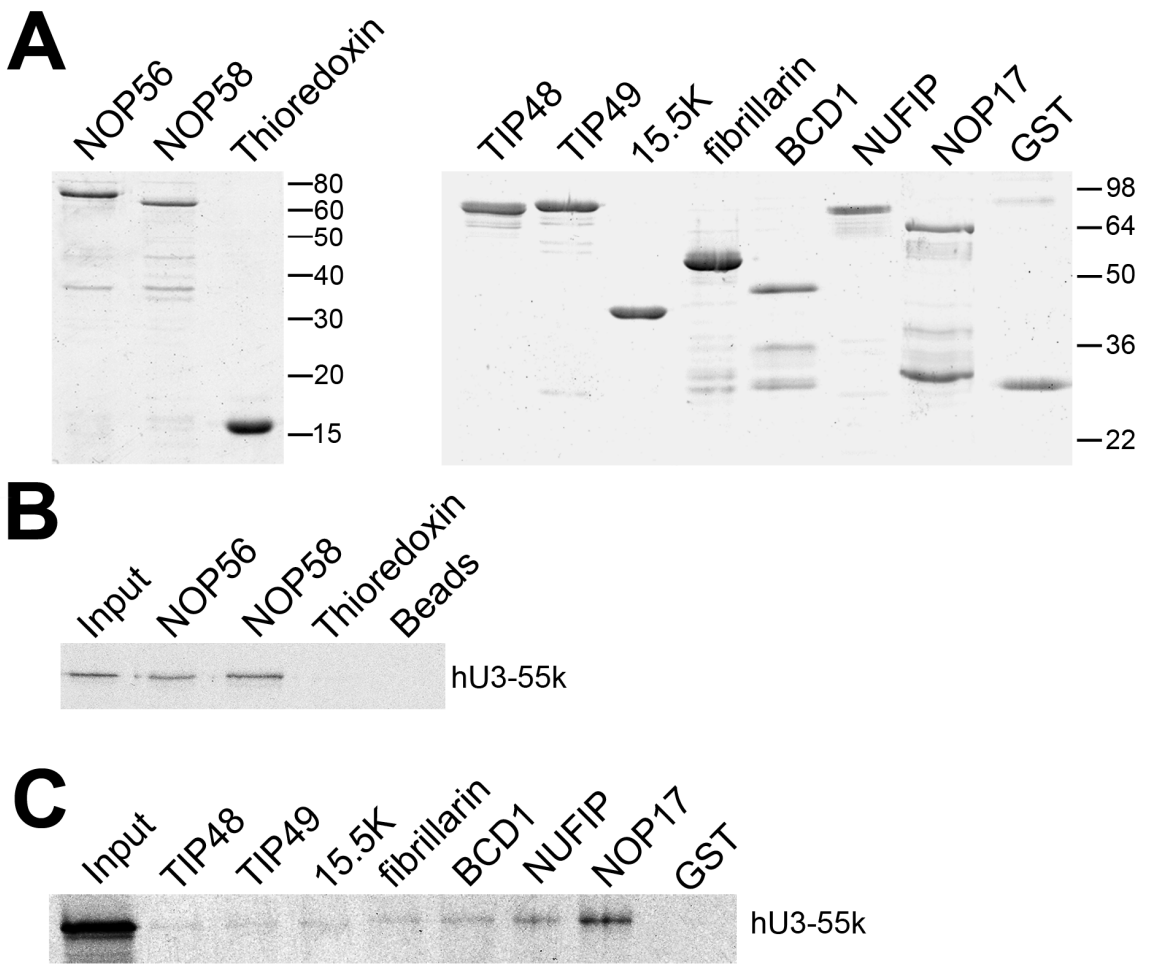


Figure 3.12 hU3-55k Interacts with NOP58, NOP56 and NOP17 *in vitro*
A) Proteins of interest were expressed in *E. coli* and purified by either their thioredoxin-tag (NOP56, NOP58, Thioredoxin; left panel) or their GST-tag (TIP48, TIP49, 15.5K, fibrillarin, BCD1, NUFIP, NOP17, GST; right panel). Purified proteins were then separated by SDS PAGE and visualised by Coomassie staining. The protein loaded is indicated at the top of each lane. The positions of the marker proteins are indicated to the right of each panel. **B)** Purified thioredoxin and thioredoxin-tagged fusion proteins of NOP56 and NOP58 were bound to anti-thioredoxin antibody-coupled protein-A sepharose beads, then incubated with [^{35}S]methionine-labelled *in vitro* translated hU3-55K. Co-precipitated complexes were separated by SDS PAGE and the presence of hU3-55k analysed by PhosphorImager. **C)** Recombinant purified GST, and GST-tagged proteins were bound to glutathione sepharose and incubated with [^{35}S]methionine-labelled *in vitro* translated hU3-55K, as in (B). Bound proteins were separated by SDS PAGE and analysed by PhosphorImager. The purified protein used in each case is indicated above each panel. Input is 10% of the [^{35}S]methionine-labelled hU3-55K protein used in the interaction assay.

3.2.8 U3 snoRNA is Specifically Down-regulated During Epithelial Cell Differentiation

U3 snoRNA levels have previously been shown to be down-regulated during myoblast differentiation, correlating with decreased rates of ribosome production in terminally differentiated myotubes (Glibetic et al., 1992). It was not clear, however, if this occurs during other cellular differentiation events, whether this is specific to the U3 snoRNA, or if the levels of all box C/D snoRNAs are reduced. We therefore investigated the changes in snoRNA levels during the differentiation of lung (CaLu-3) and intestinal (CaCo-2) epithelial adenocarcinoma cells, with differentiation of the latter said to represent loss of its tumourigenic phenotype (Stierum, 2003).

RNA was extracted from both undifferentiated and differentiated cells and analysed by Northern blotting, using probes specific for the U3, U8, U13 and U15 box C/D snoRNAs, and the U1 snRNA; a component of the spliceosome (Figure 3.13). Obtaining an accurate cell count with differentiated cells proved difficult; therefore, each lane was loaded with an equal amount of RNA. The total level of ribosomes in differentiated cells (which constitute up to 80% of the cell's RNA content), however, is expected to be between ~2 – 4-fold lower than that observed in actively growing cells, although this has never been measured in CaCo-2 or CaLu-3 cells (Bowman and Emerson, 1977; Datta et al., 1997; Poortinga et al., 2004).

Relative to the total RNA, the amount of U1 snRNA and U15 snoRNA was constant in both un-differentiated and differentiated CaCo-2 and CaLu3 cells (Figure 3.13). The levels of the U8 and U13 snoRNAs were slightly higher in the RNA isolated from differentiated cells than undifferentiated CaCo-2 and CaLu3 cells. Conversely, and most importantly, U3 snoRNA levels were significantly reduced during the differentiation of both cell types. Quantitation of this data (not shown) revealed a four fold change in the levels of the U3 snoRNA relative to that of the U8 or U13 snoRNAs, during epithelial cell differentiation.

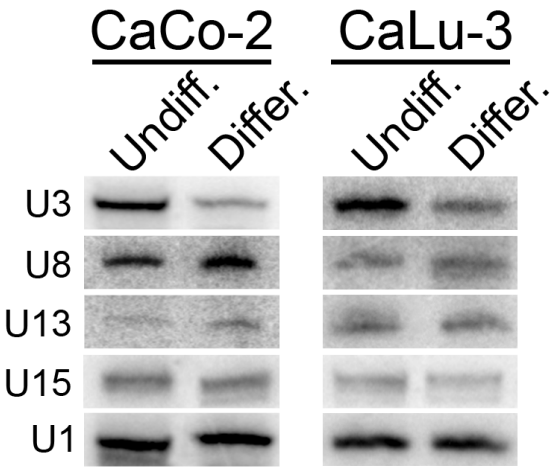


Figure 3.13 U3 snoRNA is Specifically Down-regulated upon Cellular Differentiation
CaCo-2 intestinal and CaLu-3 lung epethial adenocarcinoma cells were cultured in Transwells. RNA was extracted before (Undiff.) and after differentiation (Differ.; indicated at the top). Equal amounts of RNA were separated an 8% polyacrylamide / 7M urea gel and analysed by Northern blotting, using radiolabeled probes for the U3, U8, U13 and U15 box C/D snoRNAs, and the U1 snRNA; indicated to the left of each panel. The cell type from which the RNA was extracted is indicated above each panel.

3.2.9 hU3-55k and a Sub-set of SSU Processome Components are Specifically Down-regulated During Cellular Differentiation

Lastly, we investigated whether the levels of U3 snoRNP proteins also changed in a similar fashion to the U3 snoRNA, during cellular differentiation. Proteins from both undifferentiated and differentiated CaCo-2 and CaLu3 cells were separated by SDS PAGE and analysed by Western blotting. Protein loading was normalised to the U5-116K protein; a component of the spliceosome, comparable to the U1 snRNA loading control used previously (Figure 3.14). Western blots were probed using antibodies to core box C/D proteins and components of the SSU processome (Figure 3.14). The level of the U3-specific hU3-55K protein was significantly lower in differentiated cells than in undifferentiated cells, mirroring the reduction observed in U3 snoRNA levels. Contrastingly, the levels of the core box C/D proteins (common to all box C/D snoRNPs) NOP58, NOP56 and fibrillarin, were constant in both differentiated and undifferentiated cells. The U3 snoRNP is the most abundant snoRNA in the cell but it likely represents less than 20% of the total population of

box C/D snoRNAs in the cell. Therefore, consistent with our observations, changes in U3 snoRNP levels would not be expected to significantly alter core box C/D protein levels.

We also investigated the effect of differentiation upon the levels of other SSU processome components. Western blot analysis revealed that that the levels of KRR1 did not change during differentiation, relative to U5-116K. In contrast, the levels of the SSU processome components PNO1, MPP10, Nucleolin, UTP12 and PRP43 were all significantly reduced upon differentiation. The data therefore, indicate that a subset of SSU processome components are downregulated, together with the U3 snoRNP, during epithelial cell differentiation. This implies that these factors may be rate limiting, such that regulating their levels may control pre-rRNA processing activity. It has previously been demonstrated that the mRNAs of hU3-55k and the core box C/D proteins (along with SSU processome components) and U5-116k are co-ordinately regulated by c-Myc (Coller et al., 2000; Menssen and Hermeking, 2002; Schlosser, 2003) however, this data provides clear evidence for independent regulation of these factors.

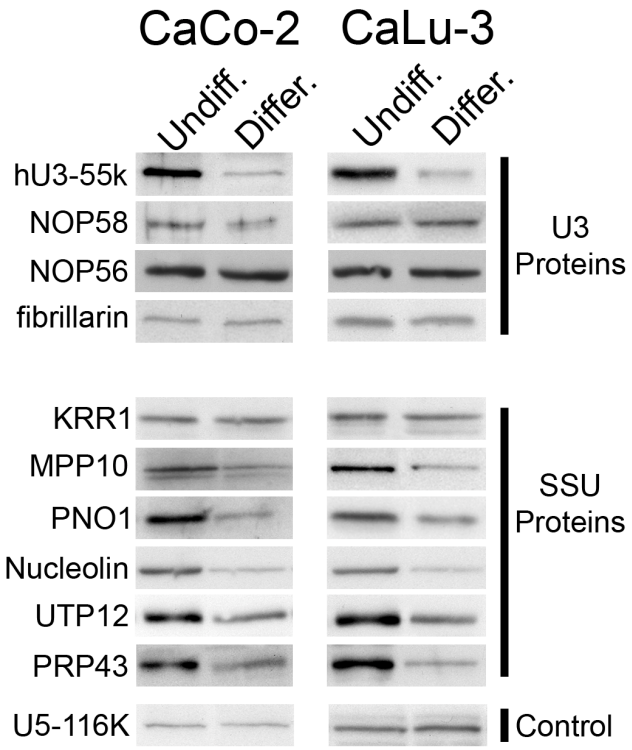


Figure 3.14 hU3-55k and Several SSU Processome Components are Specifically Down-regulated during Epithelial Cellular Differentiation
CaCo-2 intestinal and CaLu-3 lung epethial adenocarcinoma cells (indicated at the top) were cultured in Transwells. Protein was extracted from cells before (Undiff.) and after differentiation (Differ.; indicated above the panels). Equal amounts of protein were separated (loaded according to U5-116k levels) by SDS PAGE and analysed by Western blotting, using antibodies specific to a range of nucleolar proteins (indicated to the left of each panel).

3.3 Discussion

Ribosome biogenesis is inherently linked to cell growth and proliferation (Bernstein and Baserga, 2004; Bernstein et al., 2007), with transcription and processing of the rRNA up regulated during tumourogenesis (Oskarsson and Trumpp, 2005; Ruggero and Pandolfi, 2003), and down-regulated upon cellular differentiation (Bowman and Emerson, 1977; Poortinga et al., 2004). Protein translational capacity is influenced by the number of ribosomes in the cell (Burrin et al., 1997), thus regulating cellular growth. Moreover, signaling pathways that detect nutrient availability such as Ras/PKA and TOR (Guertin et al., 2006; Jorgensen et al., 2004; Soulard et al., 2009; Yang et al., 2008), the proto-oncogene c-Myc (Dai and Lu, 2008; Li et al., 2010; Shiue et al., 2009; Teleman et al., 2008), and tumour suppressors p53, pRB and ARF (Larminie et al., 1998; Oskarsson and Trumpp, 2005; Sugimoto et al., 2003; White, 1997; White, 2005) are all key regulators of both ribosome biogenesis and cell cycle control in eukaryotes. Studies have also shown cases of pre-rRNA processing, rather than pre-rRNA transcription regulation, being used to control mature ribosome levels in some instances of tumourogenesis and differentiation (Bowman and Emerson, 1977; Dudov and Dabeva, 1983; Pajic et al., 2000; Schlosser et al., 2005). Collectively, this demonstrates the importance of regulating pre-rRNA processing and ribosome biogenesis.

The U3 snoRNP is crucial to the rRNA cleavages required to produce the mature 18S rRNA small ribosomal subunit. As relatively little was previously known about control at the level of pre-rRNA processing, especially in humans, we investigated the regulation of the U3 snoRNP. We set about selectively depleting hU3-55k levels through siRNA transfections of HeLa cells, resulting in a specific decrease in U3 snoRNA levels. We subsequently created a vector and construct to inducibly and stably express FLAG-tagged hU3-55k. Upon doing so, an increase in U3 snoRNA levels could be observed by Northern blot analysis. This raised the question of how levels were being modulated by hU3-55k, as it was previously thought that only the core Box C/D proteins were required for efficient snoRNP

accumulation (Lafontaine and Tollervey, 1999; Lafontaine and Tollervey, 2000; Venema et al., 2000; Watkins et al., 2002; Watkins et al., 2004).

Utilising transfections into HeLa cells, and *in vitro* assembly assays of tagged U3 snoRNAs / mutant constructs, we have shown that the U3 snoRNP-specific protein hU3-55k is capable of modulating U3 snoRNA levels through recruitment of the core box C/D snoRNP proteins NOP58 and NOP56. This is consistent with the previous observation, that hU3-55k associates early in the biogenesis of the U3 snoRNP, at a similar time to NOP56 and NOP58 core proteins, but potentially before fibrillarin (Lukowiak et al., 2000; Watkins et al., 2004). Regulation of the U3 snoRNP by hU3-55k is specific, occurring independently of all other box C/D snoRNPs examined. Interestingly, the regulation appears to occur through the presence of the aforementioned “weak” C’ box motif which differs from the consensus sequence observed in lower eukaryotes, and in C boxes of all other box C/D snoRNAs. This non-consensus sequence appears specifically in higher eukaryotes, apparently diverging at the point of amphibian evolution. Fish display the consensus sequence, whereas frogs do not (*Drosophila* display the consensus sequence as evolution of insects occurred long before that of vertebrates). Moreover, it has been demonstrated that the yeast homologue to hU3-55k (Rrp9) is not capable of modulating U3 snoRNA levels (Venema et al., 2000), potentially due to the conserved C’ box sequence, most likely allowing NOP58 association with the U3 snoRNA in the absence of hU3-55k. Thereby, it seems hU3-55k in higher eukaryotes is unique in its ability to regulate U3 snoRNP accumulation.

We were able to observe specific interactions between NOP56 / 58 and hU3-55k, the requirement of hU3-55k association with the U3 snoRNP for NOP58 association, and a very weak interaction between fibrillarin and hU3-55k. This data, we believe, provides evidence that the U3 snoRNP functions as a single unit; with interactions between the proteins on either motif (box B/C and Box C’/D), rather than a snoRNP composed of two disparate protein-binding domains (Figure 3.15). This is consistent with the previous observation that, in vitro, the B/C motif is required for fibrillarin association, despite not being known to directly associate

here (Baserga et al., 1991; Speckmann et al., 1999). It has however, also been argued that the B/C motif is required for localisation to the nucleolus, yet not accumulation; although RNA stability was only examined 2 hours after injection into *Xenopus* oocytes (Lange et al., 1998b). Neither ourselves or others observe this upon mutation of the box C motif, instead we witness the opposite; a decrease in expression of the U3 snoRNA but retaining nucleolar localisation (Granneman et al., 2004; Speckmann et al., 1999). Nonetheless, it has been demonstrated elsewhere that hU3-55k can associate with the U3 snoRNA in the absence of fibrillarin and it is postulated that fibrillarin may interact indirectly with the U3 snoRNP, potentially through hU3-55k (Baserga et al., 1991; Lukowiak et al., 2000), supporting the idea of hU3-55k aiding the recruitment of the core proteins to the RNP structure.

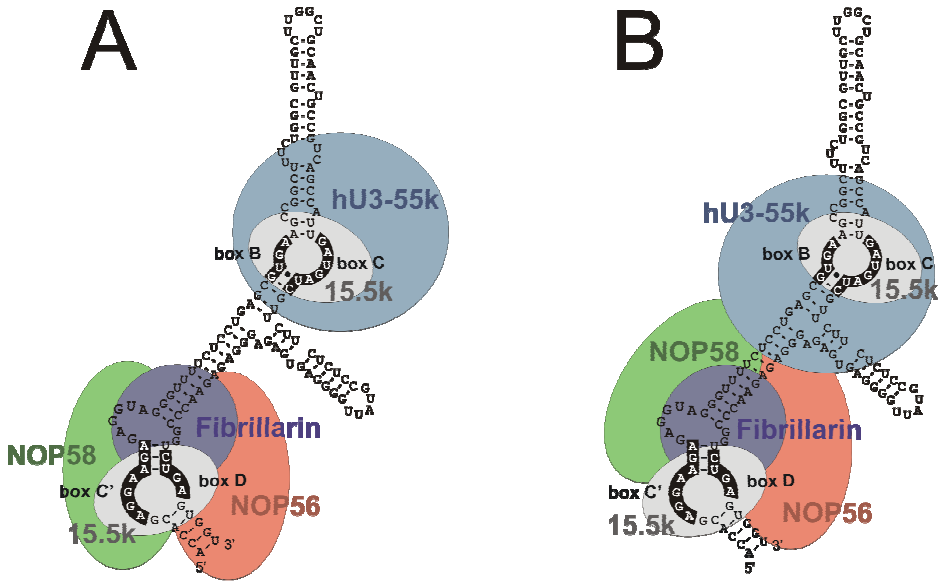


Figure 3.15 U3 snoRNP as a Single Unified RNP
A) Previous model of the U3 snoRNP, with proteins associated at box B/C motif distal to those at the C'/D box **B)** New model of the U3 snoRNP based on data presented within this study, represented as a single snoRNP structure with hU3-55k capable of NOP56/58 binding, and potentially fibrillarin binding. The conserved box motifs of the RNA are represented by white text on a black background, proteins are represented as coloured spheres with; hU3-55k blue; 15.5K grey; NOP56 red; NOP58 green. Note that the A/A' boxes have been excluded for simplicity.

It has been proposed that the proteins bound to the B/C motif play a central role in SSU processome formation (Granneman et al., 2004; Venema et al., 2000). Since

our data indicate that the proteins bound to the B/C and C'/D motifs interact to form a cohesive RNP unit, it is possible that NOP56, NOP58 and fibrillarin also participate in U3 snoRNP function. In addition, recent crosslinking data demonstrate that NOP58, NOP56 and fibrillarin (Nop1) directly contact the pre-rRNA at and around the U3 snoRNA base-pairing sites, supporting this idea (Granneman et al., 2009).

We have also demonstrated an ability of hU3-55k to interact with NOP17. It is believed that interactions between the box C/D snoRNA biogenesis factors TIP48/TIP49 (capable of ATP hydrolysis; essential for box C/D snoRNA accumulation) and NOP56/NOP58 are indirect (McKeegan et al., 2007). In yeast, NOP17 interacts with TIP48, TIP49, and NOP58 and is important for ribosome biogenesis (Gonzales et al., 2005; Zhao et al., 2005; Zhao and Houry, 2005; Zhao et al., 2008). Thus, it is thought NOP17 may bridge interactions between the TIP48/TIP49 and NOP56/NOP58 proteins. Furthermore, NOP17 has been shown to be required for the biogenesis of box C/D snoRNPs in human cells, particularly the U3 snoRNA (McKeegan et al., 2007). Moreover, we were able to detect very weak interactions between hU3-55k and NUFIP and BCD1. These proteins, along with the AAA^+ proteins TIP48, TIP49 are believed to form a scaffold which is responsible for core snoRNP protein assembly, with NUFIP thought to regulate the interactions between TIP48 / TIP49 and the core box C/D proteins (Boulon et al., 2008; McKeegan et al., 2007; McKeegan et al., 2009). Consistent with our observations, it was recently reported that hU3-55K, produced in a bacterial S30 lysate could interact with NUFIP (Boulon et al., 2008). Therefore, it would appear hU3-55k associates with a range of factors crucial to the biogenesis of the U3 snoRNP, most likely associating early in the biogenesis pathway (Watkins et al., 2004), to control production of the snoRNP complex.

It would seem that hU3-55k is stable when not associated with U3 snoRNA, as demonstrated by over-expression of the protein, yet in the absence of hU3-55k, U3 snoRNA is unstable; allowing this protein to specifically regulate levels of the snoRNP. The protein alone however, is not sufficient to significantly increase levels

of the SSU processome complex (80S), as demonstrated through glycerol gradient analysis. This was not unexpected, as we would predict that many components of the SSU processome that are required are similarly regulated, and potentially co-ordinately so, as suggested by our data from differentiating cells.

Ribosome biogenesis has been shown to be tightly linked to cell growth and proliferation; therefore we next wanted to investigate modulation of the U3 snoRNP and other related factors upon cellular differentiation. To do this, we used CaCo-2 and CaLu-3 cells, derived from heterogeneous populations of human epithelial colorectal and non-small cell lung cancer adenocarcinomas respectively. Cellular differentiation often includes cell cycle arrest at G0/G1 phase, accompanied by a reduction in cell growth and ribosome biogenesis (Poortinga et al., 2004). Furthermore, upon differentiation the adenocarcinoma cell line CaCo-2 is said to lose its tumourigenic phenotype, so represents a useful tool for examining tumour progression (Stierum, 2003). Here, we show that during these differentiation events, the levels of the U3 snoRNA and hU3-55K, but not those of other box C/D snoRNAs and core snoRNP proteins, are down-regulated. This is consistent with hU3-55K expression controlling U3 snoRNP levels in these cells. Furthermore, several other SSU processome components are co-ordinately down-regulated during the observed cellular differentiations. From this, we conclude that many of the SSU processome components, including hU3-55k, are up-regulated during tumourogenesis. Interestingly, many malignancies arise from immature cells (Heath et al., 2000) so a novel therapeutic approach is “differentiation therapy” which involves the conversion of a tumourigenic cell to a more mature phenotype with no proliferative potential. Therefore, the SSU processome components down-regulated during differentiation may represent future targets for therapeutic agents.

In a high throughput screen for factors in the bone morphogenetic protein (BMP) pathway, hU3-55k was been shown to interact with SMAD-specific E3 ubiquitin protein ligase 1 (Smurf1); an important component in the pathway, linked with tumourogenesis (Barrios-Rodiles et al., 2005; Zhu et al., 1999). Smurf1 is also known to enhance cellular responsiveness to the proto-oncogenic TGF-β signaling

pathway, and has been shown to play an important role in regulating the protrusive activity and the transformed phenotype of HEK293 cells (Wang et al., 2003; Zhu et al., 1999). In addition, hU3-55k has been observed to be mutated in a significant number of breast cancers (Sjöblom et al., 2006) although how, or if, this affects the cell is yet to be elucidated. Moreover, hU3-55k mRNA levels have been reported to significantly increase in a range of cancers (www.oncomine.org) especially when comparing tissue from pre-malignant or benign with malignant primary tissue, such as in prostate cancer (Luo et al., 2001) or germ cell tumours; suggesting it as a potential candidate for involvement in maintaining the pluripotent, undifferentiated phenotype of such cells (Sperger et al., 2003).

The U3 snoRNP is one of the few snoRNPs that is essential in both yeast and metazoans (Henras et al., 2008). This snoRNA is recruited co-transcriptionally to the pre-rRNA (Gallagher et al., 2004) with biogenesis of the small subunit believed to occur before that of the large subunit (Grandi et al., 2002) and it is therefore possible, that pre-rRNAs not bound by the U3 snoRNP/SSU processome are targeted for degradation. Therefore, controlling the levels of the U3 snoRNP and key SSU processome components would regulate the amount of pre-rRNA targeted for processing, and thus control ribosome formation. Moreover, the HIV Tat protein has been shown to affect cellular ribosome content via association with fibrillarin and modulation of pre-rRNA processing in *Drosophila melanogaster.* The authors believe this is achieved through it's association with the pre-rRNA and U3 snoRNP (Ponti et al., 2008), demonstrating that modulation of processing does change ribosome content in the cell. It would also appear that control of ribosome biogenesis through regulation of pre-rRNA processing, rather than rRNA transcription, is important in certain cases of cellular differentiation (Bowman and Emerson, 1977; Dudov and Dabeva, 1983) and tumourogenesis (Schlosser et al., 2005).

Stimulating expression of the initiator tRNA, $tRNA_i^{Met}$, has been shown to drive cell proliferation and oncogenic transformation, as it is believed to be a limiting factor for translation (Marshall et al., 2008). $tRNA_i^{Met}$ along with eIF-2, eIF-3, eIF-4E, GTP

and the small ribosomal subunit, form an initial 48S complex with target mRNA, it is only then that the 60S ribosomal subunit assembles to form the complete 80S ribosome (Dai and Lu, 2008). Therefore, it is possible that limiting the amount of the small ribosomal subunit production, through regulating the SSU processome, also aids in regulating translation initiation, and therefore cellular growth and proliferation.

Lastly, the proto-oncogene c-Myc is reported to be down-regulated during many differentiation events as part of the block in proliferation (Demeterco et al., 2002; Laurenti et al., 2009; Poortinga et al., 2004). Conversely, a range of cancers are proposed to be due to a lack of cellular differentiation, or de- / retro-differentiation which correlates with elevated c-Myc levels (Heath et al., 2000; Laurenti et al., 2009; Wall et al., 2008). Indeed, in non-small cell lung cancer (cells from which CaLu-3 cell line is derived) inhibition of c-Myc down-regulation prevents cellular differentiation (Serra et al., 2008). We were therefore surprised to find that c-Myc levels were not reduced during CaCo-2 and CaLu-3 differentiation (data not shown; experiments performed by Adele Traynor). Furthermore, upon c-Myc depletion of HeLa cells, all proteins previously discussed are depleted, with the exception of nucleolin and β-actin (data not shown; experiments performed by Adele Traynor & Nick Watkins). Curiously, and conflicting with this, it has been reported that in rat fibroblast cells, NOP56 mRNA levels are not regulated by c-Myc whereas Nucleolin mRNA levels are, although it is important to remember the mRNA levels may not always reflect protein levels (Watson et al., 2002). It is also important to note that we cannot rule out the possibility of a modified or mutated c-Myc in the differentiated epithelial cells used in our experiments, which is therefore not fully active or is altered in function. Interestingly, in human cells, NOP56, NOP58, fibrillarin and hU3-55K mRNAs, and so presumably their protein products, have all been shown to be regulated by c-Myc (Coller et al., 2000; Menssen and Hermeking, 2002; Schlosser, 2003). Therefore, the specific down-regulation of hU3-55K levels during differentiation, in contrast to steady core box C/D snoRNP protein levels, is in agreement with consistent c-Myc levels; suggesting another signaling pathway may be responsible. Moreover, signaling pathways such as

Ras/PKA and TOR (Jorgensen, Rupes et al. 2004; Guertin, Guntur et al. 2006; Yang, Yang et al. 2008; Soulard, Cohen et al. 2009), p53, pRB and ARF (White 1997; Larminie, Alzuherri et al. 1998; Sugimoto, Kuo et al. 2003; Oskarsson and Trumpp 2005; White 2005) all regulate both ribosome biogenesis and cell cycle control, and could be responsible for the specific changes in U3 snoRNP and SSU processome protein levels observed herein.

Chapter Four

Analysis of hU3-55k Mutants Reveals Regulation of SSU Processome Function through hU3-55k Phosphorylation

4.1 Introduction

hU3-55k is an important U3 snoRNA associated protein. It is known to bind the B/C motif, exclusive to the U3 snoRNA (Lukowiak et al., 2000). In the previous chapter we demonstrated that hU3-55k was also linked to the recruitment of core proteins NOP56 and NOP58 at the C'/D motif, therefore enabling the protein to regulate U3 snoRNP accumulation in human cells. The U3 snoRNP is composed of the U3 snoRNA, hU3-55k, 15.5K, NOP56, NOP58 and fibrillarin. This snoRNP a key component of the small subunit (SSU) processome, along with a host of other factors and the pre-rRNA, as described in the previous chapter (Henras et al., 2008; Turner et al., 2009). The SSU processome is crucial for guiding cleavages at A', site 1 (within the 5'ETS) and site 2 (within the ITS1), thus separating the 18S rRNA from the 28S and 5.8S precursors; essential for the production of the small subunit of the ribosome (Figure 4.1).

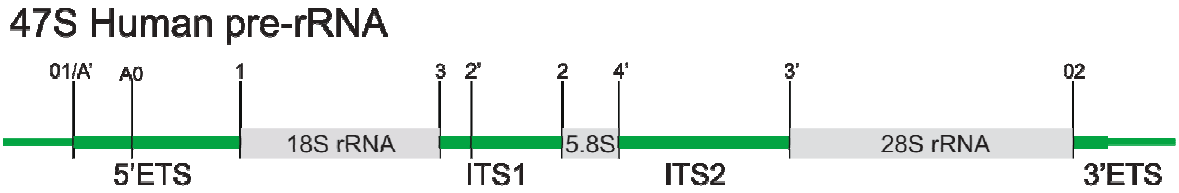


Figure 4.1 Human pre-rRNA
A full length human pre-rRNA transcript is shown. RNA is shown in green (cleaved from the mature product; named beneath) and grey (constituting the mature rRNA; named within the Box). Cleavage sites so far identified in both are indicated by vertical black lines with the cleavage site named above for each. ETS; External transcribed sequence, ITS; Internal transcribed sequence. Based on previous work (Fatica and Tollervey, 2002; Henras et al., 2008; Rouquette et al., 2005).

The U3 snoRNA does not contain the C'/D' motif of other box C/D snoRNAs but a unique box B/C motif within its 3' end, to which 15.5K and the U3-specific hU3-55k are associated (Granneman et al., 2009; Granneman et al., 2002; Venema et al., 2000; Watkins et al., 2000). U3-specific hU3-55k is therefore, the only protein of the U3 snoRNP not found associated with other box C/D snoRNPs. The U3 snoRNA also contains a unique A'/A motif within its 5' end, which is capable of base-pairing with the 5'ETS /18S pre-rRNA, and believed to act as a chaperone to the pre-rRNA, essential for its processing (Beltrame and Tollervey, 1995; Gerczei and Correll, 2004; Granneman et al., 2004). IMP3, IMP4, and MPP10, are believed to accelerate the formation of this 5'-18S pre-rRNA / U3 snoRNA duplex (Gerczei and Correll, 2004; Gérczei et al., 2009; Granneman et al., 2003; Granneman et al., 2004). These proteins, and the pre-rRNA base-pairing region of U3 snoRNA however, are thought not to be required for incorporation of the U3 snoRNP into the SSU processome whereas, the B/C motif of the snoRNA is crucial (Granneman et al., 2003; Granneman et al., 2004). It would therefore seem that the box B/C associated protein, hU3-55k, may be vital in U3 snoRNP recruitment of SSU processome factors. Core U3 snoRNP proteins may also play an important role, having been shown to crosslink directly to the pre-rRNA in yeast, suggesting they may not only be required for U3 snoRNP accumulation, but potentially for pre-rRNA association and processing (Granneman et al., 2009).

The hU3-55k protein, Rrp9 in yeast, is highly conserved amongst eukaryotes and is composed of several distinct domains. A putative bipartite NLS at residues 8-40 and a glutamic acid-rich region 64-73 (although potentially ranging from 47-105 residues) in the N-terminus were first noted upon cloning of the human cDNA (Pluk et al., 1998). These domains are conserved in *Xenopus* and yeast. When the cDNA was originally cloned, it was suggested that the glutamic acid-rich (E-) stretch in the N-terminus may be important for protein-binding (Pluk et al., 1998). However, it has been shown that the N-terminal 44 amino acid residues of the protein, and thus the NLS, are not essential for nucleolar localisation or U3 snoRNA association, with deletion of the residues leading to only slight mislocalisations. Similarly it has been demonstrated that the glutamic acid-rich

region is not required for U3 snoRNA association or nucleolar localisation (Lukowiak et al., 2000). The importance of the E-strech is therefore currently unclear.

A small 17 amino acid C-terminal deletion of hU3-55k was however, shown to drastically reduce U3 snoRNA association and nucleolar localisation (Pluk et al., 1998). The C-terminus contains 7 WD-repeat domains (as identified by UniProtKP and NCBI Conserved Domains software) which are crucial for association with the box B/C motif of U3 snoRNA, directly interacting with the U3 snoRNA as shown through crosslinking analysis (Granneman et al., 2009; Granneman et al., 2002; Granneman et al., 2004; Lukowiak et al., 2000; Pluk et al., 1998). WD40 domains are found in many eukaryotic proteins. They are known to have an array of functions including adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly. The domains are composed of a GH dipeptide at the N-terminus, 40 residues in the middle of the motif and a WD dipeptide at the C-terminus. Each WD40 motif is thought to fold into a 4-stranded anti-parallel β-sheet, with multiple WD40-domains forming a propeller-like structure, whereby each blade is composed of 3 strands from one motif, and one strand from the previous WD40 motif. This suggests hU3-55k may potentially act as a protein binding platform for assembly of SSU processome component proteins, through its WD-repeat domain in the C-terminus.

In the N-terminus of hU3-55k, there are three phosphorylatable serine residues; with at least one found conserved between yeast and humans, with similar residues found throughout eukaryotes; located at positions 50, 51 and 53 in the human protein (Figure 4.2). Recent phosphoproteome analysis experiments have provided great insight into the site-specific modifications in a host of proteins. These experiments have relied upon stable isotope labeling by amino acids in cell culture (SILAC) in conjunction with mass spectrometry analysis (Gnad et al., 2007). Results from these experiments in HeLa cells showed that the conserved serine residues (50, 51 and 53) of hU3-55k were generally phosphorylated and unaffected by treatment with epidermal growth factor (EGF) (Olsen et al., 2006). It was also

shown in mice, that both the hepatoma HEPA1-6 cell line (Pan et al., 2008) and melanoma tissue (Zanivan et al., 2008) contained hU3-55k which was highly phosphorylated at residues 50, 51 & 53. In a different study, cell cycle dependent phosphorylation was demonstrated using HeLa cells synchronised at either early S phase by a double thymidine block, or M phase by treating with nocodazole. In these experiments, the three serines appeared co-coordinately phosphorylated, specifically at mitosis (M) and growth/gap phase 1 (G_1) of the cell cycle and non-phosphorylated in synthesis (S) and gap phase 2 (G_2) (Gnad et al., 2007; Olsen et al., 2010). It is however, worth noting that this particular study was focused on the activity of kinase proteins therefore, the procedure was designed to enrich kinases using affinity ligands; potentially introducing bias into the collection method. Nonetheless, other potential phosphorylation sites have also been noted within hU3-55k at residues 57 and 475 (Dephoure et al., 2008; Mayya et al., 2009; Wang et al., 2008) although these residues are not as highly conserved throughout eukaryotes or detected as frequently to be coordinately phosphorylated.

In a different high-throughput screen, the sequence of coding exons belonging to eleven cell lines or xenografts from either breast or colorectal carcinomas was examined. This revealed a host of previously undocumented yet significant changes; 365 mutations in 236 genes (Sjöblom et al., 2006). Two of these mutations were found to be substitutions in hU3-55k; 8R->G and 342 A->E, occurring in the NLS and 5th WD repeat respectively. The effects of these mutations were however, undocumented so it was not clear how they may impact on the protein, or its role in SSU processome formation. It should be noted however, that the validity of the statistics used in the study have since been called in to question by a number of groups, suggesting that hU3-55k mutation rates in breast cancers examined in this study may fall short of achieving statistical significance (Forrest and Cavet, 2007; Getz et al., 2007; Rubin and Green, 2007). Nonetheless, we felt it was important to document the potential role these small point mutations may have on SSU processome formation.

hU3-55k is a defining feature of the U3 snoRNP and so therefore, may dictate its unique roll in the SSU processome; potentially aiding recruitment of other SSU proteins and the pre-rRNA. However, much of the data available on hU3-55k has been gleamed from high throughput screens which may have various biases attached. We therefore decided to investigate the role of hU3-55k in SSU processome assembly, and to dissect the role played by the different domains discussed here; the N-terminus including the NLS and glutamic-acid rich domains; the C-terminal WD repeats; the three highly conserved phosphoserines within the N-terminus; and the two potential breast cancer-related mutations 8R->G and 342 A->E.

4.2 Results

4.2.1 FLAG-tagged hU3-55k Mutants are Inducibly Expressed at Correct Sizes

To identify conserved regions and domains within hU3-55k, we carried out sequence alignments of the protein from a range of species (Figure 4.2 B). This demonstrated that, to varying degrees, the putative NLS, phosphorylatable residues, E-stretch and WD domains were indeed highly conserved, present in species examined from *S. cerevisiae* to *H. sapiens* (data not shown; domains indicated in the human protein in Figure 4.2 A). We were however, particularly interested in the N-terminal domain of the protein which contained the putative nuclear localisation signal (NLS; residues 8-40), phosphorylation domain (residues 50, 51 and 53) and the E-stretch (residues 64-73) as previously identified (Lukowiak et al., 2000; Pluk et al., 1998). Upon examination of the protein sequence, it appeared there was also a second cluster of aspartic acid residues in all species, aligning with residues 93 – 105 in the human protein (results not shown; in agreement with previous data (Pluk et al., 1998)). Furthermore, the NLS sequence appeared to be composed of two conserved motifs in the majority of species (Figure 4.2 B), most likely representing a bipartite NLS as previously identified in nucleoplasmin (Robbins et al., 1991). In nucleoplasmin, the sequence KR [PAATKKAGQA] KKKK gives the prototype of the bipartite nuclear localisation

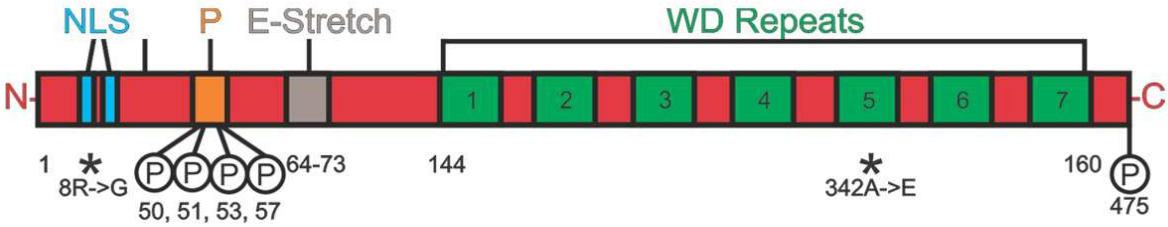
signal (NLS); composed of two clusters of basic amino acids (K/R), separated by a spacer region (Robbins et al., 1991). It is worth noting that yeast did not appear to contain this bipartite sequence (RKRGK [PASGAGAGAGAG] KRRRK in humans), but instead one closer to that of the SV40 large T antigen (PKKKRKV) composed of only one domain; KKRKRSK in yeast, suggesting evolution of the bipartite signal (Pap et al., 2001).

The residues upstream of the E-stretch, capable of being phosphorylated in humans and mice (Olsen et al., 2006; Pan et al., 2008; Zanivan et al., 2008), were very highly conserved across eukaryotes (Figure 4.2 B), with at least one of the serine residues conserved, and if not, substituted for a threonine (another phosphorylatable residue) or another charged acidic residue (E/D). This suggested that these residues may well be important in the protein's function although only one residue in the region has been recorded as being capable of phosphorylation in yeast (Albuquerque et al., 2008), suggesting an increase in phosphorylatable sites evolutionarily, potentially to enable greater control over the protein.

We therefore made mutations in the hU3-55k protein coding sequence to help characterise the significance of the domains, post-translational modifications and potential cancer-associated mutations. The hU3-55k cDNA sequence previously cloned into a modified pcDNA5 vector (pcDNA5-FLAG; Figure 2.2), was modified in a host of ways (Figure 4.3). A mutant lacking the initial 136 amino acids at the N-terminus with the addition of a minimal NLS, derived from that of SV40 to ensure nuclear localisation, was created by conventional PCR and cloning. Conversely, a mutant lacking many of the WD repeats (Del193-352 (ΔWD)) was also cloned into pcDNA5-FLAG. Mutants that either mimicked constitutive phosphorylation or which were non-phosphorylatable, were created by substituting the serine residues at positions 50, 51 and 53, to aspartic acid or alanine, respectively; mutations which have been widely used in the literature to alter phosphorylatable residues. In a similar fashion, the wild-type hU3-55k was altered to create two potential cancer mutations Δ8R->G and Δ342A->E. As before, these constructs were integrated into the genome of HEK293 cells by recombination, with the N-terminally tagged-hU3-

55k mutants of interest placed under the control of a tetracycline-regulated promoter. This allowed titratable induction of protein expression from these stably-transfected cell lines.

A



B

Species	Residues 8-29	Residues 47-57
H. sapiens (Human)	RK RGKPASGAGA GAGAGKRRRK	EEISSDSE---SES
P. troglodytes (Chimp)	RK RGKPASGAGA GAGAGKRRRK	EEISSDSE---SES
M. musculus (Mouse)	RK RAKPAPGPGA APVAGKRRRK	EEISSDSE---SES
R. norvegicus (Rat)	RK RAKPAPGPGA APVDGKRRRK	EEISSDSE---SES
B. taurus (Cow)	RK RGKPASGAGA GAEAFKRRRK	EEISSDSE---SES
X. laevis (Frog)	KK K—SGVTP--- ------RRRR	EEIESDSD---TEI
D. rerio (Zebrafish)	KK KLVSSATNKG -----DLKRK	EEISSDSE---NES
D. melanogaster (Fruit Fly)	TK KRKNPAATTK ----GKPLKK	EEIASDDEEYNSDL
A. thaliana (Thale Cress)	EK KKGIGA---- ----KRRGKK	DIESVESEEEGKVG
S. cerevisiae (Budding Yeast)	QK KR-------- ------KRSK	EEITDPSSNEDEQL
Consensus	RK RGKPASGAGA ----GKRRRK	EEISSDSE---SES

Figure 4.2 hU3-55k Protein Domains and Conservation of the N-terminus
A) Schematic of the hU3-55k protein with various domains indicated. Within the N-termins (-N) is shown the putative NLS (blue), phosphorylatable residues (orange) and the E-stretch (grey) as previously described (Granneman et al., 2002; Lukowiak et al., 2000; Pluk et al., 1998; Venema et al., 2000). In the C-terminus (-C) are shown the WD repeats (green) as predicted previously (Lukowiak et al., 2000; Pluk et al., 1998) and as identified by UniProtKP and NCBI Conserved Domains software. Residues are indicated beneath, with asterisks indicating the potential breast cancer mutations. **B)** An alignment of various hU3-55k homologue sequences (species shown on the left) was performed using CLC Seqeunce Viewer 6.3. Residues 8-29 are shown in the first panel, with negatively charged residues K/R shown in blue, emphasising that the bipartite nature of the NLS in the human protein is widely conserved. The second panel shows the region of phosphoserines at residues 50, 51, 53 and 57 (Olsen et al., 2006; Pan et al., 2008; Zanivan et al., 2008) identified in humans and mice. Phosphorylatable residues S/T are shown in orange, positively charged residues E/D are shown in red.

Induction of the hU3-55k-FLAG proteins were tested by the addition of a range of tetracycline concentrations up to 1 µg/ml, with cells induced for 48 hours before harvesting. Proteins from whole cell lysates were separated by SDS PAGE and analysed by Western blotting, using specific antibody probes to either hU3-55k or the FLAG-tag sequence (Figure 4.4). This demonstrated that all the mutants expressed inducibly, with little to no expression in the absence of tetracycline, and in a titratable manner. Antibodies to hU3-55k were able to detect both endogenous and FLAG-tagged proteins efficiently, with the exception of the Δ1-136 NLS mutant (as the antibody epitope is located within the initial 136 residues of the protein). This meant we were able to compare endogenous levels of the protein to those of the tagged at a set tetracycline concentration. Doing so demonstrated that 1:1 FLAG-tagged: endogenous hU3-55k expression levels could be achieved with the addition of 0.5 ng/ml tetracycline to the wild-type, non-phosphorylatable (-P), phosphomimic (+P) and 8R->G hU3-55k expressing cell lines (Figure 4.4 A). 1 µg/ml and 100 ng/ml tetracycline were required to give equal expression of 342A->E and ΔWD hU3-55k mutants, respectively, when compared to the endogenous protein.

As the hU3-55k antibodies were raised against peptides in the N-terminal domain of the protein, they were not able to detect expression of the Δ1-136 NLS mutant. We were however, able to demonstrate that with 0.5ng / ml tetracycline, wild-type FLAG-hU3-55k was expressed at a 1:1 ratio with the endogenous. Therefore, this cell line was used as a standard to measure Δ1-136 NLS mutant expression, as detected by α-FLAG antibodies, with 1 µg/ml tetracycline required for comparable expression levels to the endogenous protein (Figure 4.4 B). In all cases, endogenous hU3-55k levels appeared unchanged, suggesting there was no obvious feedback mechanism to limit accumulation of the protein.

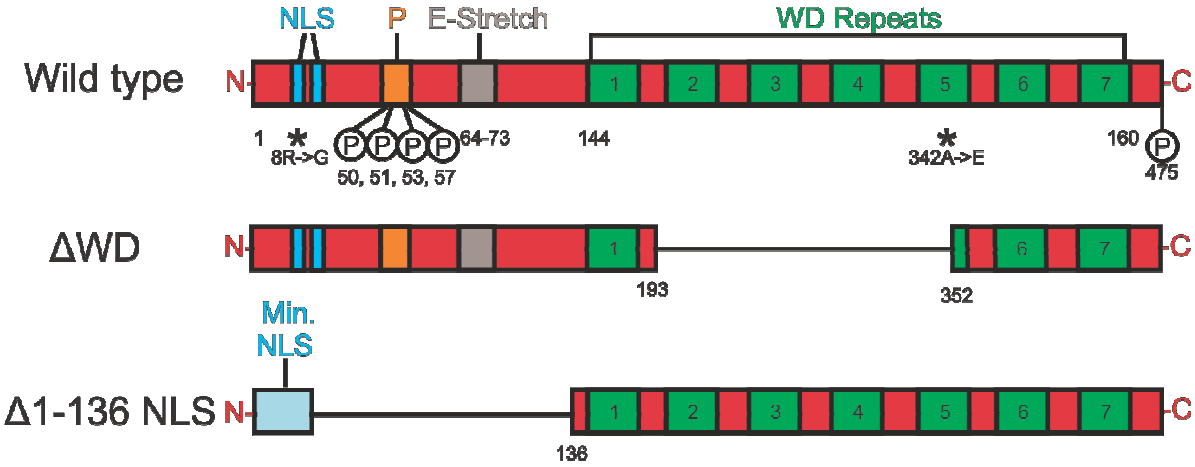


Figure 4.3 hU3-55k Mutants
Schematic diagram of the wild type hU3-55k protein and mutants cloned into pcDNA5 FLAG with various domains indicated. Within the N-termins (-N) of the wild type protein is shown the putative NLS (dark blue), phosphorylatable residues (orange) and the E-stretch (grey) as previously described (Granneman et al., 2002; Lukowiak et al., 2000; Pluk et al., 1998; Venema et al., 2000). In the C-terminus (-C) are shown the WD repeats (green) as predicted previously (Lukowiak et al., 2000; Pluk et al., 1998) and as identified by UniProtKP and NCBI Conserved Domains software. Residues are indicated beneath, with asterisks indicating the potential breast cancer mutations created in separate hU3-55k constructs. Mutations were also made whereby residues 50, 51 and 53 were changed to either aspartic acid or alanine. In two other mutants created and depicted, WD 40 repeats 2-5 were removed (Del193-352 (ΔWD)) or the initial 136 residues of the protein were removed (Δ 1-136 NLS) and replaced with a minimal NLS sequence from SV40 (light blue).

The various hU3-55k expressing cell lines were induced with the suitable amount of tetracycline to allow 1:1 expression of the tagged protein: endogenous hU3-55k. Similarly, a vector-alone containing cell line was induced with 1 µg/ml tetracycline, acting as a negative control, as described in the previous chapter. Cells were harvested, proteins separated and detected as before. This demonstrated that all hU3-55k protein expressed at comparable levels to the endogenous, with the wild-type, -P, +P, 8R->G and 342A->E proteins all resolving as the same size, as expected; approximately 64 kDa (due to the addition of the N-terminal tag). Removal of the initial 136 residues (Δ1-136 NLS) gave rise to a protein which resolved around the 40-45 kDa region which is as expected, according to calculations with ProtParam Tool software (www.expasy.ch (Wilkins et al., 1999)).

Removal of residues 193-352 (ΔWD) gave rise to a protein which resolved just below the endogenous hU3-55k protein; therefore just below 55kDa. This was initially a surprise as it was expected to resolve just above the Δ1-136 NLS at ~43 kDa, according to ExPASy ProtParam prediction software (Wilkins et al., 1999). The ΔWD mutant however, was composed of more amino acid residues than the Δ1-136 NLS and also contained many more negatively charged residues, potentially retarding its movement through the gel. Regardless of this, all of the mutants could be inducibly expressed at levels approximating that of the endogenous protein and comparable to one another, with no detectable expression from the vector only control. It is worth noting however, that in some instances the Δ1-136 NLS hU3-55k protein displayed degradation products, observed as smaller bands below that of the main band (data not shown). This indicated that the protein was unstable, suggesting a possible role for the N-terminal residues in protein stability.

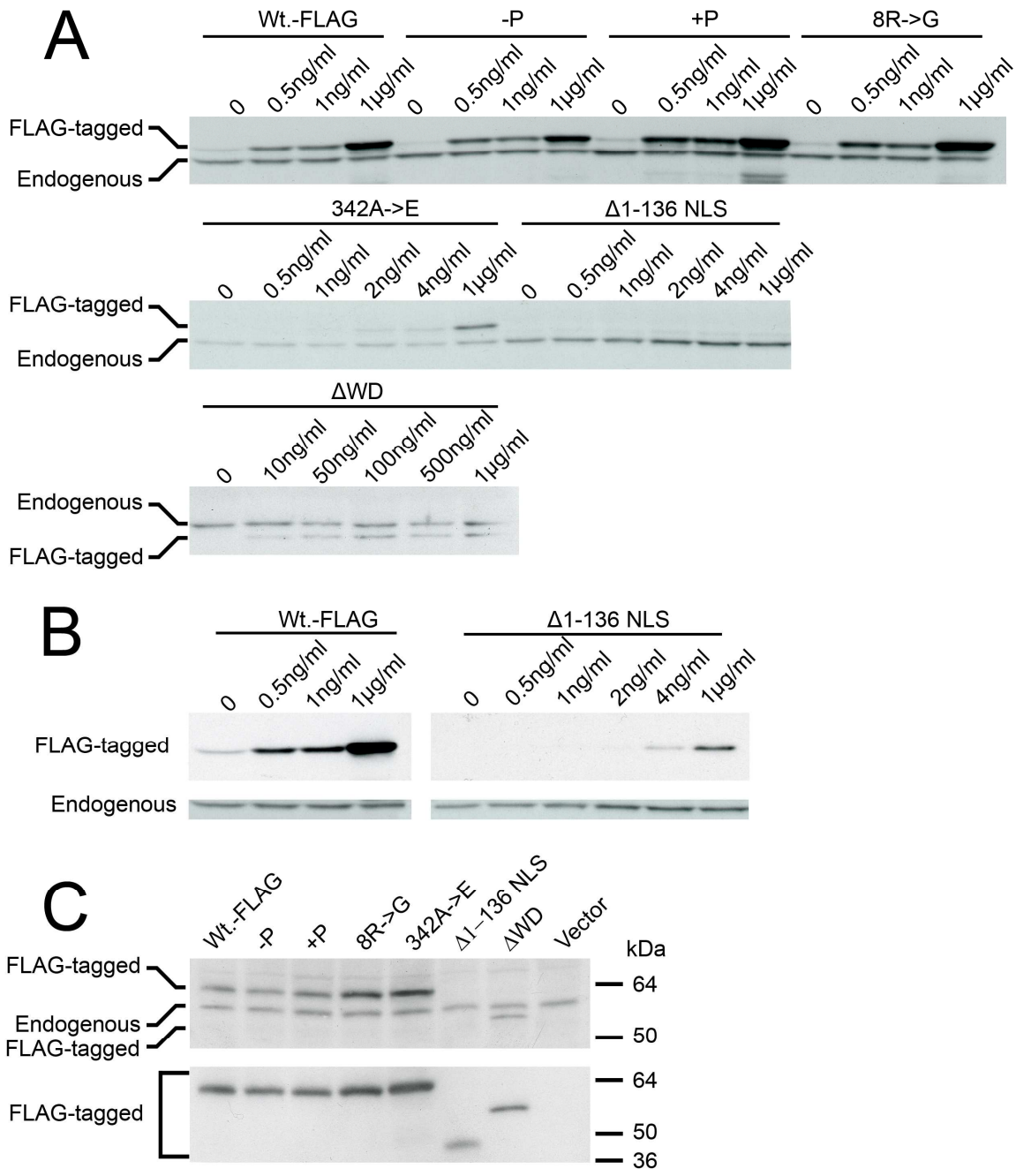


Figure 4.4 Inducible Expression of hU3-55k Mutants from HEK293 Cells
N-terminally FLAG-tagged hU3-55k constructs were stably integrated into HEK293 cells, under a tetracycline inducible promoter. **A)** Each cell line (shown above each panel) was cultured with a range of tetracycline concentrations (shown above each lane) for 48 hours and harvested, proteins separated by SDS PAGE and analysed by Western blot analysis with antibodies specific to hU3-55k; capable of detecting both endogenous and FLAG-tagged proteins (as indicated to the left of each panel). **B)** Induction of the hU3-55k mutant lacking the initial 136 amino acid residues (Δ1-136 NLS; shown above the lanes) was compared to that of the wild-type (Wt.-FLAG) tagged protein. Lysates were separated and Western blotted as in A), probing blots with antibodies specific to the FLAG-tag (top panel) and the endogenous hU3-55k protein (bottom panel). **C)** Using the data shown in A) and B) cells were induced to express FLAG-tagged proteins at 1:1 levels when compared to the endogenous protein and analysed as before; probed with antibodies specific to hU3-55k (top panel) or FLAG-tag (bottom panel).

4.2.2 The WD Domain is Required for hU3-55k Nucleolar Localisation

We next analysed localisation of the hU3-55k mutants in order to examine the requirements of the various domains for proper nucleolar accumulation. Once again, the various hU3-55k cell lines were induced so as to express comparable levels of proteins to that of the endogenous protein, as described in the previous chapter. Cells were then fixed and immunofluorescence analysis performed using antibodies specific to the FLAG-tag and either BMS1; a DFC/GC marker (Turner et al., 2009; Wegierski et al., 2001), or fibrillarin; a DFC marker, as previously described (Ochs et al., 1985), with the nucleus visualised by staining with DAPI (Figure 4.5).

The immunofluorescence data showed that as expected, the tagged wild-type hU3-55k protein localised throughout the DFC and GC of the nucleolus. The vector alone cell line did not express a detectable FLAG product, as previously discussed. The majority of the hU3-55k mutants localised in a similar manner to the wild-type protein, with the exception of the ΔWD mutant. The ΔWD mutant appeared excluded from the nucleolus; unable to co-localise with either BMS1 or fibrillarin, but was specifically retained in the nucleus; co-localising with the DAPI staining. This is in accordance with previous data whereby disruption of the WD motifs resulted in mis-localisation of the protein (Lukowiak et al., 2000; Pluk et al., 1998). Furthermore, loss of the N-terminus containing the putative NLS and E-stretch (replaced with a minimal NLS) did not appear to affect hU3-55k localisation. The E-stretch, or indeed the N-terminal 44 residues, have both previously been reported to be unnecessary for nucleolar localisation (Lukowiak et al., 2000; Pluk et al., 1998), with this data suggesting that the N-terminal 136 residues are not required for nucleolar localisation in the presence of a minimal NLS.

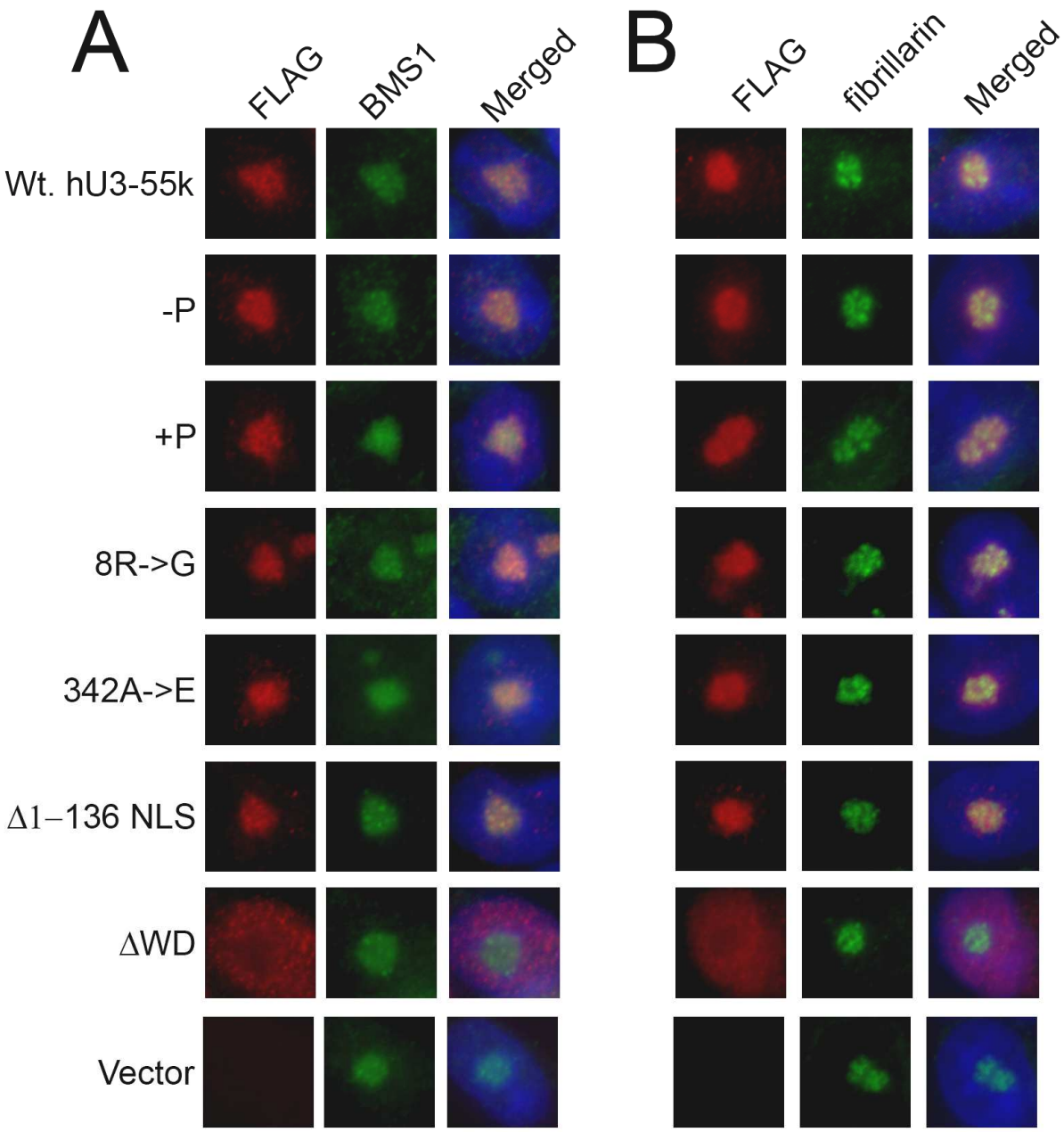


Figure 4.5 Localisation of FLAG-tagged hU3-55k Proteins in HEK293 Cells
The expression of hU3-55k proteins or vector alone expression (left of each panel) from HEK293 cells was induced with the suitable amount of tetracycline to give 1:1 expression of the tagged protein: endogenous wild-type protein, as described in the text. Cells were subsequently fixed for analysis. **A)** Cells were assayed by immunofluorescence using antibodies that specifically detected the FLAG-tag (red) and BMS1 (green), the nucleus was stained with DAPI (blue; only included in the merged image, as indicated above each panel). **B)** Cells were similarly assayed by immunofluorescence but with antibodies that specifically recognised fibrillarin (green) instead of BMS1 in this instance. All other conditions were the same as in (A). The protein (or vector alone) expressed in each instance is indicated to the left of each panel where Δ1-136 NLS indicates the removal of the initial 136 residues and the addition of a minimal SV40 NLS sequence, ΔWD indicates removal of C-terminal WD repeats 2-5 (residues 193-352).

4.2.3 The WD Domain alone is Required to Increase U3 snoRNP Levels

We were next interested to examine if the hU3-55k mutants had the same ability to increase accumulation of U3 snoRNA levels, as described for the wild-type protein in the previous chapter. Unfortunately, as shown in Figure 4.4, not all of the proteins were capable of over-expression, so it was decided to express the proteins at comparable levels to one another, and to that of the endogenous protein. Therefore, cells were incubated in tetracycline as before and harvested after 48 hours. Nucleic acid was extracted, total RNA levels quantified and equal amounts analysed by Northern blotting using probes specific to U3 and U8 snoRNAs, and the U1 snRNA (Figure 4.6).

The data demonstrated that upon induction of the wild-type hU3-55k, as in the previous chapter, levels of the U3 snoRNA increased by 2.5 fold in comparison to the vector control cell line, relative to U1 snRNA levels (Figure 4.6). Similarly, expression of hU3-55k mutant proteins resulted in a 2-2.5 fold increase in relative U3 snoRNA levels with the exception of the ΔWD mutant, which did not appear to aid U3 snoRNA accumulation. Importantly, expression of the Δ1-136 NLS mutant did result in increased U3 snoRNA accumulation, demonstrating that these residues are most-likely not required for recruitment of core box C/D proteins to the U3 snoRNA. Furthermore, this shows that the WD motif is crucial for U3 snoRNA accumulation, and potentially the association of the other core proteins.

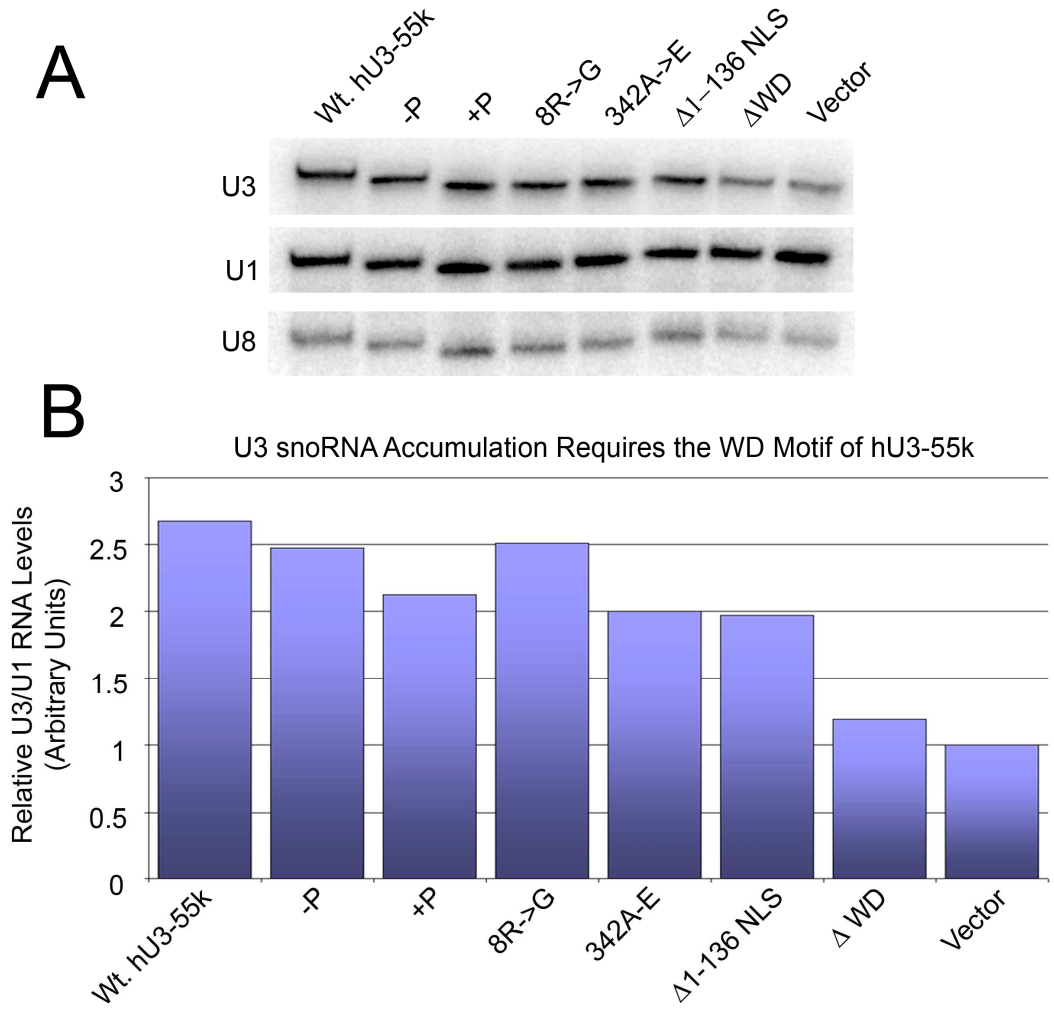


Figure 4.6 The WD Motif of hU3-55k is Required for U3 snoRNA Accumulation

Expression from HEK293 cells of stably integrated hU3-55k constructs or vector alone was induced with the suitable amount of tetracycline to give comparable expression levels to the endogenous wild-type protein. **A)** RNA was extracted from each cell line (indicated above each lane), quantified and equal amounts separated on a denaturing polyacrylamide gel. RNA was transferred by Northern blotting and analysed using radiolabeled probes, specific to U3 or U8 snoRNA, or the U1 snRNA, as indicated to the left of each panel. **B)** Quantitation was performed on the data in (A) showing the level of U3 snoRNA accumulation from each cell line. Analysis was performed using ImageQuant TL (GE Healthcare) software with background substitution. Relative U3/U1 RNA levels are plotted on the vertical axis and the protein expressed in each instance plotted on the horizontal axis.

4.2.4 The WD Domain is Required for U3 snoRNA Association

The effect of the various hU3-55k mutations were investigated further by examining their association to the U3 snoRNA. As before, the HEK293 cells were cultured with tetracycline for 48 hours. Cells were harvested and nucleolar disruption was ensured by sonication in buffer lacking magnesium. Insoluble debris was removed by centrifugation and the lysate supernatant incubated with anti-FLAG antibodies conjugated to Protein G Sepharose beads. Co-precipitated protein and RNA were separately extracted, separated by denaturing gel electrophoresis, and then analysed by Western or Northern blotting, respectively. Western blots were probed using anti-FLAG specific antibodies to detecte the FLAG-tagged proteins whilst radiolabeled probes specific to the U3 snoRNA and U1 snRNA were used to probe Northern blots.

Neither RNA nor protein were co-precipitated from extracts derived from control cells expressing the FLAG-tag alone (Vector; Figure 4.7). In contrast, the U3 box C/D snoRNA was co-precipitated with the tagged hU3-55K proteins, whilst U1 snRNA was not. The exception to this was the ΔWD mutant, which was unable to associate with the U3 snoRNA, as previously described (Lukowiak et al., 2000). This confirmed that the majority of the FLAG-tagged hU3-55K mutants were associated with the box C/D U3 snoRNP, with the WD motifs crucial for U3 snoRNA association. It should be noted however, that expression levels of the FLAG-tagged proteins Δ1-136 NLS and ΔWD appeared lower than that of the other hU3-55k proteins, yet no drastic decrease in precipitation of U3 snoRNA with Δ1-136 was observed. This suggested that some of the tagged-hU3-55k protein in each cell may not have been associated with the U3 snoRNA, but was most likely free protein. Nonetheless, it would appear that the entire N-terminal domain of the protein is redundant for U3 snoRNA association. The N-terminal domain is highly conserved however, suggesting that it may play a regulatory role in SSU processome formation, as opposed to a structural role in U3 snoRNP accumulation.

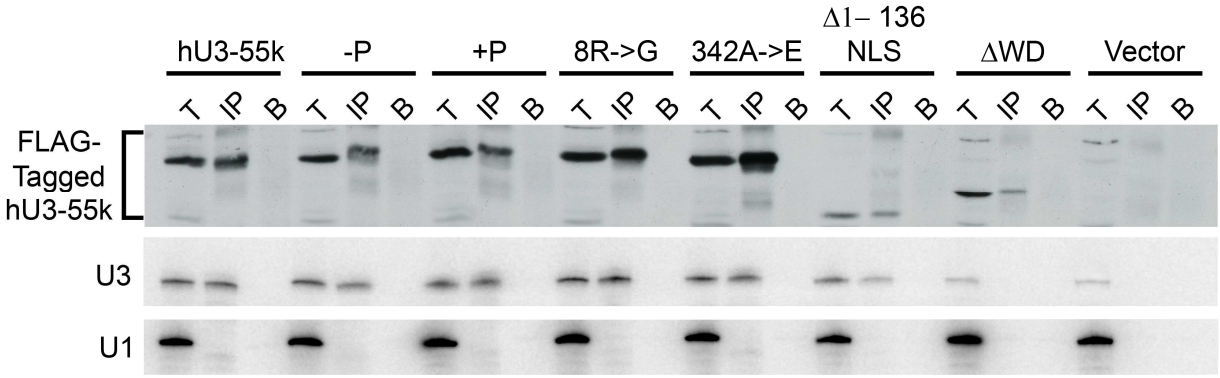


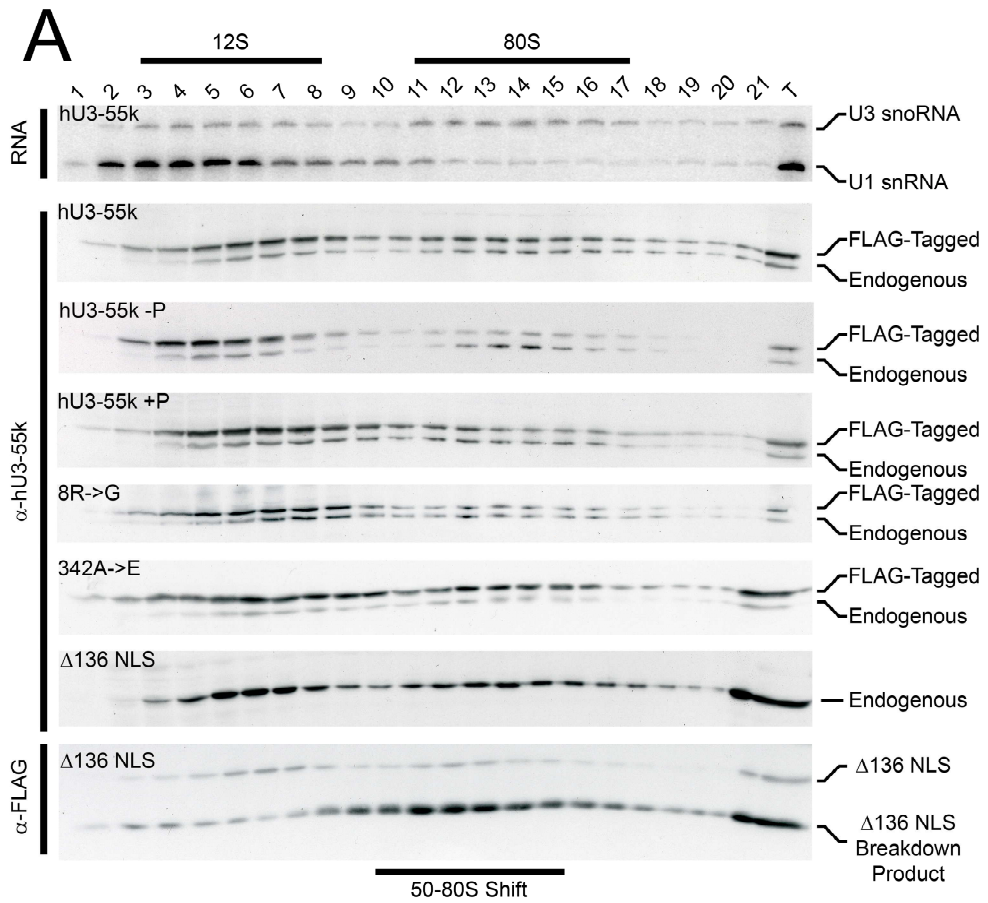
Figure 4.7 The hU3-55k WD Motif Alone is Required for U3 snoRNA Association
Expression of FLAG-tagged proteins from HEK293 cells was induced by the addition of tetracycline for 48 hours before cells were harvested. Immunoprecipitation reactions were performed using murine anti-FLAG coated Protein G Sepharose beads, and co-precipitated protein and RNA separated by gel electrophoresis. The cell line used is shown at the top. Western blot analysis was performed using rabbit anti-FLAG antibodies (as shown to the left of each panel) to detect the FLAG-tagged hU3-55k proteins. Northern blots were performed using radiolabeled probes specific to U3 snoRNA or U1 snRNA. T represents 10% total input; IP immunoprecipitation; B beads alone control with no antibody.

4.2.5 N-terminal hU3-55k Residues Capable of being Phosphorylated are Required for Efficient Incorporation into the SSU Processome

We next examined whether any of the mutants affected the integration of the U3 snoRNP into the SSU processome. Extracts were prepared from cells expressing wild-type and mutant hU3-55K-FLAG proteins at equimolar amounts to the endogenous protein and separated by glycerol gradient centrifugation. hU3-55k ΔWD was not included in this experiment as we had previously demonstrated that it did not associate with the U3 snoRNA. Protein and RNA was extracted from the individual fractions and analysed by Western and Northern blotting respectively. Western blots were probed using antibodies specific to hU3-55k, capable of detecting both endogenous and FLAG-tagged hU3-55k proteins, with the exception of Δ1-136 NLS whereby antibodies specific to the FLAG motif were used. Northern blot hybridisation was performed using probes specific for the U3 snoRNA and U1 snRNA.

As discussed in the previous chapter, it has been demonstrated that the U3 snoRNA localises to either a 12S peak; representing the U3 monoparticle containing only the core proteins and hU3-55k, or an 80S peak; representing the SSU processome complex containing the U3 snoRNP with accessory factors and the pre-rRNA (Grandi et al., 2002; Granneman et al., 2004; Turner et al., 2009). In all instances, the U3 snoRNA was found distributed between the 12S U3 monoparticle and 80S SSU processome, co-migrating with the endogenous hU3-55k protein (shown only for the wild-type hU3-55k; Figure 4.8 A). Similarly, the FLAG-tagged wild-type hU3-55k, or mutants +P; 8R->G; 342A->E were evenly distributed between the two complexes and co-migrated with the endogenous protein (12S fractions 3-8, 80S fractions 11-17). The hU3-55k mutant 342A-E however, was slightly over expressed so also accumulated as free protein (fractions 1-2; Figure 4.8 A). In contrast, the non-phosphorylatable hU3-55K mutant –P, showed a significant reduction in 80S incorporation, mainly associating with the 12S U3 monoparticle (Figure 4.8 A and B). This is contrast to the wild-type and phosphomimic +P which both followed the profile of the endogenous protein. This difference was highlighted when the ratios of tagged- to endogenous-hU3-55K protein were compared in the 12S and 80S complexes (Figure 4.8 B). Phosphorylation of the N-terminus of hU3-55K is therefore required for efficient incorporation of the U3 snoRNP into the SSU processome.

Δ1-136 NLS was also less able to be incorporated into the 80S SSU processome but was highly unstable, with loss of a portion of the C-terminus resulting in an approximately 50-80S peak (fractions 10-15; Figure 4.8 A), potentially similar to the 50S SSU processome precursor previously described (Turner et al., 2009) although it is difficult to draw conclusions from this data. Furthermore, it should be noted that it was not possible to determine when degradation occurred, although it was often observed upon western blot analysis of Δ1-136 NLS from whole cell lysates, as discussed previously, suggesting it was not due to the process of glycerol gradient centrifugation. Nonetheless, the degradation suggests that the N-terminal motif of the protein is required for stability of the protein whilst both the N- and C-termini may be required for efficient incorporation into the SSU processome.



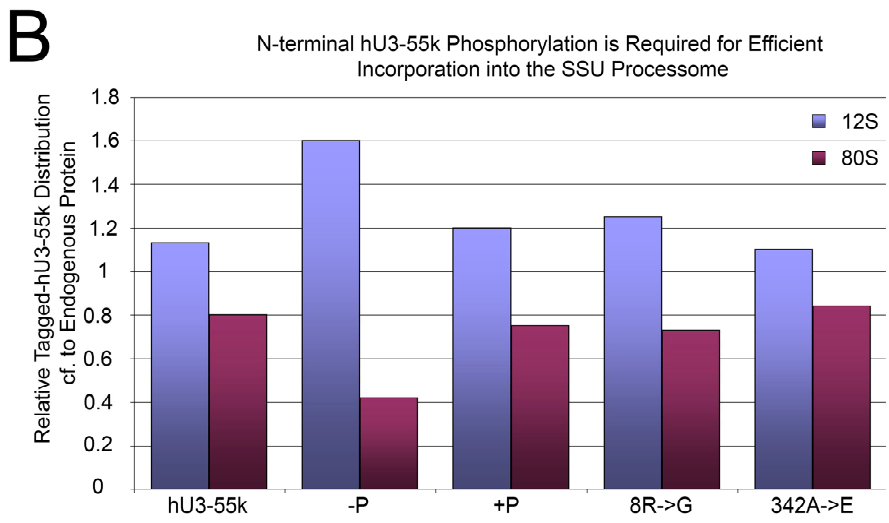


Figure 4.8 Glycerol Gradient Analysis of hU3-55k Mutants Illustrates that Phosphorylation at the N-terminus is Required for Efficient Incorporation into the SSU Processome

A) Extracts were prepared from cells expressing wild-type and mutant hU3-55K-FLAG proteins (indicated at the top left of each panel) at equimolar amounts to the endogenous protein and separated by glycerol gradient centrifugation. Protein and RNA was extracted from the individual fractions and analysed by Western and Northern blotting respectively. Western blots were probed using antibodies specific to either hU3-55k or the FLAG motif (left of each panel) and U3 and U1 RNAs detected using radiolabeled probes. FLAG-tagged and endogenous proteins, and U1 and U3 RNAs are indicated to the right of each panel. 12S monoparticle and 80S SSU processome indicated at the top, as are fraction numbers. T; 10% total input **B)** A bar chart showing the quantitation of tagged-protein distribution relative to that of the endogenous protein across the 12S (blue) and 80S (purple) peaks.

4.2.6 N-terminal Phospho-residues are required for an Active Processome at the A' Cleavage Site of pre-rRNA

The reduced incorporation of the non-phosphorylated hU3-55k into the SSU processome was thought to be either due to inefficient SSU processome formation or the generation of a defective complex. To test these hypotheses, we examined the association of the U3 snoRNP complexes, containing either the wild-type or mutant tagged hU3-55k proteins, with the pre-rRNA. This was done by expressing hU3-55k and mutant proteins from HEK293 cells, immunoprecipitation of the tagged proteins and associated RNA, and subsequent analysis of the pre-rRNA by S1 nuclease protection, using a probe spanning the initial A' cleavage site (Turner et al., 2009).

The pre-rRNA, both uncleaved and cleaved at the A' site, was co-precipitated with wild-type, +P, 8R->G and 342A->E hU3-55k proteins with equal efficiencies (Figure 4.9). No significant amount of pre-rRNA was co-precipitated with the C-terminal mutant ΔWD, most likely due to its lack of association with the U3 snoRNA and similarly, pre-rRNA was not co-precipitated with the vector alone cell line. Uncleaved pre-rRNA however, co-precipitated with the non-phosphorylated (-P) and Δ1-136 NLS hU3-55k proteins to a much higher level than seen with the wild-type or constitutive phosphorylation mimic +P. Furthermore, pre-rRNA that had been cleaved at the A' locus was only weakly co-purified with the –P and Δ1-136 NLS mutants. This demonstrates that the C-terminus is crucial for association with the U3 snoRNA and therefore, the pre-rRNA, whereas the N-terminus is required to support efficient cleavage at the initial A' site within the 5' ETS. Moreover, it is specifically the phosphorylation of conserved residues within the N-terminus that appear to be important in regulating the U3 snoRNP's association with A' cleaved pre-rRNA.

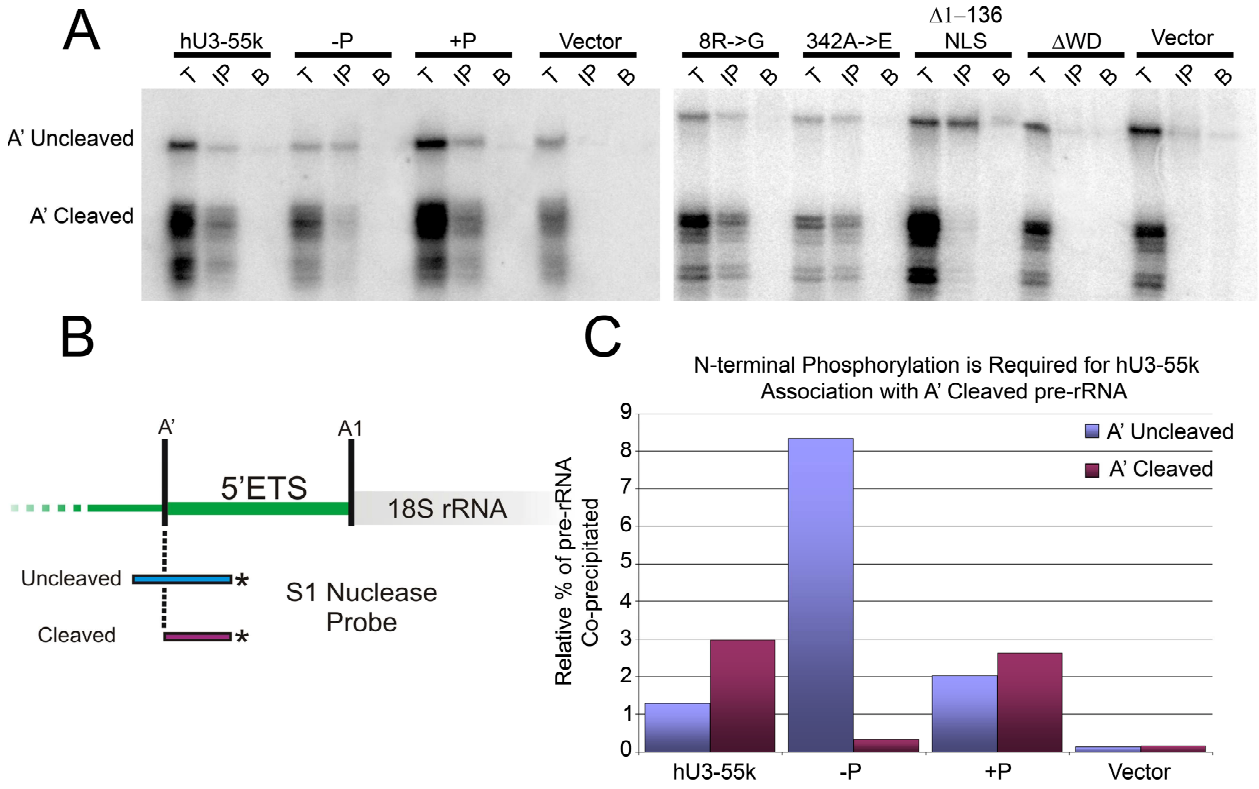


Figure 4.9 hU3-55k's C-terminus is Required for Association with pre-rRNA, and N-terminal Phosphorylation is Required for an Active Processome at the A' Cleavage Site

hU3-55k expressing HEK293 cells were incubated with the appropriate amount of tetracycline to induce comparable levels of expression to that of the endogenous protein. **A)** Cells were lysed and protein-RNA complexes immunoprecipitated using anti-FLAG coated Protein G Sepharose beads. RNA was extracted and analysed by S1 nuclease protection, using a radiolabeled probe spanning the initial A' cleavage site of the pre-rRNA, yielding either an uncleaved probe (protected by the pre-rRNA) or a partially degraded probe (due to only a section being protected after the initial pre-rRNA cleavage, see (B); as indicated to the left of the panel). RNA was subsequently separated upon a denaturing gel and exposed to a PhosphorImager screen. The protein expressed and precipitated is indicated above each panel. T represents 10% total input; IP immunoprecipitation; B beads alone control with no antibody. **B)** Schematic diagram of the S1 nuclease protection assay. Pre-rRNA is shown in green, 18S rRNA in grey with the A' and A1 cleavage sites indicated. The uncleaved probe is shown in blue, the cleaved probe in purple. Asterisks indicate the radiolabel on the probe. **C)** A bar chart indicating that N-terminal phosphorylation of hU3-55k is required for association with A' cleaved pre-rRNA. The relative percentage of probe protected by the pre-RNA co-precipitated (the vertical axis) from each cell line (wild-type hU3-55k; -P; +P or vector alone, as indicated on the horizontal axis) was derived from the known 10% input (T). A' uncleaved; blue, A' cleaved; purple.

4.3 Discussion

In the previous chapter we demonstrated that hU3-55k is essential in regulating levels of the U3 snoRNP present in human cells. The protein however, was originally hypothesised to play a key role in defining the U3 snoRNP as functionally distinct from other box C/D snoRNPs, and was believed to aid recruitment of other SSU processome factors (Granneman et al., 2004; Samarsky and Fournier, 1998; Venema et al., 2000). Furthermore, the protein contains highly conserved domains, with the potential for post-translational modifications within the N-terminus, which had previously been shown to be unnecessary for nucleolar localisation (Pluk et al., 1998). Moreover, with potential regulation of the protein during tumourogenesis (Luo et al., 2001; Pan et al., 2008; Sjöblom et al., 2006; Sperger et al., 2003; Zanivan et al., 2008) and cell cycle progression (Olsen et al., 2010), we decided to investigate the role of some of these conserved domains with respect to U3 snoRNA association, localisation and SSU processome formation.

Here we have demonstrated that deletion of the C-terminal residues 193-352, removing WD domains 2-5 (ΔWD), resulted in loss of U3 snoRNA association and therefore, mis-localisation of the protein to the nucleus, as previously demonstrated (Lukowiak et al., 2000). Conversely, the other hU3-55k mutants examined had no discernable affect on U3 snoRNA association or on the localisation of the protein; with all other hU3-55k proteins localising to the nucleolus. Most interestingly, the N-terminal serine residues 50, 51 and 53, found to be phosphorylated in a range of cells (Olsen et al., 2006; Pan et al., 2008; Zanivan et al., 2008), were required to be phosphorylated for efficient U3 snoRNP integration into the SSU processome (80S complex) and cleavage of the pre-rRNA at the initial (A') site, with processing blocked at this site in pre-rRNAs associated with the unphosphorylatable hU3-55k. This was further emphasised by the similarity of the results if the entire N-terminal 136 residues were removed. Curiously in this case though, the protein was unstable, being degraded at the C-terminus and forming what appeared to be ~50S complex; similar to that previously described as an SSU processome precursor (Turner et al., 2009).

It was previously demonstrated that the N-terminal 44 residues were not required for nucleolar localisation (Pluk et al., 1998), and elsewhere that the E-stretch (residues 64-74) was similarly, not required for nucleolar localisation or U3 snoRNA association (Lukowiak et al., 2000). Here we demonstrate that in the presence of a minimal NLS, the entire 136 residues of the N-terminus are not required for nucleolar localisation of the protein, binding the U3 snoRNA or association with pre-rRNA. They are however, required for efficient accumulation into the 80S SSU processome complex and for association with A' cleaved pre-rRNA, although due to protein degradation at the C-terminus, only limited conclusions may be drawn from these results.

Base pairing of the U3 snoRNA within the 5' ETS of the pre-rRNA and the subsequent A' cleavage event are thought to be essential for movement of the U3 snoRNA from the DFC to the GC (Granneman et al., 2004). It may therefore, be expected that preferential (-P) hU3-55k association with the uncleaved pre-rRNA may result in mis-localisation of the protein with retention in the DFC, however this was not observed. This may be due to the A' cleavage event occurring in the GC, or blocked processing complexes lacking a component required to tether them to the DFC. Moreover, the tagged protein was expressed at low levels, with the endogenous wild-type protein expected to remain functioning normally. For this reason, we would not expect to observe any major changes in RNA localisation or pre-rRNA processing within the cell. Nonetheless, phosphorylation of this N-terminus at residues 50, 51 and 53, is shown here to be a key regulatory event in allowing SSU processome formation and most likely modulating the A' cleavage event.

The two potential cancer mutations did not appear different to the wild-type hU3-55k protein in any of the assays performed, suggesting that either any alterations in function occurred elsewhere in ribosome biogenesis or were not measureable by our means. As mentioned previously however, the original methodology and statistical analysis used in the study identifying these mutations has been questioned by a number of other groups after we created these mutants,

suggesting these mutations may not occur at a statistically significant rate (Forrest and Cavet, 2007; Getz et al., 2007; Rubin and Green, 2007).

Interestingly, the C-terminal domain of UBF is made up almost exclusively of blocks of aspartic and glutamic acid residues, each terminating in serine residues. This domain binds and recruits SL1, with binding enhanced by phosphorylation of the serine residues, probably by casein kinase II (CK2) (Moss et al., 2007; Tuan et al., 1999). This is thought to be important in formation of the pre-initiation complex on the DNA, and is required for transcription of the pre-rRNA in eukaryotes (Lin et al., 2002; Moss et al., 2007). This would appear to have striking similarity with hU3-55k, whereby the protein is phosphorylated, potentially by CK2 (Gnad et al., 2007), adjacent to the glutamic acid / E-stretch, which in turn may recruit other proteins required for the A' cleavage of the pre-rRNA. Moreover, glutamic acid is frequently used as a phospho-mimic, similar to the use of aspartic acid used in our study due to their negative charge. This suggests that phosphorylation of these serine residues may act to enhance or regulate the function of the charged motif. Indeed, the glutamic acid-rich domain was previously hypothesized to facilitate protein-protein interactions (Pluk et al., 1998) which, when our data is taken into account, may be enhanced by phosphorylation of adjacent serines in a similar fashion to the UBF protein.

There are also many examples of phosphorylation / dephosphorylation regulating the nuclear import and export of protein factors. Entry into mitosis is regulated by the subcellular relocalisation of Cdc2/cyclin B, which is rapidly imported into the nucleus at the end of G2. The constitutive phosphorylation mimic S191D of Cdc25C has been shown to enhance accumulation of Cdc25C in the nucleus, whilst the unphosphorylatable S191A mutant facilitates nuclear exclusion of the protein (Bahassi el et al., 2004). Under similar control is β-catenin, an important protein present in two separate pools within the cell. It is involved in either cell-cell adhesion, binding the cytoplasmic domain of cadherin-type adhesion receptors, or within the nucleus, associating with transcription factors as part of the Wnt signalling pathway. It has been found that phosphorylation of specific residues

control its nuclear accumulation, which in turn is thought to enhance its role in transcription (Maher et al., 2010). It may therefore be the case that phosphorylation of hU3-55k may be responsible for differences in localisation of the protein, although we did not see evidence to support this.

Although phosphorylation of these residues has been reported in a range of cells, it is not clear how the phosphorylation is regulated, although it appears unaffected by treatment with epidermal growth factor (EGF) (Olsen et al., 2006; Pan et al., 2008; Zanivan et al., 2008). Nonetheless, whilst it has not been demonstrated experimentally, it is predicted that residue 51 may potentially by phosphorylated by CK2 or GSK3, whereas residue 53 may be phosphorylated by CK1, CK2 or GSK3, based upon the amino acid motif, all of which are linked to the Wnt signaling pathway (Gnad et al., 2007; Olsen et al., 2010). Although thought to be constitutive, CK2 and the serine-threonine kinase GSK3 also have opposing actions on β-catenin. GSK-3 phosphorylation of the N- terminus of β-catenin promotes degradation; whilst phosphorylation by CK2 in a different domain protects it. This allows expression of the downstream target gene, cyclin D1; required for cell cycle progression through the G_1 restriction point (Farago et al., 2005; Seldin et al., 2005). It does not appear that this is the case however in our study, as neither protein (-P or +P hU3-55k) appeared prone to degradation, although this may be due to the use of phosphomimic motifs. Intriguingly, the UTP-C complex contains the proteins RRP7, UTP22 and the four subunits of casein kinase 2 CKA1, CKA2, CKB1, CKB2 (Krogan et al., 2004). The UTP-C complex is believed to associate with the pre-rRNA at a similar time point to the U3 snoRNP, after tUTP association in yeast, suggesting that this could well be the complex responsible for hU3-55k phosphorylation (Krogan et al., 2004; Perez-Fernandez et al., 2007).

If these phosphorylation events are regulated to control ribosome biogenesis, it would also be expected that their de-phosphorylation is regulated. There are a range of different phosphatase families, with serine / threonine specific phosphatases and dual specificity phosphatases, capable of targeting both

phosphotyrosine and phosphoserine/phosphothreonine residues (Camps et al., 2000; Mumby and Walter, 1993). Both of these families of phosphatases are crucial in regulating cell growth and division (Camps et al., 2000; Mumby and Walter, 1993), although it is not clear which may be responsible for de-phosphorylating hU3-55k, or when this occurs.

It has been reported that the residues are phosphorylated in a cell cycle dependent manner in human cells, most highly at M and G_1 phases when compared to asynchronous cells (Olsen et al., 2010). The data from the study suggests that in tumourigenic cells, hU3-55k residues 50, 51 & 53 are phosphorylated during mitosis, at the same time as more than half of all the identified phospho-residues of kinase proteins. This includes many major kinase regulators of cell-cycle progression (Olsen et al., 2010). During the subsequent G_1 phase, the checkpoint of which is deregulated in virtually all cancers (Yao et al., 2008), hU3-55k was also found to be highly phosphorylated. In this phase, the biosynthetic activities of the cell resume to produce components required for growth and DNA replication in S phase. The results from this study (Olsen et al., 2010) are surprising however, and may be flawed. The procedure used was designed to enrich kinase proteins using affinity ligands, therefore potentially biasing the collection method. It is also unclear how material used in the study was collected or lysate made. It is often the case that purification of nucleolar material can prove difficult due to inaccessibility of the components therefore, the study may not have been able to collect representative amounts of each protein. Furthermore, in some instances, nuclear extracts were used which have been shown to lack nucleolar material (Watkins et al., 2004). This problem is made worse when considering collection of material through the cell cycle, as in M phase there are no nucleoli; changing the abundance and accessibility of the nucleolar proteins. Moreover, the study reported phosphorylation during M phase which our data predicts, would increase pre-rRNA processing, yet this is part of the cell cycle where RNA polymerase I transcription and processing are repressed (Hernandez-Verdun et al., 2002). Conversely, it should be noted that A' cleaved, 45S pre-rRNAs are thought to be present in M phase so hU3-55k may become phosphorylated to ensure that all pre-rRNAs

present are processed to this point. Moreover, pre-rRNA processing is believed to restart as soon as nucleoli begin to reform at late M / early G_1 phase, with the recruitment processing complexes potentially aiding nucleolar assembly (Hernandez-Verdun et al., 2002). Nonetheless, it is not clear how reliable the data from phosphorylation studies may be regarding nucleolar proteins, especially when considering the methodology was designed to examine kinases. Regardless of this, our data indicate that phosphorylation of hU3-55k may play a key regulatory role in SSU processome assembly and facilitation of cleavage at the A' site of pre-rRNA.

The C-terminal domain of hU3-55k has been shown to contain 7 WD repeat elements, believed to be important in protein-protein interactions in other proteins. In the case of hU3-55k though, these motifs are required for U3 snoRNA association, making any subsequent role in protein-protein interactions, such as with SSU processome components, difficult to discern. One possible way to investigate the role of the WD 40 domains in protein-protein interactions may be to generate either a crystal structure, or computational model based on known structures. These in turn would be used to design point mutations on the surface of the protein that would not disrupt the propeller-like structure and aid in characterising the various domains with greater resolution.

Association of hU3-55k with the U3 snoRNA is required for integration of the U3 snoRNP into the SSU processome complex, as shown here through a lack of association of the ΔWD mutant with pre-rRNA. For this reason, we created the Δ1-136 NLS mutant, containing a minimal NLS to ensure maximal nucleolar localisation, so as not to limit potential association with the U3 snoRNA. Although this protein was able to associate efficiently with the U3 snoRNA and pre-rRNA, it appeared unstable, with degradation at the C-terminus. Nonetheless, this hinted that none of the N-terminal residues may be required for integration of the U3 snoRNP into the SSU processome precursor described previously (Turner et al., 2009). To demonstrate this more fully, further experiments would be required such

as the immunoprecipitation of SSU processome proteins from this peak, as performed in the previous study (Turner et al., 2009).

Nonetheless, it would appear that the C-terminus is required for U3 snoRNA association, accumulation and potentially the recruitment of the initial SSU processome factors. Conversely, the N-terminus may play a regulatory role in recruiting factors required for the A' cleavage step and subsequent association with the pre-rRNA; modulated by phosphorylation at residues 50, 51 and 53. This demonstrates hU3-55k as a crucial protein for ribosome biogenesis, with regulation at the point of U3 snoRNP accumulation and pre-rRNA processing.

Chapter Five

RNA Binding / Modifying Proteins Involved in 18S rRNA Processing

5.1 Introduction

In eukaryotes the 47S pre-rRNA (35S in yeast) is transcribed by RNA pol. I in the nucleolus. This is in contrast to the 5S rRNA which is transcribed by RNA pol. III in the nucleoplasm of the cell (Nazar, 2004). The 47S pre-rRNA contains the 18S, 5.8S and 28S (25S in yeast) rRNAs within a single precursor which also contains two internal transcribed sequences (ITS1 and ITS2), and two external transcribed sequences (5' ETS and 3' ETS), all of which are removed from the mature rRNA through processing events (Figure 5.1).

There are however, a number of differences between the processing steps in yeast, and those in humans. In *S. cerevisiae*, the initial processing steps that produce the 18S precursor have been demonstrated to occur co-transcriptionally, although it is not known if this also occurs in humans (Kos and Tollervey, 2010; Osheim et al., 2004).

In yeast, initial pre-rRNA processing occurs within the 5'ETS at site A_0 (Figure 5.1) (Granneman and Baserga, 2004) followed by A_1 (5' ETS / 18S 5' boundary) which occur to remove the 5'ETS. Cleavage at A_2 subsequently occurs within ITS1, separating the pre-18S (20S) from the 25S and 5.8S precursors. The large and small subunit precursors are then processed separately. The 20S precursor is transported to the cytoplasm where cleavage at the D site produces the mature 18S rRNA (Fatica et al., 2003; Schafer et al., 2003). In contrast, the processing of the large precursor occurs within the nucleolus and nucleus through two alternative pathways, resulting in either long or short forms of the 5.8S rRNA; $5.8S_L$ or $5.8S_S$ respectively, and 25S rRNA (Henras et al., 2008).

In higher eukaryotes pre-rRNA processing has been shown to differ from that in yeast. Initial pre-rRNA processing occurs within the 5'ETS at site 01/A' in mammalian cells, at a site upstream of the A_0 locus in yeast pre-rRNA (Enright et al., 1996; Henras et al., 2008; Kass et al., 1990). Processing at A' occurs at two adjacent sites, with processing at site 02 (3'ETS), leading to two discrete 45S pre-rRNAs that differ by only a few nucleotides at the 5' end (Kass et al., 1987). Nonetheless, these cleavages remove the 5'ETS and separate the 18S rRNA from the 28S and 5.8S precursors (Figure 5.1; (Nazar, 2004)).

It has however, recently been demonstrated that *Xenopus laevis*, *Trypanosoma brucei,* mice and humans may also cleave pre-rRNA at a site comparable to that of site A0 in yeast (Borovjagin and Gerbi, 2001; Hartshorne et al., 2001; Kent et al., 2009; Rouquette et al., 2005). Therefore, it would appear that in higher eukaryotes there may be 3 alternative pathways for 45S processing. Cleavage at site A0 in the 5' ETS leads to a 43S product; cleavage at site 1 at the 5'ETS/18S border leads to a 41S product; or cleavage at site 2 within ITS1, leading to a 32S (5.8S and 28S) and 30S (18S) product (Figure 5.1) (Hadjiolova et al., 1993; Rouquette et al., 2005). These pathways then reconverge upon subsequent cleavages leading to the 18S precursor (21S) and the 5.8S and 28S precursor (32S).

The 21S pre-rRNA is then cleaved 24 nucleotides into ITS1 (called site 2' here) to produce 18S-E, a step not present in yeast. Subsequently, site 3 cleavage produces mature 18S rRNA (Hadjiolova et al., 1993; Rouquette et al., 2005). The 32S pre-rRNA is cleaved at site 3' and 4' to produce mature 28S and 5.8S rRNA.

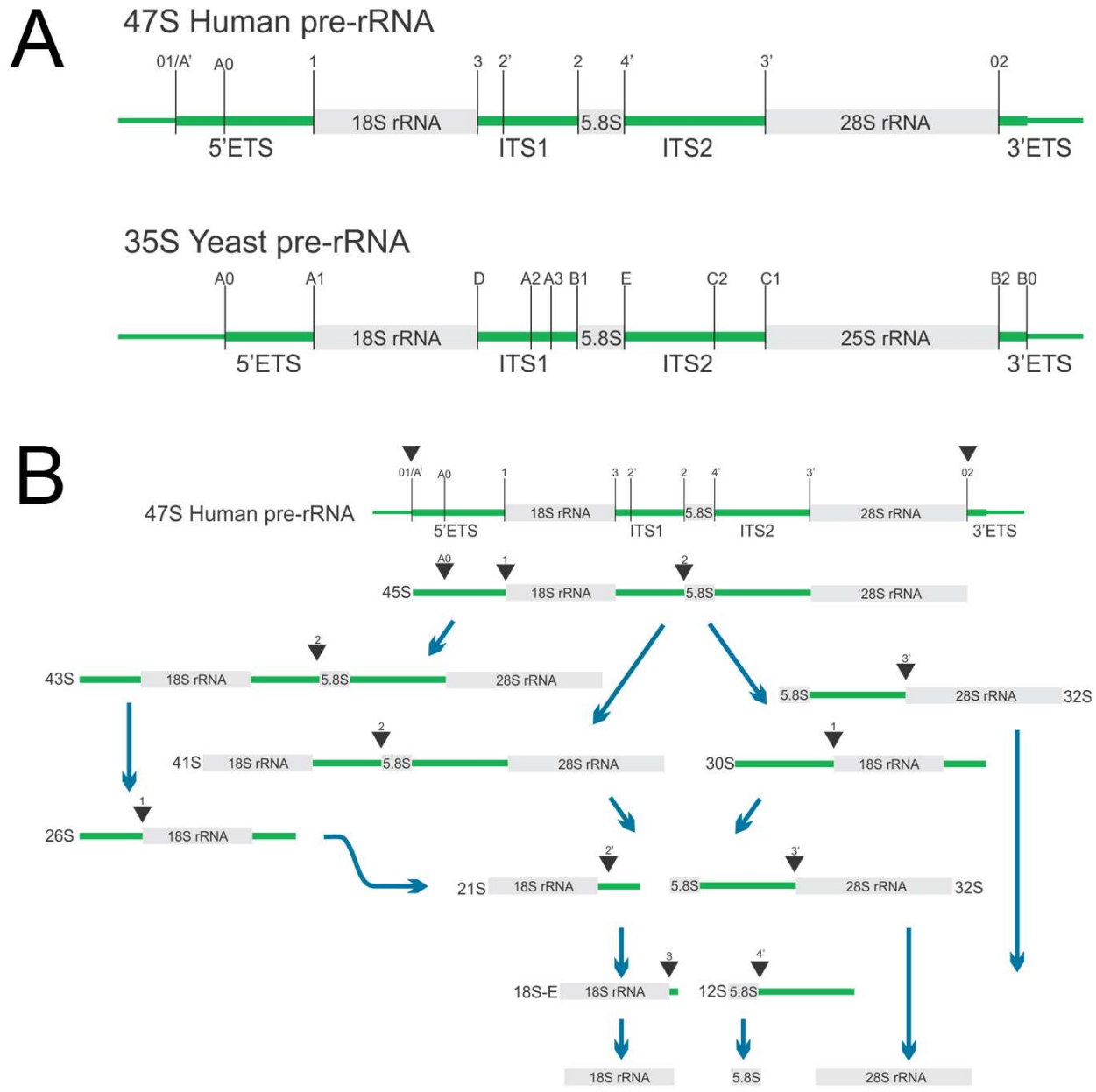


Figure 5.1 Pre-rRNA Processing in Humans and Yeast
A) Full length pre-rRNA transcripts are shown for both human and yeast (*S. cerevisiae*). RNA is shown in green (cleaved from the mature product; named beneath) and grey (constituting the mature rRNA; named within the box). Cleavage sites so far identified in both human and yeast pre-rRNA are indicated by vertical black lines with the cleavage site named above. ETS; External transcribed sequence, ITS; Internal transcribed sequence. **B)** Processing of human pre-rRNA, as in (A) with blue arrows indicating processing steps, black triangles indicating the sites of cleavage. Figure not to scale, derived from previous work (Fatica and Tollervey, 2002; Hadjiolova et al., 1993; Henras et al., 2008; Rouquette et al., 2005).

It would appear that in human cells, there is cleavage at both A0 and A', in contrast to yeast which is only known to cleave at A_0. In addition, the cleavage of 21S pre-rRNA in human cells at around nucleotide 24 of ITS1, producing 18S-E pre-rRNA, is not known to occur in yeast (Figure 1.7) (Hadjiolova et al., 1993; Rouquette et al., 2005). This demonstrates that there may be at least two additional steps in 18S pre-rRNA processing in human cells. This may add yet another layer of complexity to pre-rRNA processing in higher eukaryotes (Rouquette et al., 2005).

A number of snoRNPs are involved in regulating pre-rRNA processing rather than directly facilitating base modifications (Maxwell and Fournier, 1995). The U3 Box C/D snoRNPs is required for the initial cleavage steps of pre-rRNA; crucial for production of the 18S rRNA. It functions as part of a multi-protein complex termed the small subunit (SSU) processome. This is required for cleavages at sites A', 1 and 2 (A_0, A_1 and A_2 in yeast). Moreover, U3 snoRNA has also been shown to be required for cleavages at A0 in *X. laevis* and *T. brucei*, suggesting that it may also be required for such cleavage in human cells (Borovjagin and Gerbi, 2001; Hartshorne et al., 2001; Kent et al., 2009; Rouquette et al., 2005).

The U3 snoRNP is observed in two complexes, the 12S free U3 snoRNP and the 80S SSU processome (90S in yeast) (Granneman et al., 2003). The 12S U3 snoRNP contains the Box C/D core proteins NOP56, NOP58, fibrillarin and 15.5K, but also the U3-sepcific protein hU3-55K (Pluk et al., 1998; Watkins et al., 2000). The association of hU3-55k (Rrp9 in yeast) at the B/C Box is required for U3 snoRNP association with the rRNA, and also for cleavage at sites A', 1 and 2 in humans / A_0, A_1, A_2 in yeast (Granneman et al., 2002; Granneman et al., 2004; Venema et al., 2000). Furthermore, it may not only be required for incorporation of the U3 snoRNP into the 80S SSU processome but may also aid recruitment of processing factors responsible for these cleavages to the complex (Granneman et al., 2002; Granneman et al., 2004).

Base-pairing between the 5' end of the U3 snoRNA and the 3' terminus of the pre-rRNA (Figure 1.10) is thought to prevent premature pseudo-knot structure within

the small-subunit rRNA; crucial in the overall folding of the mature rRNA (Hughes, 1996; Sharma and Tollervey, 1999) and for the cleavages surrounding the 18S pre-rRNA (Borovjagin and Gerbi, 2001; Gerbi et al., 2003; Hughes, 1996; Sharma and Tollervey, 1999). This supports a model whereby U3 snoRNA acts as a bridge to draw together the 5' and 3' ends of the 18S rRNA pre-rRNA to coordinate their cleavage and aid SSU biogenesis (Borovjagin and Gerbi, 2001; Gerbi et al., 2003).

U3 snoRNA – pre-rRNA base-pairing is thought to be aided by protein factors, potentially an MPP10 complex containing MPP10, IMP3 and IMP4, with IMP3 responsible for mediating interactions with the U3 snoRNA (Granneman et al., 2003; Granneman et al., 2004; Wehner et al., 2002). This is thought to ensure 18S – 5'ETS interactions are not rate limiting for the cell (Gerczei and Correll, 2004; Gérczei et al., 2009).

The mammalian nucleolus is comprised of three distinct structures, which correlate to the stages of ribosome biogenesis (Figure 1.12). Pre-rRNA processing is most widely documented in yeast, although work has also been carried out in vertebrates with distinct differences discovered between higher and lower eukaryotes (Granneman and Baserga, 2004; Prieto and McStay, 2005; Rouquette et al., 2005; Terns and Terns, 2002). A significant difference is the spatial organising of pre-rRNA processing. Yeast are believed to only contain two nucleolar compartments; a network of fibrillar strands (F) embedded within granules (G). This is in contrast to higher eukaryotes which contain three distinct nucleolar compartments; a fibrillar centre (FC), dense fibrillar component (DFC) and granular component (GC) where processing has been shown to occur in a vectorial fashion (Thiry and Lafontaine, 2005).

In *S. cerevisiae*, it is thought that pre-rRNA is transcribed within the fibrillar strands where cleavages A_0-A_3 also occur to separate the large and small subunit precursors. The pre-rRNA then moves to the granules where cleavages B_1, C_2 (separating 5.8S and 25S precursors) and B_2 (3' ETS) occur. This is followed by

cleavage at sites E and C_1 in the nucleus and cleavage at site D in the cytoplasm ((Figure 5.1 A (Henras et al., 2008)).

The exact timings of these cleavages are however, not known. Nonetheless, it is thought that in human cells, 47S pre-ribosomal RNAs (rRNAs) are transcribed at the FC / DFC border of the nucleoli (Cheutin et al., 2002; Huang, 2002; Puvion-Dutilleul et al., 1997). Elongating 47S pre-rRNA is thought to move rapidly into the surrounding DFC (Cheutin et al., 2002) where early processing of pre-18S rRNA (at the A' site within the 5'ETS) is thought to occur (Derenzini et al., 1990; Granneman et al., 2004) before movement to the granular component (GC). It is here that removal of the core 5'ETS sequence (site 1 cleavage) is then thought to take place (Gerbi and Borovjagin, 1997; Lazdins et al., 1997). The pre-rRNAs then move into the nucleoplasm before finally being exported through nuclear pores to the cytoplasm (Lei and Silver, 2002) where the final 18S 3' cleavage occurs (Rouquette et al., 2005)).

During maturation, the 18S rRNA is incorporated into the small 40S ribosomal subunit (SSU) and the 28S and 5.8S rRNAs are incorporated into the large 60S ribosomal subunit (LSU), along with the 5S rRNA (transcribed separately by RNA pol. III). These are then assembled at the site of transcription with initiation factors to form a functional ribosome (Dai and Lu, 2008).

Consistent with roles in both the initial and later cleavage steps of pre-rRNA processing, the U3 snoRNA is known to localise throughout the DFC and GC regions of the nucleolus (Gerbi and Borovjagin, 1997; Granneman et al., 2004). It is, therefore, suggested that in the DFC, U3 snoRNA mediates cleavage at A' within the 5' ETS and remains associated in the GC to facilitate cleavages at sites 1 and 2 on either side of the 18S rRNA (Gerbi and Borovjagin, 1997; Granneman et al., 2004).

A number of other proteins and sub-complexes are associated with the SSU processome in a dynamic fashion. This has been best documented through

proteomic studies in yeast (Figure 1.11), although in the majority of cases, precise roles are yet to be elucidated (Dragon et al., 2002; Grandi et al., 2002; Krogan et al., 2004; Perez-Fernandez et al., 2007; Schafer et al., 2003). In contrast to yeast, large-scale proteomic analysis has not been performed on human cells. Therefore, the composition of the human SSU processome is relatively unknown, apart from studies examining specific homologous proteins (Gerus et al., 2010; Granneman et al., 2003; Granneman et al., 2002; Prieto and McStay, 2007; Rouquette et al., 2005; Turner et al., 2009).

In yeast, five main sub-complexes of the SSU processome have been identified. These are thought to associate independently of the rRNA and to be loaded onto the SSU processome in a modular manner (Perez-Fernandez et al., 2007). Detected through proteomics, the U-three interacting proteins (UTPs) have been shown to be required for efficient pre-rRNA processing (Dragon et al., 2002). The UTPs form distinct complexes; the UTP-As/tUTPs (transcriptional UTPs), UTP-Bs and UTP-Cs (Figure 1.11) (Dosil and Bustelo, 2004; Krogan et al., 2004; Perez-Fernandez et al., 2007). It has been suggested that the tUTP complex binds to the pre-rRNA first, followed by the UTP-B and UTP-C complexes, with UTP-B, U3 snoRNP and MPP10 complexes potentially associating at the same time (Dunbar et al., 1997; Granneman et al., 2003; Lee and Baserga, 1999; Perez-Fernandez et al., 2007).

In humans, it is has been shown that the U3 snoRNP, tUTP, UTP-B and MPP10 complexes are present in the SSU processome and it has been suggested that these may function in a similar way to their counterparts in yeast (Gerus et al., 2010; Granneman et al., 2003; Granneman et al., 2002; Prieto and McStay, 2007; Rouquette et al., 2005; Turner et al., 2009). It should be noted however, that study has been limited to only a few SSU processome proteins in human cells. Nonetheless, the tUTPs have been shown to be required for efficient transcription of rDNA genes in humans, linking transcription and pre-rRNA processing (Prieto and McStay, 2007).

It has been stated that the 90S pre-ribosome complex (SSU processome) contains at least 32 non-ribosomal proteins in yeast, many of which associate with and / or modify the rRNA such as RNA helicases, GTPases, RNA annealing factors, methyltransferases, and potential chaperones (Bernstein et al., 2004; Dragon et al., 2002; Henras et al., 2008). RNA binding proteins include PNO1, ESF2, MRD1, RRP5, KRR1 and RRP7, all of which are thought to either aid the association or release of other processing factors. Proteins believed to modify the RNA include the di-methylatransferase DIM1 and the RNA cyclase-like RCL1. Putative rRNA nucleases include UTP23 and UTP24 (Table 5.1). If these proteins are conserved in humans and the roles they may play are however, mostly unknown.

It is predicted that following cleavage at sites A_0, A_1 and A_2, many of the SSU processome factors dissociate from the pre-rRNA (Fatica and Tollervey, 2002). The 20S pre-rRNA in yeast (18S-E in humans) is subsequently exported to the cytoplasm where it is processed to the 18S rRNA (Fatica and Tollervey, 2002; Henras et al., 2008). In yeast, once in the cytoplasm, the pre-rRNA is methylated at residues A1779 and A1780, at the 3' end of the 18S sequence, by the DIM1 methylase (Brand et al., 1977; Lafontaine et al., 1994). Subsequently, the 20S pre-rRNA of yeast is cleaved at site D by the NOB1 endonuclease, a PINc domain protein (Lamanna and Karbstein, 2009; Pertschy et al., 2009). Again, if this also occurs in human cells has not been documented.

Late processing of 20S (18S-E in humans) to the mature 18S rRNA requires a range of proteins. This pathway appears however, to be conserved between yeast and humans. The exportin CRM1/XPO1 (chromosomal region maintenance) / exportin 1) has been shown to be required for the nuclear export of the pre-40S complexes to the cytoplasm (Leger-Silvestre et al., 2004; Moy and Silver, 1999; Rouquette et al., 2005). RIO1 and RIO2 are shuttling proteins which most likely associate with pre-40S particles in the nucleus (Rouquette et al., 2005; Vanrobays et al., 2003). Whilst they are not necessary for export of the pre-40S complexes, they are required for the cytoplasmic maturation of 20S pre-rRNA at site D in yeast / 18S-E maturation and cleavage site 2' in humans (Rouquette et al., 2005;

Vanrobays et al., 2003; Vanrobays et al., 2001; Zemp et al., 2009). Moreover, in human cells, RIO2 kinase activity is essential for the recycling of PNO1, LTV1 (an export factor), and NOB1 (an endonuclease) (Zemp et al., 2009).

Despite a relatively large volume of work on ribosome biogenesis, the majority of research on SSU processome components has focused on the processes in yeast. Whilst this is a useful model system, it is almost certain that there may be key differences between this and humans. Furthermore, due to the vast number of proteins involved, and the transient nature of the SSU processome complex, little has been done in some cases beyond identification of the proteins. Crucially, very little documentation exists in either yeast or humans, of where or how the vast majority of these RNA-binding or RNA-associated factors bind the pre-rRNA. Recently in yeast, a novel protein-RNA cross-linking analysis technique has demonstrated the binding sites for a number of proteins including the core U3 snoRNP proteins (Granneman et al., 2009), and six late-acting 40S synthesis factors (Granneman et al., 2010). Unfortunately, no binding sites for human proteins have been reported using this technique.

Table 5.1 SSU Processome Factors to be Examined

Protein	Domains and Associations	References
PNO1 (Partner of NOB1, also known as DIM2)	U3 associated, crucial for cleavages A_1 and A_2, pre-40S nucleocytoplasmic export. RNA-binding KH-domain, directly binds the pre-rRNA in ITS1, shuttles between the nucleolus and the cytoplasm. Trafficking is tightly regulated by growth. Binds NOB1 and DIM1. Homologous to KRR1.	(Gromadka et al., 2004; Gromadka and Rytka, 2000; Tone et al., 2000; Vanrobays et al., 2004; Vanrobays et al., 2008)
DIM1 (Di-Methyltransferase)	Required for cleavage at A_1, A_2 and D. Methylates A1779 and A1780 (*S. cerevisiae*) at the 3' end of 18S pre-rRNA, occurring in the cytoplasm, prior to the site D cleavage although largely nucleolar in localisation. Associates with early and intermediate 40S pre-ribosomes, association may be incompatible with translation. Binds PNO1.	(Brand et al., 1977; Granneman et al., 2010; Lafontaine et al., 1994; Lafontaine et al., 1995; Lafontaine et al., 1998b; Schafer et al., 2003; Zemp et al., 2009)
NOB1	PINc domain, essential for processing of the 20S pre-rRNA to 18S rRNA. D-site endonuclease in yeast, requires RNA helicase PRP43. Localises in the cytoplasm, associates with the pre-40S ribosomal complex containing ribosomal proteins and late-assembly factors DIM1, PNO1 and RIO2. Binds PNO1.	(Fatica et al., 2003; Fatica et al., 2004; Lamanna and Karbstein, 2009; Pertschy et al., 2009; Stevens et al., 1991)
KRR1	KH-domain containing protein, highly expressed in dividing cells, expression is down regulated in stationary phase. Possible role in the S phase check point. Required for the synthesis of 18S rRNA and for the assembly of 40S ribosomal subunit. Possibly paralog of PNO1. Binds KRI1 (KRR1 interaction protein).	(Grandi et al., 2002; Gromadka et al., 2004; Gromadka and Rytka, 2000; Kondoh et al., 2000; Sasaki et al., 2000; Vanrobays et al., 2004)
RCL1 (RNA terminal phosphate cyclase-like)	Involved in rRNA processing at sites A_0, A_1, and A_2; associates with BMS1 (contains G-domain; possible molecular switch to initiate cleavage). May associate with BMS1, SOF1 and DHR1.	(Billy et al., 2000; Wegierski et al., 2001)
UTP23	Essential protein in yeast, required pre-rRNA processing at sites A_0, A_1, and A_2 / 18S rRNA maturation. Contains potential nuclease PINc domain. Associates with UTP24 and Faf1.	(Bleichert et al., 2006; Dammann et al., 1993; Lee and Nazar, 2003; Rempola et al., 2006)
UTP24 (Fcf1)	Essential protein in yeast, required for pre-rRNA processing at sites A_0, A_1, and A_2 steps / 18S rRNA maturation. Contains potential nuclease PINc domain required for A_1 and A_2 cleavages. Associates with UTP23 and Faf1.	(Bleichert et al., 2006; Rempola et al., 2006; Russell and Zomerdijk, 2005)
RRP7	Component of the UTP-C complex. Required for cleavages at A_0, A_1 and A_2, 20S pre-rRNA production, incorporation of ribosomal protein S27 into the pre-ribosome.	(Baudin-Baillieu et al., 1997; Denissov et al., 2007; Krogan et al., 2004; Perez-Fernandez et al., 2007)
tUTP4	Component of the transcriptional UTP sub-complex (tUTP). tUTPs associate with pre-rRNA and U3 snoRNA, are required for subsequent association of the U3 snoRNP, the UTP-B and UTP-C complexes, and pre-rRNA processing.	(Gallagher et al., 2004; Perez-Fernandez et al., 2007; Prieto and McStay, 2007)
UTP6	Component of the UTP-B sub-complex, required for cleavage at A_0, A_1 and A_2. Associated with U3 snoRNA and 5' ETS of pre-rRNA.	(Gallagher et al., 2004; Krogan et al., 2004; Perez-Fernandez et al., 2007)
ESF2 (ABT1; an enhancer of basal transcription)	Associates with the pre-rRNA 5'ETS and U3 snoRNA. Required for cleavages A_0-A_2 and release of U3 snoRNA from the pre-rRNA. May bring DBP8 helicase to the pre-rRNA and stimulate its enzymatic activity. Co-purifies with PWP2 and MPP10.	(Granneman et al., 2006b; Hoang et al., 2005; Oda et al., 2000)
MRD1	Contains five RNA binding domains (RBDs). Binds pre-rRNA during transcription in yeast and is required for compaction of the pre-18S rRNA into SSU processome and dissociation of the U3 snoRNP. Interacts with PWP2, MPP10 and U3 snoRNP sub-complexes	(Lundkvist et al., 2009; Segerstolpe et al., 2008)

Of particular interest is the recruitment of U3 snoRNP and SSU processome protein sub-complexes to the pre-rRNA. It is known in human cells, that the protein-binding element of U3 snoRNA, but not U3 snoRNA – pre-rRNA base-pairing interactions are required for SSU processome assembly (Granneman et al., 2004; Lukowiak et al., 2000). This suggests that protein-protein interactions may play a significant role in U3 snoRNP recruitment to the pre-rRNA, and thus SSU processome assembly, raising the important question of which protein(s) may be responsible for this? We can however, discount the MPP10 complex, which is known to be involved in U3 base-pairing which is not key to the initial recognition of the pre-rRNA (Granneman et al., 2003; Granneman et al., 2004). Intriguingly, it has been demonstrated in yeast that snoRNP proteins NOP1, NOP58 and NOP56 are capable of directly binding the rRNA, although it is not known which other proteins are capable of this. Furthermore, it is not known which proteins are responsible for recruitment of the known sub-complexes to the pre-rRNA or if orthologous components are present in the human SSU processome.

We therefore considered it necessary to examine a wide range of putative SSU processome RNA-binding proteins, taking representative components of each known sub-complex involved in the SSU processome. This was with the exception of the MPP10/IMP3/IMP4 complex which has been extensively studied and not required for production of the 80S SSU processome (Granneman et al., 2003). All of the proteins we examined were chosen as they were believed to be potentially important factors involved in 18S pre-rRNA processing. This involved establishing a range of tools to facilitate the study of these proteins in human cells, allowing us to examine protein localisation and protein-protein / protein-RNA associations.

5.2 Results

5.2.1 Protein Expression and Purification of UTP23, UTP24, ESF2, RCL1, KRR1, NOB1 and PNO1

Many interesting SSU processome proteins have recently been identified in yeast which are required for efficient pre-rRNA processing. Most have orthologs in the human genome but these are not well characterised in terms of function, localisation and U3 snoRNA or pre-rRNA interactions. Therefore, we chose a range of these proteins with putative RNA binding and RNA cleavage/modification motifs and identified their human homologs. These were cloned into protein expression vectors, expressed and purified from *E. coli* (Table 5.2-1, see Table 5.1-2 for more details). The aim of this was to raise antibodies against these proteins of interest to detect proteins on immunoblots and to provide a means to develop RNA binding and cleavage assays.

The human orthologs of the yeast proteins were found using BlastP and relavent cDNAs (purchased from imgGenes) PCR-amplified (and errors repaired where applicable; see materials and methods) and were purified by immobilised metal (Ni^{2+}) affinity chromatography. Proteins were purified further and concentrated, with the exception of PNO1 and RCL1, using either Resource Q (cationic; NOB1, ESF2, UTP24) or S (anionic; KRR1, UTP23) columns and desalted. Purified proteins were then separated by SDS PAGE and visualised by Coomassie staining (Figure 5.2 A). The recombinant proteins were all approximately the anticipated sizes, with the exception of UTP24 which is highly charged (Figure 5.2 A).

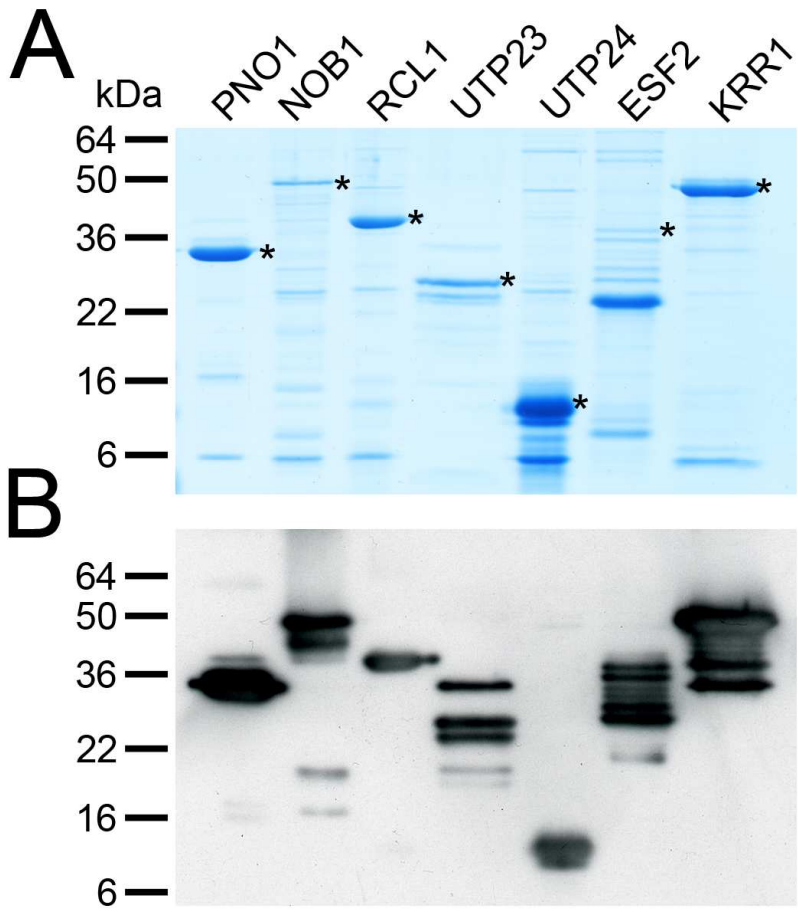


Figure 5.2 Recombinant SSU Processome Protein Expression and Purification
A) Proteins of interest were expressed in *E. coli* and purified by their N-terminal poly-His-tags. Purified proteins were then separated by SDS PAGE and visualised by Coomassie staining. The protein loaded is indicated at the top of each lane. The positions of the marker proteins are indicated to the left of each panel. Black asterisks indicates the purified protein of interest. **B)** Purified proteins were separated as in (A), Western blotted and probed using antibodies that recognise the 6xHis tag to identify the presence of the tagged proteins.

The identities of the purified proteins were verified by Western blotting analysis, using antibodies against the poly-His tag at the N-terminus (Figure 5.2 B). This demonstrated that all purified proteins contained the N-terminal tag although UTP23, ESF2 and KRR1 appeared to have suffered some proteolysis; observed as several bands below that of the band at the anticipated size. Nonetheless, Bradford assay indicated sufficient protein was present in all cases with the exception of UTP23, to raise antibodies (data not shown). Antibodies to the purified proteins were generated in rabbits by Eurogentec, tested against the relevant FLAG-tagged proteins from HEK293 cell lines (data not shown) and used to identify PNO1, KRR1 (Figures 5.9 & 5.15) and NOB1 (data not shown).

5.2.2 Creation and Characterisation of Stable HEK293 Cell Lines Containing Tagged SSU Processome Components

We next wanted to examine the various RNA-binding and modifying proteins alongside markers for the sub-complexes of the SSU processome, all of which are thought to have potentially interesting functions in yeast. A range of putative RNA-binding, RNA modifying and cleavage proteins were chosen (Table 5.1), all of which have been implicated in 18S pre-rRNA processing in yeast. tUTP4, UTP6 and RRP7 are representative components of the tUTPs, UTP-Bs and UTP-Cs respectively, with RRP7 thought to be an RNA-binding protein.

In order to provide a useful system for studying these components, HEK293 stable cell lines were generated, capable of inducible expression of FLAG-tagged proteins of interest (as previously; Figure 3.3). cDNAs (PNO1, NOB1, RCL1, UTP23, UTP24, ESF2, KRR1, DIM1, RRP7, UTP6, tUTP4, and MRD1) were identified by BlastP and cloned into the pcDNA5-FLAG vector (see Chapter 3 for details).

In order to enable comparable expression of the tagged proteins, the stably transfected HEK293 cells were incubated in a range of tetracycline concentrations (0 – 1 µg/ml). As a comparison, FLAG-hU3-55k expressing cells were induced with 0.5ng/ml tetracycline to achieve a comparable level of expression to the endogenous protein. This was used as a marker to indicate the desired expression level of the other SSU processome proteins. The cells were harvested after 48 hours of incubation with tetracycline, separated by SDS PAGE and analysed by Western blotting. Blots were probed for the N-terminal FLAG tag and either fibrillarin or hU3-55k (Figure 5.3 A). Fibrillarin and hU3-55k levels appeared constant across all the lanes, indicating equal loading. Two hU3-55k bands of equal intensity were observed in the FLAG-hU3-55k expressing cells; representing the endogenous and tagged forms of the protein. This showed that FLAG-tagged expression was comparable to that of the endogenous protein. Western blot analysis with anti-FLAG antibodies of cells grown in the absence of tetracycline revealed that no bands were present, indicating that none of the tagged proteins

were detectably expressed. All cell lines however, expressed proteins of the anticipated sizes upon the addition of tetracycline.

Different concentrations of tetracycline were required for each cell line to achieve similar expression levels of tagged protein to that of hU3-55k (Figure 5.3 and see Materials and Methods, Table 2.12). To allow a direct comparison between the proteins, cells induced at approximately the same level of expression (as estimated from Figure 5.3 A) were analysed side by side, again by separating the proteins by SDS PAGE and examination by Western blot analysis (Figure 5.3 B). Tagged protein expression from the various cell lines was achieved with the addition of tetracycline, for 48 hours before harvesting.

In Figure 5.3 B, hU3-55k antibodies once again detected two bands of equal intensity from the FLAG-hU3-55k cell line, indicating equal expression of the endogenous and tagged proteins. When probed with anti-FLAG antibodies, the tagged proteins of interest could be visualised as bands of the correct sizes (as previously discussed) with the level of intensity approximately equal between them. This allowed us to compare results directly between the various proteins in subsequent experiments.

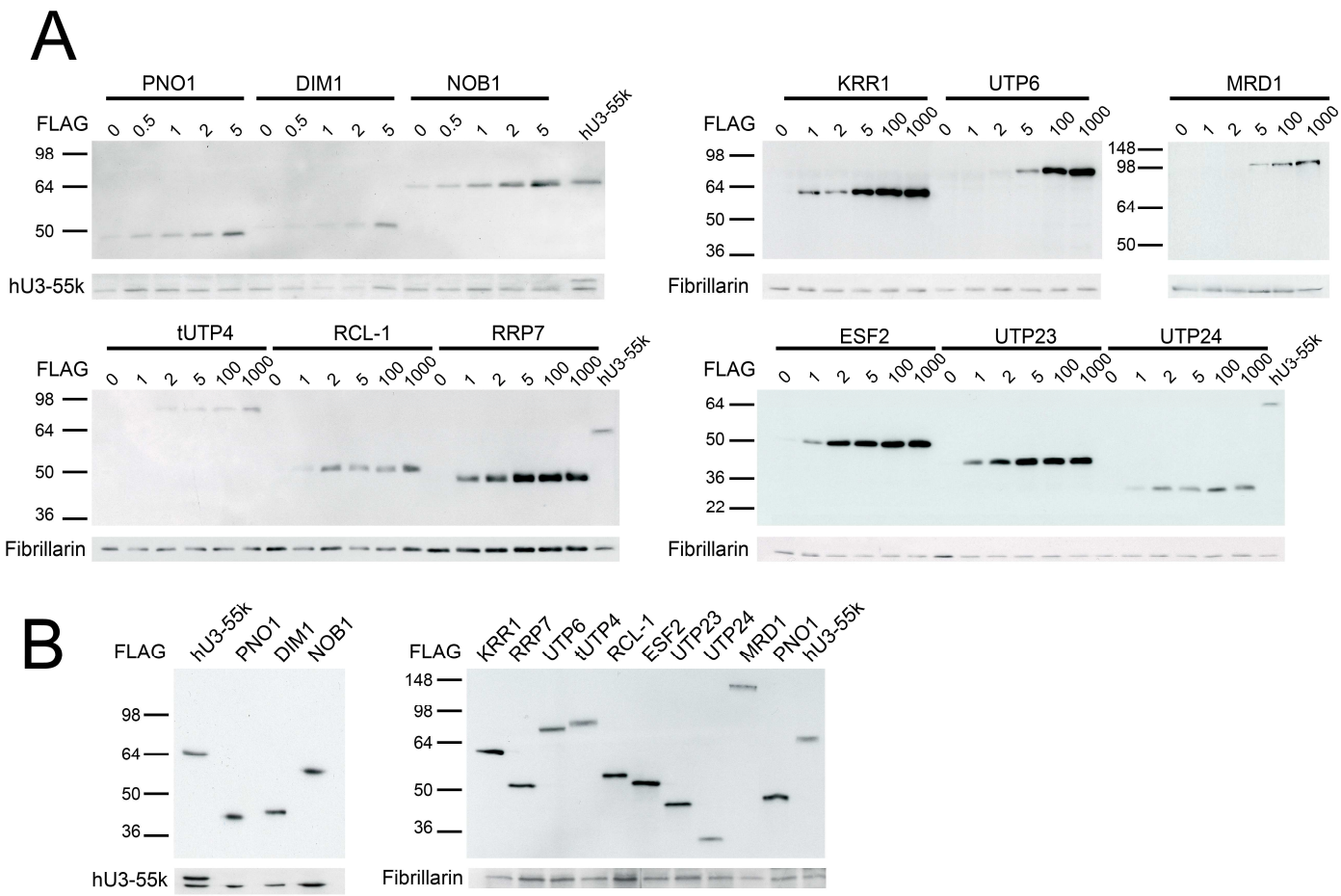


Figure 5.3 Expression of FLAG-tagged SSU Processome Proteins in HEK293 Cells
A) HEK293 cell lines with stably integrated N-terminally tagged SSU processome proteins (above each panel) were induced by the addition of tetracycline (shown above each lane in ng/ml, or 0.5ng/ml for hU3-55k) for 48 hours. Cells were harvested and proteins separated by SDS PAGE. Western blot analysis was performed using anti-FLAG, anti-hU3-55k or anti-fibrillarin antibodies (indicated to the left of each panel). Approximate molecular masses are indicated to the left of each panel. **B)** Similarly to (A), protein expression was induced at comparable levels from each cell line using the relevant concentrations of tetracycline (see the text for details) and as in (A) Western blot analysis performed.

5.2.3 Protein-Protein and Protein-RNA Associations within the SSU Processome

Both the building of the SSU processome and its role in the cleavage steps surrounding the 18S pre-rRNA are reliant on a range of protein-protein and protein-RNA interactions. Furthermore, these proteins are believed to associate and dissociate in a temporal fashion as the pre-rRNA is processed. In order confirm that the proteins of interest were components of the SSU processome in human

cells and to examine potential protein-protein and protein-RNA associations, we performed immunoprecipitation experiments; purifying components associated with our FLAG-tagged proteins of interest to allow a range of analyses.

Firstly, we wished to examine the association of each of the proteins with the U3 snoRNA. HEK293 stable cells were induced with the suitable amount of tetracycline to give approximately a 1:1 stoichiometric ratio of tagged protein to endogenous hU3-55k, as discussed previously. 48 hours later the cells were harvested and extracts prepared. Immunoprecipitation reactions were performed using anti-FLAG antibodies conjugated to Protein G Sepharose beads. Co-precipitated RNAs were separated by denaturing gel electrophoresis and analysed by Northern blotting, using radiolabeled probes to U3 snoRNA and the unrelated U1 snRNA as a control.

This demonstrated that the U3 snoRNA was efficiently co-precipitated with the protein hU3-55k, a known component of the U3 snoRNP (IP; Figure 5.4). U3 snoRNA was also specifically co-immunoprecipitated with all of the FLAG-tagged proteins examined, with the exception of NOB1 which was not reliably co-precipitated above background levels. Minimal amounts of signal were detected in the absence of antibodies (beads alone; B) and antibodies failed to co-precipitate the spliceosomal U1 snRNA. It should be noted however, that U3 snoRNA was only weakly co-precipitated with the majority of proteins (UTP6, UTP23, UTP24, ESF2, KRR1, RCL1, RRP7, MRD1, PNO1 and DIM1). It is possible that the association of the SSU processome factors may be limited by competition with the endogenous proteins or that the FLAG tag motif at the N-terminus of the protein may limit associate of the proteins with the U3 snoRNP, by causing steric hindrance. Nonetheless, their specific associations with the U3 snoRNA indicates that the tagged proteins examined were indeed components of the SSU processome.

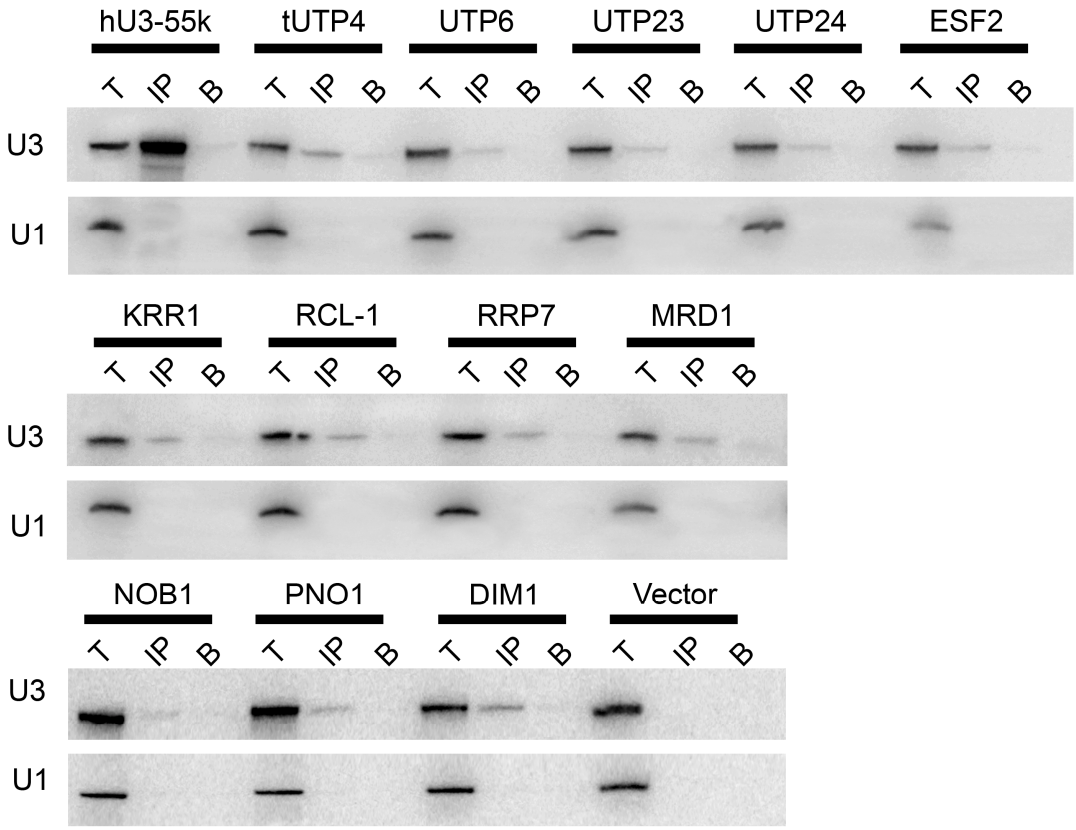


Figure 5.4 Association of the SSU Processome Proteins with the U3 snoRNA
FLAG-tagged proteins in HEK293 cells were induced by the addition tetracycline for 48 hours. Cells were harvested, extracts prepared and immunoprecipitation reactions performed using anti-FLAG antibodies, with co-precipitated RNA separated by gel electrophoresis. The cell line used is shown above each panel, where “Vector” contained only the FLAG tag sequence. Gel electrophoresis and Northern blot analysis was performed on RNA samples, using radiolabeled probes specific to U3 snoRNA or U1 snRNA. T; 10% of the total input, IP; Immunoprecipitated material, B; Beads alone control (no antibody).

To further investigate the timing of interactions within the SSU processome, we examined proteins associated with FLAG-tagged proteins by Western blotting. As before, extracts from induced HEK293 stable cells were used in immunoprecipitation reactions. Co-precipitated proteins were separated by SDS PAGE and analysed by Western blotting; using antibodies specific to the FLAG-tag, PNO1 or KRR1 proteins.

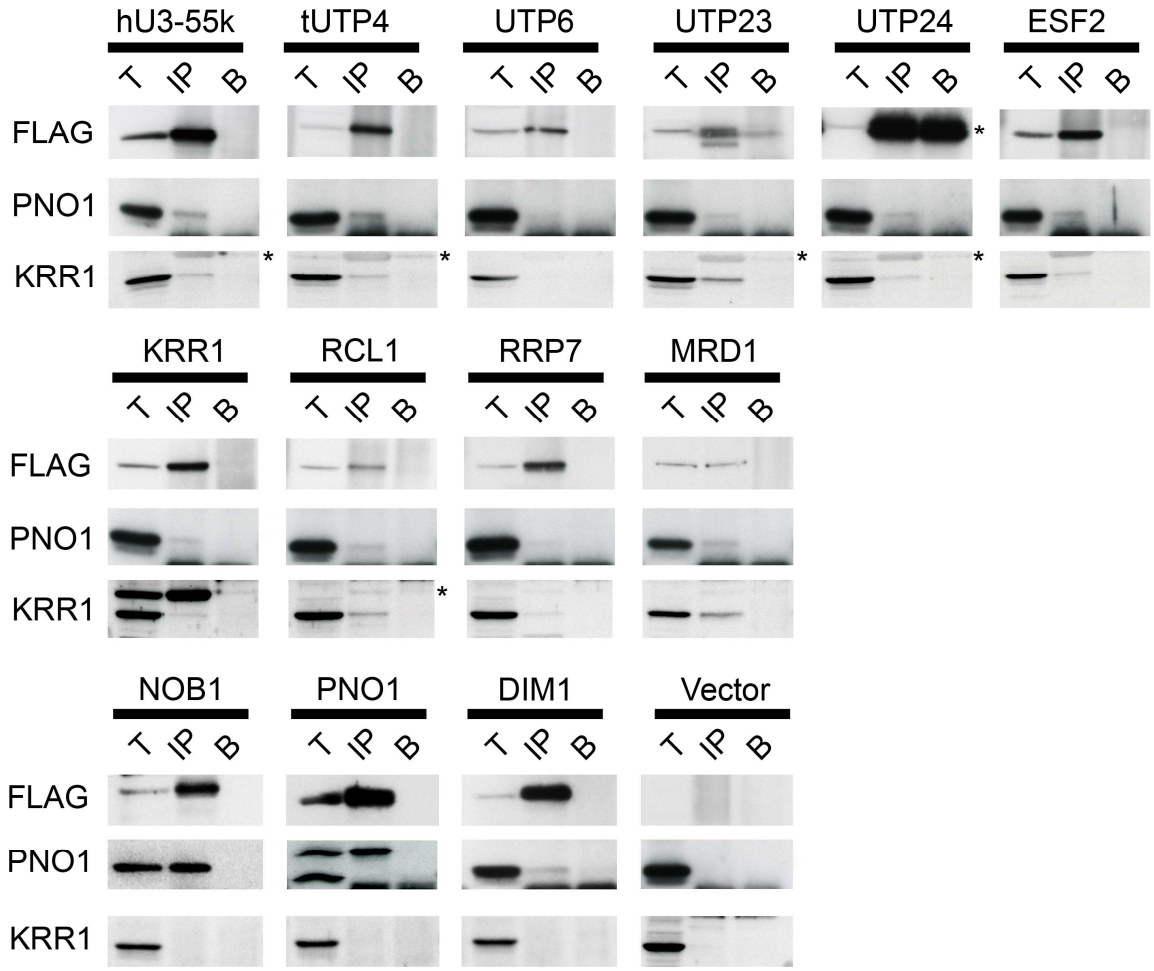


Figure 5.5 Association of the SSU Processome Proteins with PNO1 and KRR1
FLAG-tagged proteins in HEK293 cells induced by the addition tetracycline (described in the text) for 48 hours. Cells were harvested and immunoprecipitation reactions performed using anti-FLAG antibodies, with co-precipitated proteins separated by SDS PAGE. The cell line used is shown above each panel, where “Vector” contained only the FLAG tag sequence. Western blot analysis was performed on protein samples, using antibodies specific to the FLAG-tag sequence, PNO1 or KRR1 proteins. T; 10% of the total input, IP; Immunoprecipitated material, B; Beads alone control (no antibody). Note, UTP24 resolved at the same size as a background band present in all immunoprecipitations, thought to be due to the protein coated beads used, background bands indicated by asterisks.

Western blot analysis of the immunoprecipitations indicated that the FLAG-tag (Vector) was not measurably expressed or precipitated by anti-FLAG antibodies. In contrast, the FLAG-tagged proteins were all expressed at similar levels; comparable to the expression of hU3-55k or below (Figure 5.5). PNO1 and KRR1 input levels were consistent between samples, indicating that loading was equal, and therefore, comparable across the samples. Bands of equal (RCL1 and MRD1) or greater intensity (hU3-55k, tUTP4, UTP23, ESF2, KRR1, RRP7, NOB1, PNO1

and DIM1) than those of the inputs were detected by the anti-FLAG antibodies in immunoprecipitations of all proteins. Unfortunately, UTP24 resolved at the same size as a background band present in all immunoprecipitations (not shown), most likely due to Protein G from the sepharose beads as the band was observed in the beads alone control lane.

Neither PNO1 nor KRR1 were co-precipitated by anti-FLAG antibodies from the FLAG-tagged (Vector) cell line, indicating that subsequent precipitations of these proteins were specific. PNO1 and KRR1 were co-precipitated to varying degrees by anti-FLAG antibodies from cell extracts containing FLAG-tagged SSU processome proteins, tUTP4, UTP23, UTP24, ESF2, KRR1, RCL1 and MRD1. PNO1 and KRR1 were not however, co-precipitated with UTP6 or PNO1 and only low levels were co-precipitated with RRP7. KRR1 was precipitated with neither NOB1 nor DIM1, whereas PNO1 was efficiently co-precipitated with NOB1 and more weakly with DIM1. Interestingly, a small amount of endogenous KRR1 (lower band) was co-precipitated with the FLAG-tagged form of the protein (upper band), potentially indicating that there may be multiple KRR1 proteins present in each SSU processome. In contrast, endogenous PNO1 did not co-precipitate with the FLAG-tagged protein (not shown). It should be noted that whilst KRR1 was not co-precipitated from PNO1 expressing cells, the reciprocal association was observed however, this was only very weak and was not observed with a shorter exposure time, similar to that of FLAG-PNO1 immunoprecipitations (not shown). Nonetheless, the data indicated that the majority of these proteins were able to associate with an SSU processome in which PNO1, KRR1 and U3 snoRNP were all present. Furthermore, despite their lack of association with KRR1, it appeared that NOB1, PNO1 and DIM1 were also SSU processome components as observed through their associations with the U3 snoRNP and / or PNO1.

The association of these proteins with the U3 snoRNA and other SSU processome proteins appeared variable, suggesting a dynamic SSU processome complex. These observations raised questions as to if and when the SSU processome proteins associate with the pre-rRNA. This was examined by the analysis of RNAs

immunoprecipitated with the different FLAG-tagged proteins by S1 nuclease protection, using a probe spanning the initial A' cleavage site within the 5' ETS (Prieto and McStay, 2007; Turner et al., 2009). This allowed indirect detection of co-precipitated pre-rRNA and the ability to visualise whether it was cleaved or uncleaved at the A' site (Figure 5.6).

No significant amount of pre-rRNA was co-precipitated from the beads alone controls (B), and similarly only background amounts were precipitated from the control FLAG-tag (Vector) cell line (Figure 5.6), by anti-FLAG antibodies. Both A' uncleaved and cleaved pre-rRNA were co-precipitated from cells expressing either hU3-55k, tUTP4, UTP23, UTP24, ESF2, KRR1, RCL1, RRP7 or MRD1. The pre-rRNA was however, only weakly co-precipitated with UTP6; potentially due to limited association with the processing complex as previously discussed. Worth noting is the bias of RCL1 and RRP7 to associate with A' uncleaved pre-rRNA. This suggested they may function early in pre-rRNA processing and leave the SSU processome complex not long after, whereas other components may remain associated for a longer period through pre-rRNA processing.

NOB1 did not appear to co-precipitate pre-rRNA reliably above background. This is in agreement with its role in yeast whereby it associates only very weakly with 35S pre-rRNA and U3 snoRNA, with the majority associating after the initial cleavages, with the 20S pre-rRNA (Fatica et al., 2003). Intriguingly, PNO1 and DIM1 were both biased in their precipitations of pre-rRNA; PNO1 weakly associating with that cleaved at the A' site, and DIM1 with pre-rRNA uncleaved at the A' site. Similarly, in yeast DIM1 is believed to associate with pre-rRNA in the nucleolus (Lafontaine et al., 1998b). It should also be noted that very low levels of pre-rRNA co-precipitation were observed for PNO1 and DIM1, suggesting either inefficient or very transient associations with the pre-rRNA. Nonetheless, the data imply that all the proteins associate with pre-rRNA containing 5'ETS sequences, with the exception of NOB1, although interestingly, RCL1, RRP7, PNO1 and DIM1 display a bias in their associations with A' cleaved / uncleaved pre-rRNA, suggesting temporal associations / dissociations dependent on the initial cleavage event.

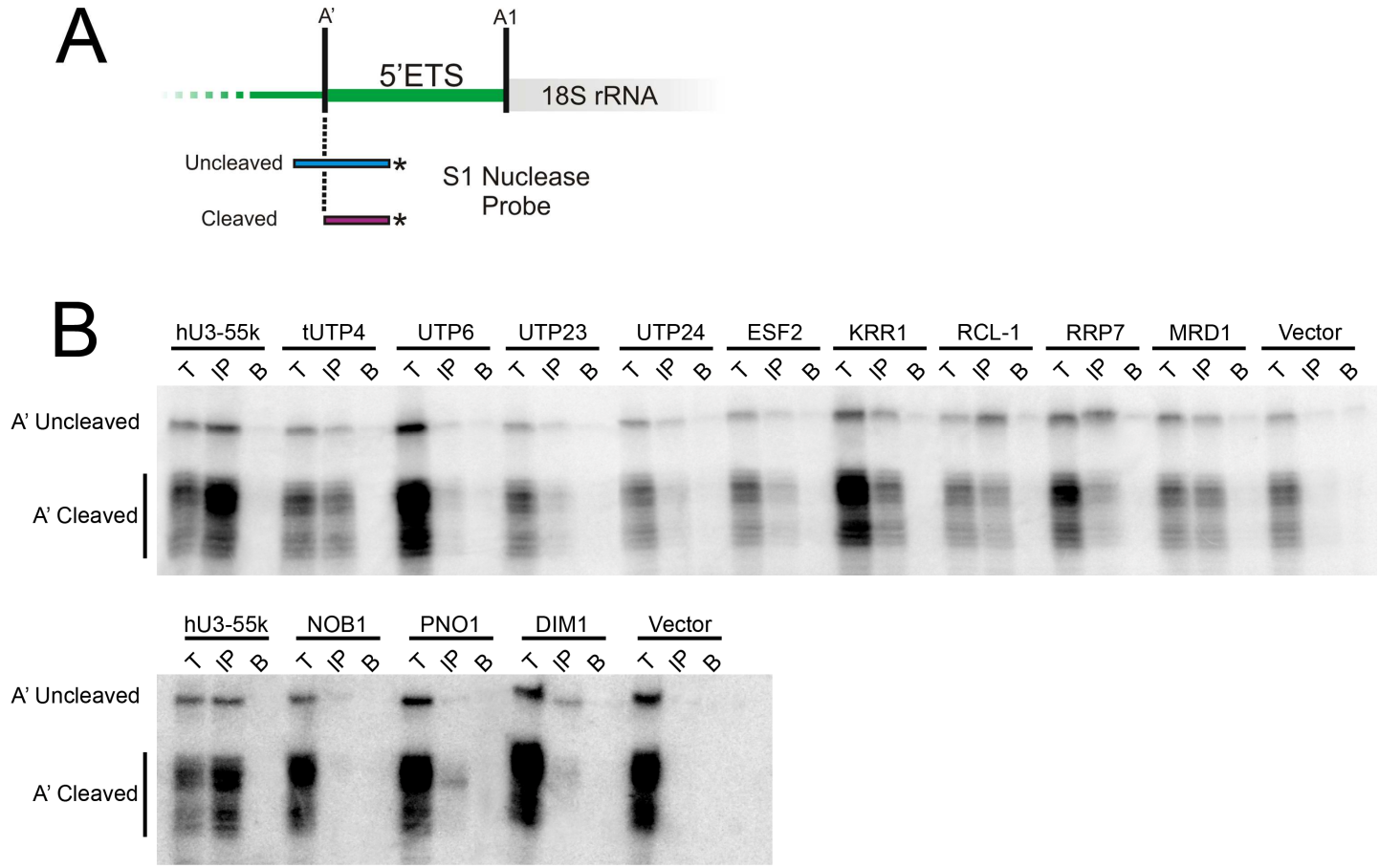


Figure 5.6 Association of SSU Processome Components Before and After pre-rRNA Cleavage at the A' Site
A) Schematic diagram of the S1 nuclease protection assay. Pre-rRNA is shown in green, 18S rRNA in grey with the A' and A1 cleavage sites indicated. The uncleaved probe is shown in blue, the cleaved probe in purple. Asterisks indicate the radiolabel on the probe. **B)** Cell extracts derived from HEK293 cells expressing proteins of interest were immunoprecipitated using anti-FLAG antidboies. RNA was extracted and analysed by S1 nuclease protection, using a radiolabeled probe spanning the initial A' cleavage site of the pre-rRNA, yielding either an uncleaved probe (protected by the pre-rRNA) or a partially degraded probe (due to only a section being protected after the initial pre-rRNA cleavage; as indicated to the left of the panel). RNA was subsequently separated upon a denaturing gel and exposed to a PhosphorImager screen. The protein expressed is indicated above each panel. T represents 10% of the total input; IP immunoprecipitation; B beads alone control with no antibody. Vector indicates the control FLAG-tag expressing cell line.

5.2.4 Sub-cellular Localisation of SSU Processome Proteins

The sub-cellular localisation of SSU processome components is widely uncharacterised in human cells. To examine this, HEK293 cells were induced to express tagged proteins of interested at levels comparable to endogenous hU3-55k, as this is a key SSU processome component. This was done to prevent mis-localisation of the tagged proteins and limit secondary effects of exogenous protein expression. After induction of tagged protein expression, the cells were fixed with paraformaldehyde and proteins visualised by immunofluorescence. Antibodies to the FLAG-tag sequence (N-terminal to each protein of interest) and to the core Box C/D snoRNP protein fibrillarin were used, along side DAPI staining of the nucleus.

No signal could be detected in the FLAG-tag alone expressing cell line (Vector; Figure 5.7 A), as described in previous chapters. Fibrillarin localised in a punctate nucleolar fashion, typical of DFC/FC localisation. hU3-55k is a key component of the U3 snoRNP and as such, co-localised with the snoRNP throughout the GC and DFC/FC, as previously described (Leary et al., 2004; Pluk et al., 1998).

The human ortholog of the site D endonuclease, NOB1, was observed to localise mainly to the cytoplasm (Figure 5.7 B). This is somewhat in keeping with previous data from humans where it has been observed to localise cytoplasmically although notably, inconsistent nuclear localisation was also previously reported (Zemp et al., 2009). This is in contradiction to data from yeast, whereby it has been said to localise evenly throughout the cell (Fatica et al., 2003). PNO1 was observed to localise throughout the cell although appearing more concentrated in the nucleolus. DIM1 was observed throughout the nucleolus, with weak nuclear localisation but no signal was detected in the cytoplasm. PNO1 has been shown to localise throughout the cell and to bind DIM1 in yeast (Vanrobays et al., 2004), with DIM1 previously observed in pre-40S particles (Lafontaine et al., 1998b; Schafer et al., 2003; Zemp et al., 2009). In HeLa cells however, PNO1 has been observed to localise to the nucleolus in untreated cells, with cytoplasmic localisation observed only upon depletion of the kinase RIO2 (Zemp et al., 2009). This indicates that the

proteins of this potential sub-complex are localising somewhat as would be expected but with some notable differences.

The remaining SSU processome proteins studied (ESF2, KRR1, MRD1, RCL1, RRP7, tUTP4, UTP6, UTP23 and UTP24) all localised throughout the nucleolus, in the DFC/FC and in the GC regions of the nucleolus. It is known that much of the pre-rRNA processing and association with ribosomal proteins, occurs in the GC of the nucleolus (Gerbi and Borovjagin, 1997; Grandi et al., 2002; Kruger et al., 2007). Similarly, the U3 snoRNP, a key SSU processome component, is known to localise throughout the DFC/FC and GC. Therefore, these SSU processome components also appear to be localising where they may be predicted to function.

Actinomycin D (ActD) is frequently used to block RNA polymerase I transcription therefore blocking ribosome biogenesis (Perry, 1963). Specifically, at low concentrations ActD inhibits RNA pol. I activity alone (Perry, 1963). It has been shown that blocking rRNA transcription blocks the co-transcriptional recruitment of the SSU processome, giving rise to a 50S U3 snoRNP-containing SSU processome complex (Turner et al., 2009). Furthermore, when treated with ActD, mammalian nucleoli exhibit a segregated phenotype; potentially a method of stopping ribosome biogenesis under stress conditions (Leary et al., 2004). We therefore decided to use this chemical at a low concentration to specifically disrupt RNA pol. I transcription and further examine the co-localisation of proteins after nucleolar disruption.

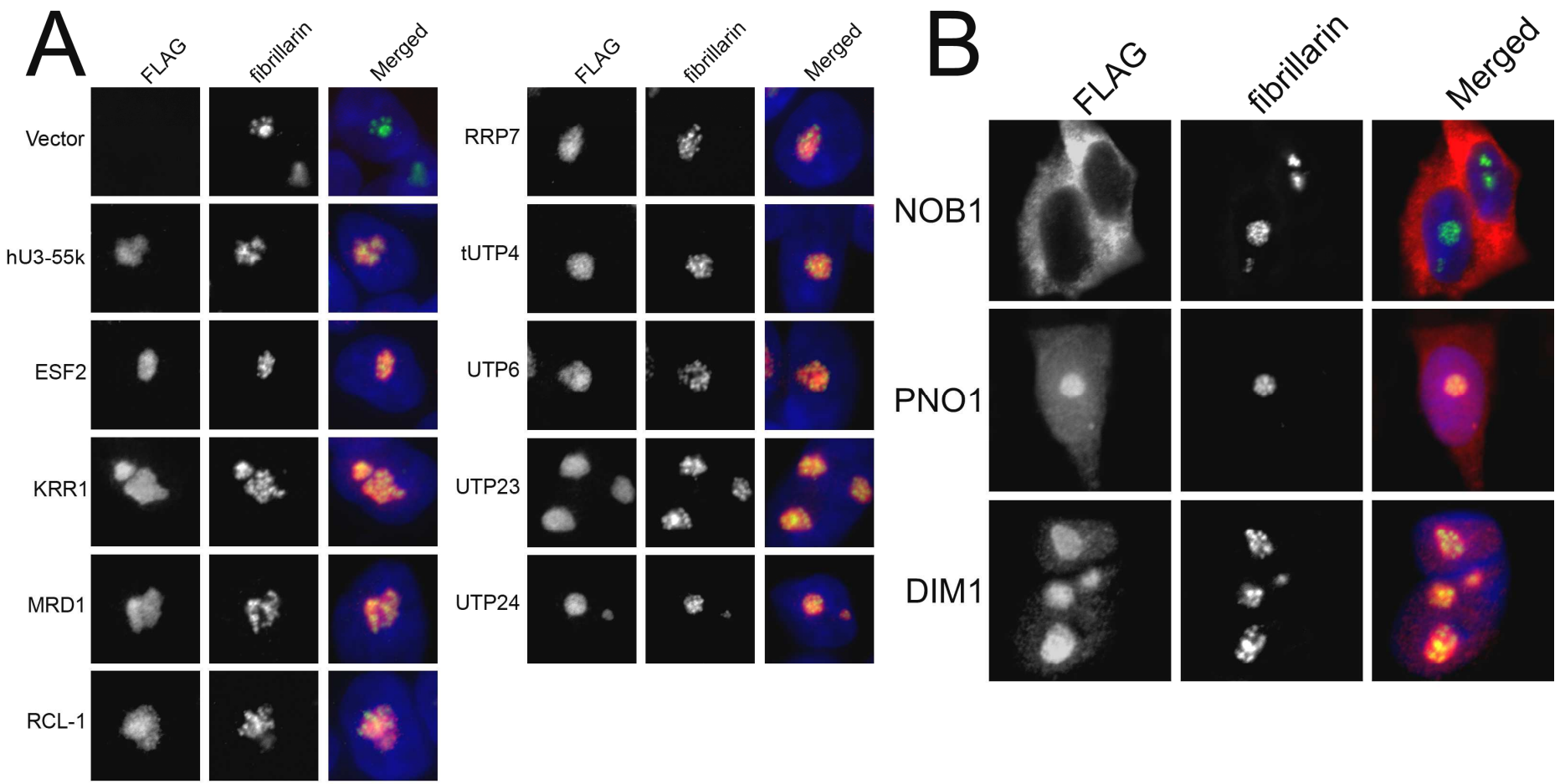


Figure 5.7 Sub-cellular Localisation of SSU Processome Proteins

A) The expression of FLAG-tagged SSU processome proteins was induced at comparable levels to that of endogenous hU3-55k from HEK293 cells by the addition of tetracycline for 48 hours (see the text for tetracycline concentrations). Cells were fixed with paraformaldehyde and proteins visualised by immunofluorescence. Antibodies against the FLAG-tag sequence (N-terminal to each protein of interest; shown in red) and to the core Box C/D snoRNP protein fibrillarin (green) were used, along side DAPI (blue) staining of the nucleus; shown above each panel. The FLAG-tagged protein expressed is shown to the left of each panel where "Vector" signifies a FLAG-tag alone expressing cell line. The nucleus of a representative cell from each cell line is focused on; all panels are of the same sized area **B)** The entire cell(s) of FLAG-tagged NOB1, PNO1 and DIM1 expressing cells are shown to illustrate the cytoplasmic localisation of the tagged NOB1 and PNO1 proteins, otherwise cells treated as in panel (A).

HEK293 cells were induced to express tagged proteins of interested for 48 hours. Two hours before fixing the cells, 0.1 µg/ml ActD was added to block pre-rRNA transcription as previously described (Turner et al., 2009). Subsequently, cells were fixed and proteins visualised by immunofluorescence using antibodies against the FLAG-tag or against fibrillarin, along side DAPI staining of the nucleus (Figure 5.8). The FLAG-tag (Vector) cell line did not express a detectable amount of the FLAG protein, whilst fibrillarin appeared in cap-like structures. This disrupted fibrillarin localisation was similar to that described previously in HE-p2 (human esophageal epithelial type 2) and HeLa cells (Chen and Jiang, 2004; Leary et al., 2004). It is believed that upon ActD treatment of mammalian cells, the granular component remains in the center of the nucleolus, whereas the fibrillar components move to the periphery of the nucleoli to form these nucleolar caps (Chen and Jiang, 2004; Leary et al., 2004; Shav-Tal et al., 2005).

The U3-snoRNP protein hU3-55k appeared to co-localise with fibrillarin and was also observed throughout the nucleoplasm, co-localising with the DAPI staining (Figure 5.8). This is in agreement with previous data from HeLa cells which shows NOP58 and hU3-55k displaying similar localisation profiles upon ActD treatment; co-localising in caps with fibrillarin and more diffusely throughout the nucleus (Leary et al., 2004).

NOB1 localisation appeared slightly altered upon the addition of ActD, localising through the nucleus of the cell, although excluded from the nucleolus and still largely present in the cytoplasm (Figure 5.8 B). PNO1 was seen throughout the nucleus and nucleolus, yet it was excluded from the cytoplasm. Similarly, upon ActD treatment, DIM1 was distributed throughout the nucleoplasm and nucleolus, appearing enriched in nucleolar caps but was excluded from the cytoplasm.

ESF2 was also observed throughout the nucleoplasm and nucleolus, with some concentration in caps, co-localising with fibrillarin. This is in contrast to the KH-domain protein KRR1 that became concentrated within the granular body of the nucleolus, where other GC components co-localise, surrounded by the fibrillarin-

containing nucleolar caps. MRD1 became excluded from the granular body upon ActD treatment, localising throughout the nucleoplasm but also co-localising with fibrillarin, suggesting that it was present in nucleolar (fibrillar) caps. Similarly, RCL1 was observed to be excluded from the granular body, although was present throughout the nucleoplasm and cytoplasm, again co-localising with fibrillarin suggesting limited fibrillar cap localisation.

The UTP-C component RRP7 was observed to localise in a similar fashion to that of ESF2; appearing throughout the nucleoplasm and nucleolus, with some concentration in caps, co-localising with fibrillarin. The tUTP protein tUTP4 co-localised in caps precisely with fibrillarin and was excluded from the rest of the cell. In contrast, the UTP-B component UTP6 localised throughout the nucleoplasm; being excluded from the cytoplasm and granular body, similarly to MRD1, with some co-localisation with the fibrillar caps. The potential nucleases UTP23 and UTP24 were also absent from the cytoplasm, localising throughout the nucleoplasm and appearing concentrated in the nucleolus with some co-localisation with fibrillarin; similar to RRP7.

This demonstrates that many of the SSU processome components do not co-localise within disrupted nucleoli. Furthermore, ActD treatment does not appear to result in a common mis-localisation profile for the SSU processome proteins. This suggests that whilst they may all function in SSU biogenesis, they may exist in separate complexes when not involved in pre-rRNA processing.

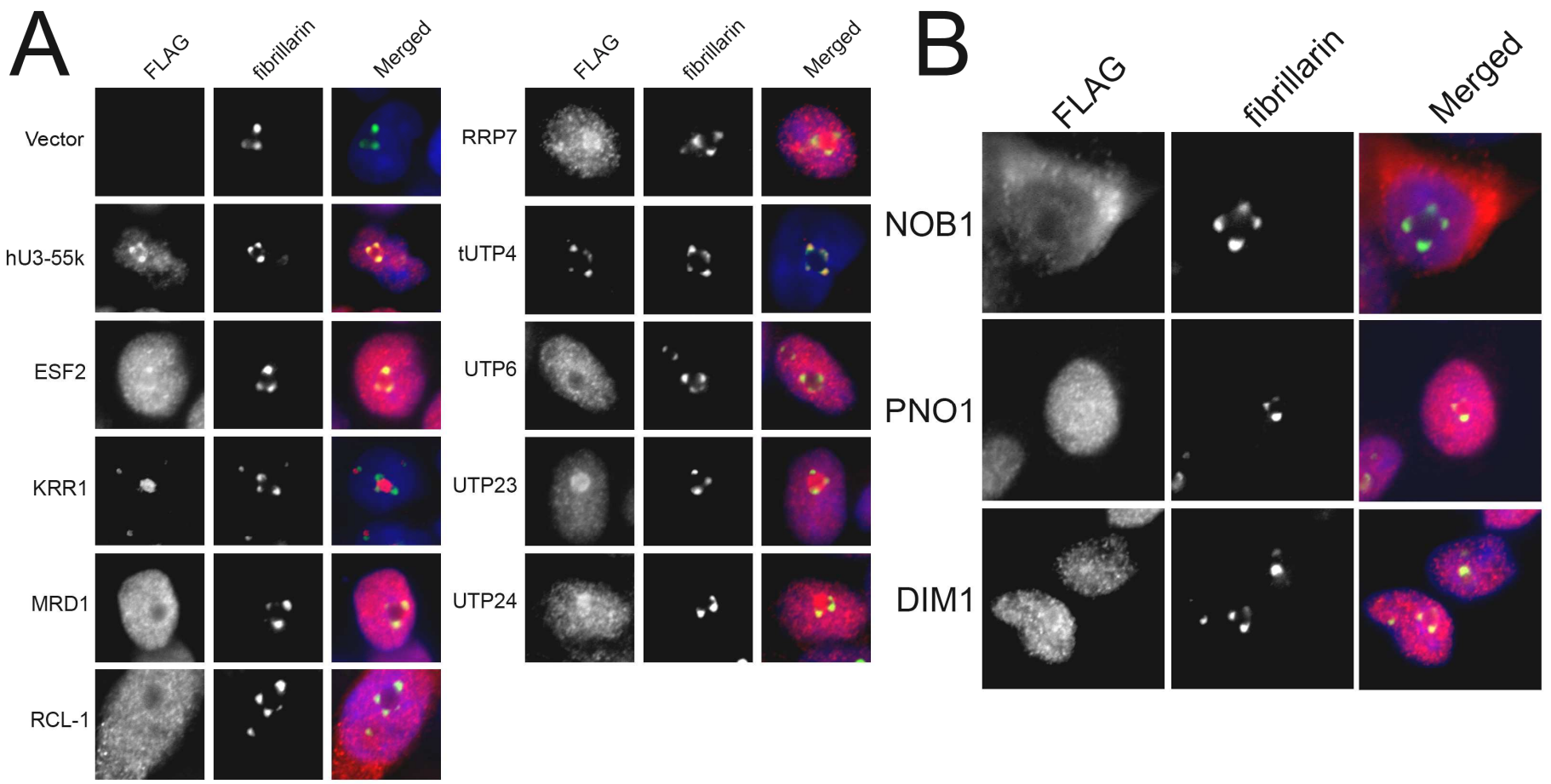


Figure 5.8 Sub-cellular Localisation of SSU Processome Proteins when pre-rRNA Transcription is Blocked
A) Expression of FLAG-tagged SSU processome proteins were induced at comparable levels to that of endogenous hU3-55k from HEK293 cells by the addition of tetracycline for 48 hours. Cells were treated with 0.1 µg/ml Actinomycin D (ActD) for 2 hours to inhibit pre-rRNA transcription. Subsequently, cells were fixed with paraformaldehyde and proteins visualised by immunofluorescence. Antibodies to the FLAG-tag sequence (N-terminal to each protein of interest; shown in red) and to the core Box C/D snoRNP protein fibrillarin (green) were used, along side DAPI (blue) staining of the nucleus; indicated above each panel. The FLAG-tagged protein expressed is shown to the left of each panel where "Vector" signifies a FLAG-tag alone expressing cell line. The nucleus of a representative cell from each condition is focused on; all panels are of the same sized area. **B)** The entire cell(s) of FLAG-tagged NOB1, PNO1 and DIM1 expressing cells are shown, otherwise cells treated as in panel (A).

5.2.5 NOB1 and PNO1 Shuttle Between the Nucleus and Cytoplasm

Our data indicated that DIM1 and NOB1 proteins may function at temporally and physically separate points in the pre-rRNA processing pathway, in contrast to their closely related counterparts in yeast. We therefore wanted to further investigate the potential 3' end 18S-pre-rRNA processing factors PNO1, DIM1 and NOB1. These proteins have been proposed to shuttle between the nucleus and cytoplasm in yeast (Lafontaine et al., 1994; Pertschy et al., 2009; Vanrobays et al., 2008). In the previous section however, DIM1 association was biased towards uncleaved pre-rRNA and was not observed in the cytoplasm. Conversely, PNO1 displayed a bias towards association with A' cleaved pre-rRNA and was associated with NOB1, both of which proteins were observed within the cytoplasm. It has been reported that in humans, RIO2 and NOB1 shuttle between the nucleolus and cytoplasm in a CRM1 dependent manner (Zemp et al., 2009). Moreover, inhibition of CRM1 with leptomycin B (LMB) prevents nuclear export of the pre-40S particle, preventing processing of 21S pre-rRNA to mature 18S rRNA in the cytoplasm (Rouquette et al., 2005; Zemp et al., 2009).

Shuttling was therefore further investigated by examining if NOB1, PNO1 or DIM1 were affected by CRM1 inhibition. We induced our FLAG-tagged expressing HEK293 cells at the same levels as previously discussed in this chapter and then incubated them in the absence or presence of 30 nM LMB for 2 hours (as was previously described (Muro et al., 2008; Rouquette et al., 2005)) before fixing with paraformaldehyde. Immunofluorescence analysis was used to detect protein localisation, using anti-FLAG antibodies to detect the proteins of interest, anti-fibrillarin antibodies to identify the core Box C/D snoRNP protein and DAPI staining to visualise the nucleus (Figure 5.9 A & B).

Upon treatment with LMB, similar to non-drug treated cells (Figure 5.9 A & B), the FLAG-tag alone cells did not express a detectable amount of the FLAG tag (data not shown). In contrast to non-drug treated cells, fibrillarin became concentrated in a few foci (Figure 5.9 B), similar to those observed in HeLa cells upon depletion of

the RNA pol. I subunit RPA135 (Turner et al., 2009). This was also observed for hU3-55k which co-localised with fibrillarin. In untreated cells, NOB1 was observed largely in the cytoplasm however, upon LMB treatment, localisation was observed also within the nucleus, yet excluded from the nucleolus. This was in agreement with data from HeLa cells whereby depleting CRM1 or RIO2 has been shown to result in a similar localisation profile (Zemp et al., 2009). PNO1 was observed throughout control cells, appearing concentrated in the nucleolus although with reduced co-localisation with fibrillarin; observed as “holes” in the staining pattern where fibrillarin foci can be observed to localise. Importantly, PNO1 was excluded from the cytoplasm when treated with LMB, indicating that the treatment prevented nuclear – cytoplasmic shuttling of this protein, as was observed for NOB1. In contrast, DIM1 was observed to remain present throughout the nucleolus with some nucleoplasmic staining.

In yeast, PNO1, DIM1 and NOB1 are all believed to be shuttling proteins, most likely associated with the pre-40S particle (Fatica et al., 2003; Lafontaine et al., 1998b; Pertschy et al., 2009; Schafer et al., 2003; Vanrobays et al., 2008). Our data suggests that similarly, NOB1 and PNO1 are capable of shuttling between the nucleus and cytoplasm in human cells. Whilst a complete block in PNO1 shuttling was observed upon LMB treatment; no longer localising in the cytoplasm, NOB1 was still observed in the cytoplasm despite displaying some nuclear localisation. This may indicate that not all of the NOB1 proteins shuttle. Furthermore, DIM1 was only observed within the nucleolus and nucleus in both control and LMB treated cells, indicating that DIM1 does not shuttle in a CRM1 dependent manner in human cells. This is supported by evidence from HeLa cells whereby the protein is not observed in late pre-40S complexes (Zemp et al., 2009).

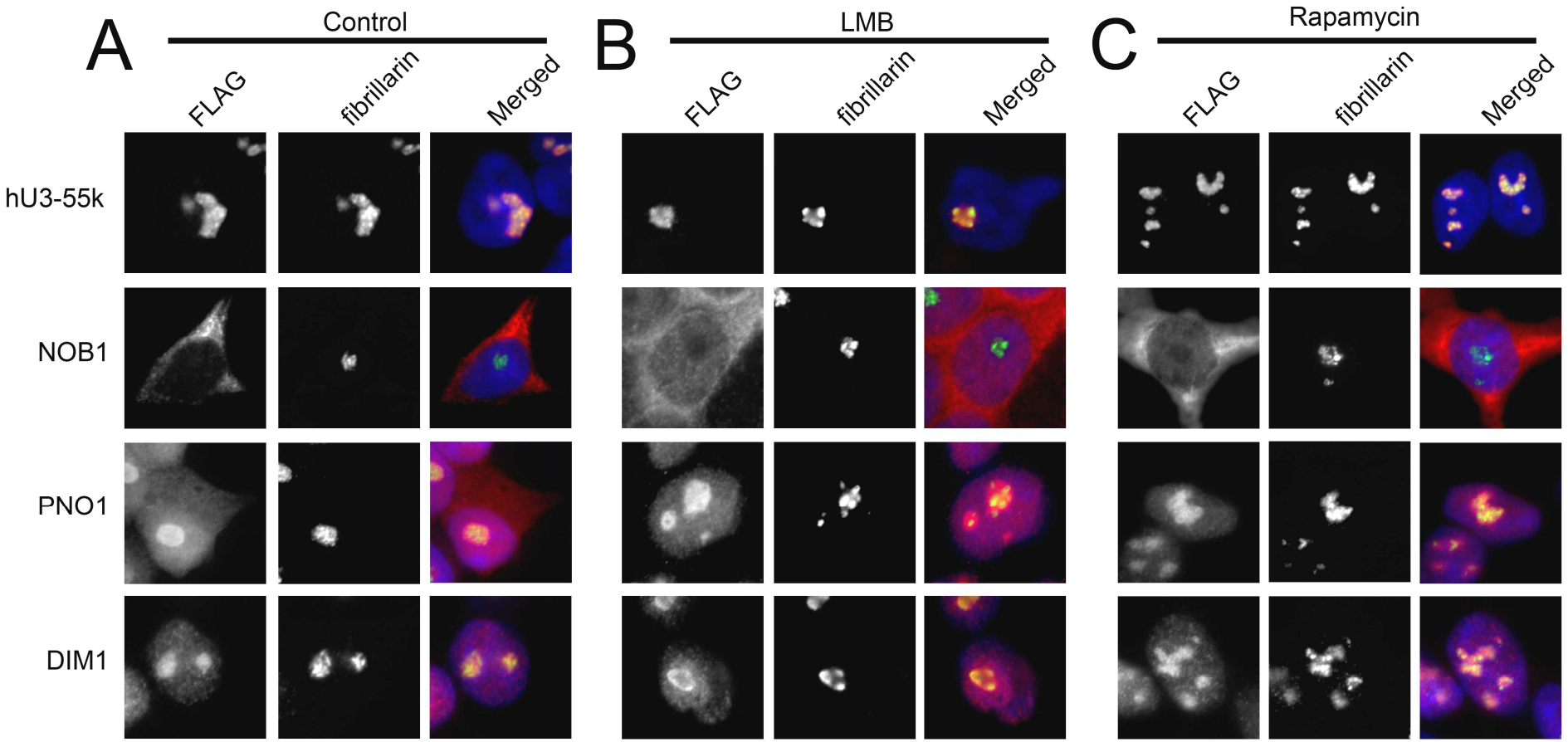


Figure 5.9 Sub-cellular Localisation of SSU Processome Proteins in HEK293 Cells, with and without LMB and Rapamycin
Expression of FLAG-tagged SSU processome proteins was induced at comparable levels to that of endogenous hU3-55k from HEK293 cells by the addition of tetracycline for 48 hours. Cells were treated **A)** with no drug, **B)** with 30 nM LMB for 2 hours to inhibit CRM1 mediated export or **C)** with 100 nM rapamycin for 4 hours to inhibit the mTOR complex mTORC1, as indicated at the top. In all instances, cells were subsequently fixed with paraformaldehyde and proteins visualised by immunofluorescence. Antibodies to the FLAG-tag sequence (N-terminal to each protein of interest; shown in red) and to the core Box C/D snoRNP protein fibrillarin (green) were used, along side DAPI (blue) staining of the nucleus; shown above each panel. The FLAG-tagged protein expressed is shown to the left of each panel where “Vector” signifies a FLAG-tag alone expressing cell line. A representative cell from each condition is focused on; all panels are of the same sized area.

In yeast, it has been demonstrated that the target of rapamycin (TOR) pathway is responsible for regulating the nuclear-cytoplasmic shuttling of PNO1 (Vanrobays et al., 2008). This was observed by treating cells with the TOR inhibitor, rapamycin, which acts by directly binding the TOR Complex 1 (TORC1, mTORC1 in mammals) (Hay and Sonenberg, 2004; Vanrobays et al., 2008). TOR / mTOR is known to act as a nutrient sensor stimulated by a range of factors, affecting cell growth and proliferation (Hay and Sonenberg, 2004; Kim et al., 2002; Loewith et al., 2002). We decided to examine the effect of mTOR inhibition through the use of rapamycin on the localisation of our SSU processome proteins of interest, as we were interested to see if PNO1 in human cells, or other related proteins, were regulated by the mTOR pathway.

HEK293 cells were induced to express the FLAG-tagged proteins hU3-55k, NOB1, PNO1 and DIM1. 4 hours before harvesting, 100 nM of rapamycin was added to the cells to inhibit the mTORC1 pathway, as previously described (Pradelli et al., 2009; Vanrobays et al., 2008). Subsequently, cells were fixed and analysed by immunofluorescence as before (Figure 5.9 C). Fibrillarin localisation appeared unaffected by the drug treatment, giving a DFC staining pattern which matched that observed in the absence of drugs (Figure 5.9 C). hU3-55k was observed throughout the nucleolus, indicating its localisation was unaffected by the rapamycin treatment. Similarly, DIM1 was observed mainly in the nucleolus, with some nucleolar localisation in both the presence and absence of rapamycin. This was not the case for NOB1, which was observed to localise in the cytoplasm and the nucleus upon rapamycin treatment, with exclusion from the nucleolus. This was in contrast to its cytoplasmic localisation in untreated cells. This further indicates that NOB1 is capable of nucleocytoplasmic shuttling although the block observed was not complete, suggesting that either insufficient rapamycin was used, or that not all NOB1 proteins are capable of shuttling. Conversely, PNO1 was not found in the cytoplasm upon rapamycin treatment, this is in contrast non-drug treated cells, where PNO1 was observed throughout the cell (Figure 5.9).

The data suggests that of the proteins examined, only the localisation of NOB1 and PNO1 were affected by inhibition of the mTORC1 pathway; limiting PNO1's localisation to the nucleolus and nucleoplasm and preventing cytoplasmic export of the protein. This is in agreement with previous work on PNO1 in yeast, where TOR has been shown to regulate the subcellular distribution of PNO1, however, it is unclear if this is direct (Vanrobays et al., 2008). Nonetheless, this in turn may limit nucleocytoplasmic shuttling of NOB1, causing it to appear in the nucleus. It is of course, possible that other processes or aspects of the nucleoli which we did not examine are also disrupted by the drug, therefore resulting in the observed mislocalisation of PNO1 and NOB1.

5.2.6 The Association of PNO1 and DIM1 Proteins with the U3 snoRNA is Disrupted by Actinomycin D

We decided to further investigate the potential 3' end pre-18S rRNA processing factors NOB1, PNO1 and DIM1 as their localisation profiles differed somewhat from those previously documented, with PNO1 and NOB1 both specifically affected by LMB and rapamycin. This suggested that the proteins may shuttle together between the nucleus and cytoplasm. Furthermore, it appeared that DIM1 may not act in the cytoplasm, supported by our observation of bias towards association with A' uncleaved pre-rRNA. We decided to utilise the RNA pol. I inhibitor, Actinomycin D and the CRM1 inhibitor, LMB to further examine the protein-protein and protein-RNA associations of NOB1, PNO1 and DIM1 and the requirements for both active pre-rRNA transcription and nucleolar export of the pre-40S to maintain these associations.

The relevant HEK293 stable cells were induced with tetracycline to give approximately a 1:1 stoichiometric ratio of tagged protein to endogenous hU3-55k, and treated with either ActD or LMB. The cells were then harvested and extracts prepared. Immunoprecipitation reactions were performed using anti-FLAG antibodies and co-precipitated RNA separated upon a denaturing gel as before.

Northern blotting analysis was performed using radiolabeled probes to U3 snoRNA and the unrelated U1 snRNA (Figure 5.10).

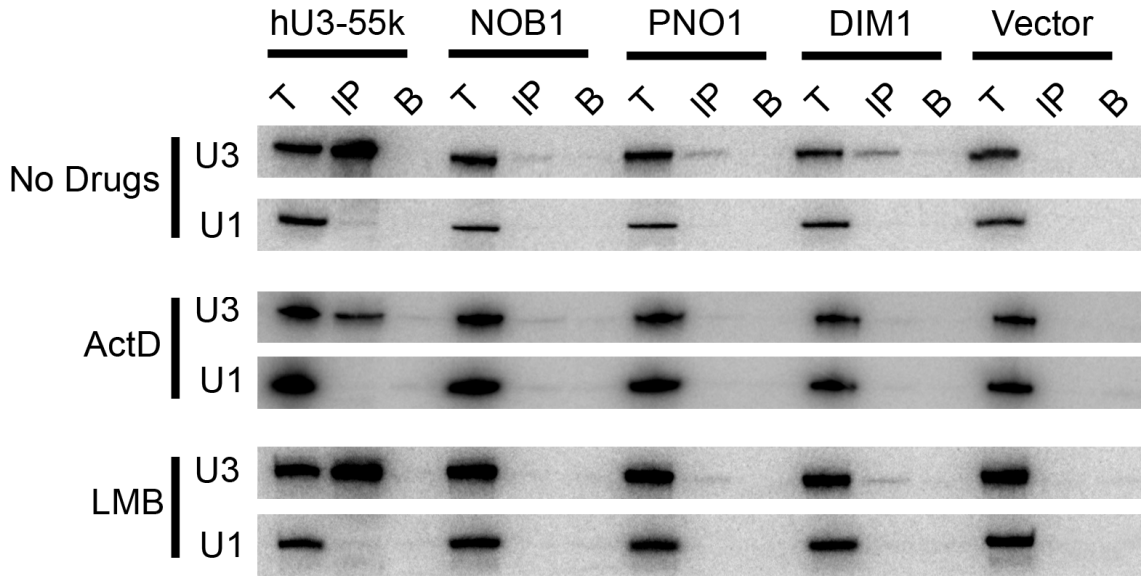


Figure 5.10 Association of the SSU Processome Proteins with U3 snoRNA in the Absence of pre-rRNA Transcription or CRM1 Nucleoplasmic Export
FLAG-tagged proteins in HEK293 cells induced by the addition tetracycline for 48 hours. Cells were incubated with either no drugs, ActD (0.1 µg/ml, 2 hours) or LMB (30 nM, 2 hours). Subsequently, cells were harvested and immunoprecipitation reactions performed using anti-FLAG antibodies, with co-precipitated RNA separated by gel electrophoresis. The cell line used is shown above each panel, whereby "Vector" contained only the FLAG tag sequence. Northern blot analysis was performed on RNA samples, using radiolabeled probes specific to U3 snoRNA or U1 snRNA. T; 10% of the total input, IP; Immunoprecipitated material, B; Beads alone control (no antibody).

Anti-FLAG antibodies efficiently co-immunoprecipitated U3 snoRNA with FLAG-tagged hU3-55k, precipitating more than the 10% input (T) in the absence of drug treatments (Figure 5.10). Although the treatment of cells with ActD led to a slight reduction in U3 snoRNA – hU3-55k association, this was not consistently observed and is not seen with the native protein (Turner et al., 2009), therefore is not believed to be significant. No U1 snRNA was co-precipitated under any condition, and neither U3 nor U1 was RNA precipitated from the FLAG-tag (Vector) cell line, indicating immunoprecipitations were specific to the tagged proteins of interest. In all instances, U3 snoRNA co-precipitation with NOB1 was not reliably above

background. In contrast, PNO1 and DIM1 could be observed to co-precipitate a moderate amount of U3 snoRNA from control cell extracts, but this was reduced in extracts from LMB treated cells, and absent from ActD treated cell extracts.

The results suggest that NOB1 does not significantly associate with the U3 snoRNP whereas active RNA pol. I transcription is required for the association of both PNO1 and DIM1 with the U3 snoRNP. In comparison, blocking CRM1-mediated nuclear export merely decreases PNO1 and DIM1 associations with U3 snoRNA, suggesting that cytoplasmic export of the pre-40S particles is not essential for their association with the U3 snoRNA.

To examine if the NOB1, PNO1 and DIM1 proteins were part of the same complex, and examine their association with the SSU processome further, FLAG-tagged proteins were again expressed from HEK293 cells which were subsequently drug treated with ActD or LMB as before. Proteins were immunoprecipitated from each, separated by SDS PAGE, transferred by Western blotting and probed using antibodies to the FLAG-tag, PNO1 or KRR1 proteins. In all instances, no protein was precipitated from the control beads alone (no antibodies; B) or the FLAG-tag (Vector) control cell line; indicating that co-precipitated proteins were specific to the FLAG-tagged proteins of interest (Figure 5.11). In all instances, the tagged proteins were precipitated at levels equal to or greater than the 10% total (T) input. This did not appear to significantly change with drug treatments.

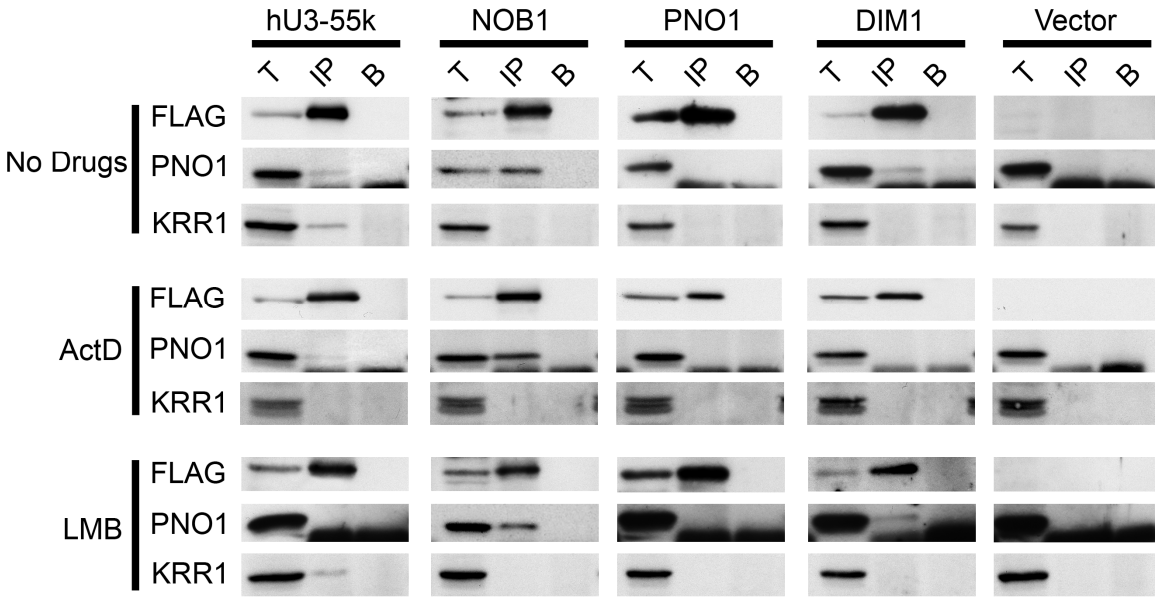


Figure 5.11 Association of the SSU Processome Proteins with PNO1 and KRR1 in the Absence of pre-rRNA Transcription or CRM1 Nucleoplasmic Export

FLAG-tagged proteins in HEK293 cells induced by the addition tetracycline (described in the text) for 48 hours. Cells were incubated with either no drugs, ActD (0.1 µg/ml, 2 hours) or LMB (30 nM, 2 hours). Subsequently, cells were harvested and immunoprecipitation reactions performed using anti-FLAG antibodies, with co-precipitated proteins separated by SDS PAGE. The cell line used is shown above each panel, whereby "vector" contained only the FLAG tag sequence. Western blot analysis was performed on protein samples, using antibodies specific to the FLAG-tag sequence, PNO1 or KRR1 proteins. T; 10% of the total input, IP; Immunoprecipitated material, B; Beads alone control (no antibody).

A small amount of PNO1 and KRR1 were co-precipitated with the core U3 snoRNP component hU3-55k from untreated cells. The association of PNO1 with hU3-55k remained in the presence of ActD but was lost in LMB treated cells. Conversely, the hU3-55k – KRR1 association remained in LMB treated cells but was lost in ActD treated cells. PNO1 was efficiently co-precipitated (~10% of the input) with NOB1, with the association remaining, albeit at a reduced level, upon ActD or LMB treatment. KRR1 was not detectibly precipitated with NOB1 under any condition. PNO1 was not co-precipitated with itself, nor was KRR1 co-precipitated with PNO1 in any condition examined. PNO1 was weakly co-precipitated with DIM1 in the absence of drugs and under LMB treatment, although association was lost upon ActD treatment. KRR1 was not co-precipitated with DIM1 in any condition. The absence of KRR1 co-precipitation was somewhat unexpected due to the previously

demonstrated KRR1 associations with other SSU processome proteins (Figure 5.5) however, these were very weak.

The data indicate that in untreated cells, PNO1 and DIM1 can associate with the U3 snoRNP, that hU3-55k can associate with PNO1 and KRR1, and that PNO1 can associate with NOB1 and DIM1. Importantly, NOB1 – PNO1 associations were maintained throughout, in agreement with their maintained co-localisation upon ActD and LMB treatment.

5.2.7 Human pre-rRNA Contains an A0 Cleavage Site that is Blocked by LMB Treatment, and 18S-E pre-rRNA is Accumulated upon ActD Treatment.

It appeared that NOB1 and PNO1 were shuttling between the nucleus and cytoplasm together, with their association independent of RNA pol. I transcription or nuclear export. This suggested that they may associate in the absence of pre-rRNA transcription, potentially upon a partially processed precursor. We therefore investigated pre-rRNA processing both with and without nucleolar disruption. ActD is frequently used to block RNA polymerase I transcription of rRNA. As the SSU processome assembles on nascent pre-rRNA, blocking rRNA transcription prevents SSU processome recruitment; giving rise to a 50S pre-SSU processome complex (Turner et al., 2009). ActD treatment of HeLa cells has also been shown to decrease all pre-rRNA species examined, although no difference in the level of mature 28S and 18S rRNAs has been observed (Shcherbik et al., 2010; Turner et al., 2009). Specifically, at low concentrations, ActD inhibits RNA pol. I activity alone (Perry, 1963). For this reason, we used a low concentration of the drug to specifically disrupt RNA pol. I transcription.

Inhibition of pre-rRNA processing has also been reported in HeLa cells upon treatment with LMB, whereby 18S-E rRNA accumulates and pre-rRNA processing is blocked at a site equivalent to the A0 cleavage site in yeast (Rouquette et al., 2005). Leptomycin B (LMB) is an inhibitor of CRM1; a protein required for nuclear export of proteins containing a nuclear export sequence (NES) (Fukuda et al.,

1997). Moreover, late-stage pre-ribosomal particles are exported to the cytoplasm via a CRM1-mediated pathway (Moy and Silver, 2002; Zemp et al., 2009). We therefore decided to use these chemicals to further examine pre-rRNA processing in the presence of nucleolar disruption.

HEK293 cells were treated with either no chemicals, LMB (30 nM for 2 hours) or ActD (0.1 µg/ml for 2 hours). RNA was isolated, quantified and separated by glyoxal agarose gel electrophoresis. The RNA was visualised by UV transillumination to reveal the mature 28S and 18S rRNAs (Figure 5.12 A). The RNA was subsequently blotted onto a nylon membrane and probed for rRNA, using a specific radiolabeled probe which recognised species containing the 5' ITS1 sequence (Figure 5.12 B).

None of the drug treatments appeared to affect levels of mature rRNAs (Figure 5.12 A). This was not surprising as relatively short treatments were used and the rRNAs are believed to be highly stable once bound with proteins, forming the mature ribosomal subunits. We could reliably detect the 47/45S, 41S, 30S, 21S and 18S-E precursors in control cells, as described previously (Rouquette et al., 2005). Similarly, upon LMB treatment these species were still detected, with the added presence of the 26S pre-rRNA band, indicating a failure of cleavage at the A0 site however, a 43S band (A0 – site 2 pre-rRNA) was not resolved as it may have been too close to the 45S band (Rouquette et al., 2005). Nonetheless, the presence of the 26S band verifies the results previously described in HeLa cells and indicates that this site was blocked from processing in our experiments when LMB was used. Upon ActD treatment, none of the pre-rRNA species examined were present, with the exception of 18S-E. This is a novel observation as it was previously believed that no pre-rRNA precursors remained after the inhibition of pre-rRNA transcription (Rouquette et al., 2005). However, it should be noted that a lower concentration of ActD was used for a shorter time period in the previous study (Rouquette et al., 2005). We however, believe that our observation may suggest a novel mode of action for ActD; not only blocking pre-rRNA transcription,

as indicated by the lack of other pre-rRNA precursors, but also blocking cleavage of site 3 in human pre-rRNA.

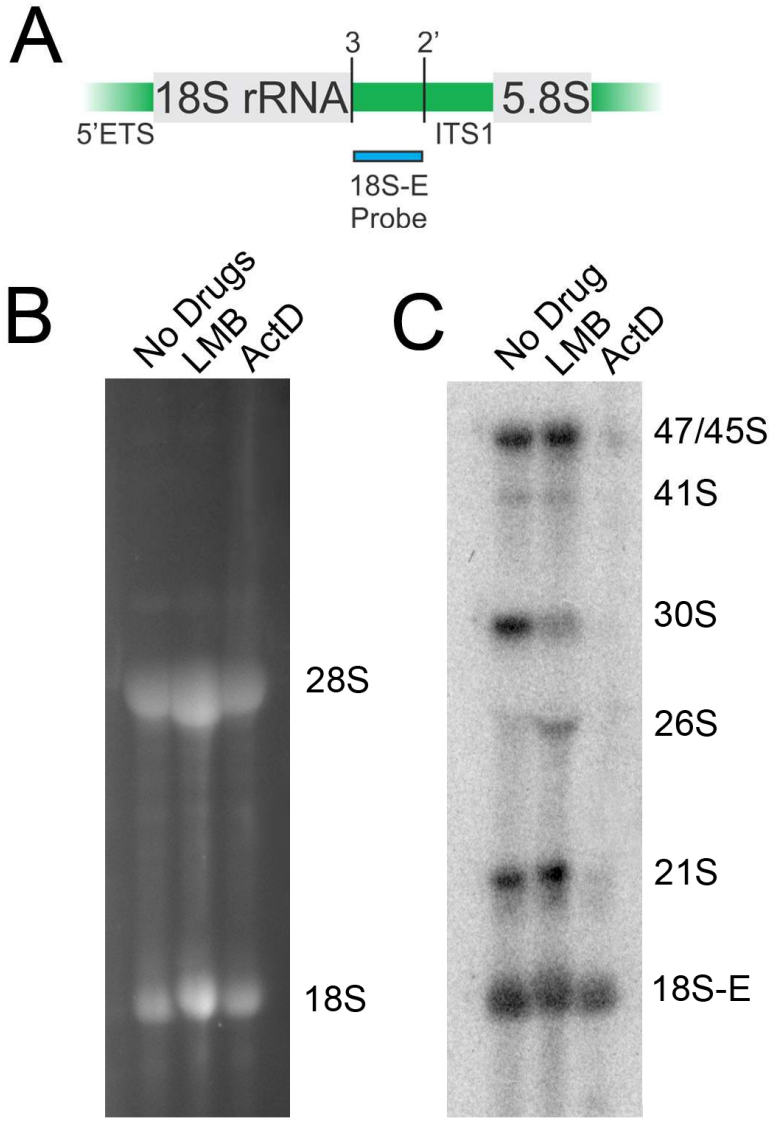


Figure 5.12 Presence of an A0 Cleavage Site that is blocked by LMB Treatment, and Accumulation of an 18S-E rRNA precursor upon ActD treatment

A) Model of human pre-rRNA and the location of the 18S-E radiolabeled probe (blue). The pre-rRNA excised by processing steps (indicated above) is shown in green, the mature rRNA shown in grey. The 5'ETS and ITS1 are indicated. Only the relevant cleavages sites and sections of the 47S pre-rRNA are shown. **B)** HEK293 cells were treated with no drug, LMB or ActD as described in the text, indicated above each lane. Extracts were prepared from cells expressing the FLAG-tag alone, ensuring no shearing of the RNA. RNA was mixed with ethidium bromide, separated by agarose glyoxal gel electrophoresis and RNA visualised by a UV transilluminator. The mature 28S and 18S rRNA are indicated to the right of the panel. **C)** RNA separated by glyoxal gel electrophoresis was transferred to a nylon membrane and probed for pre-rRNAs containing the 3' ITS1 sequence using a specific radiolabeled probe to the 18S-E as shown in A. Approximate sizes of the fragments are indicated to the right of each lane.

5.2.8 Pre-rRNA Processing Factors, pre-rRNA and the 80S SSU Processome

We had thus far observed a range of associations between the proteins involved in 3' pre-18S rRNA processing, the pre-rRNA and the U3 snoRNA. We therefore wished to investigate their integration into the 80S and pre-40S processing complexes. The U3 snoRNP monoparticle is known to sediment at 12S whereas the U3 snoRNP-containing SSU processome sediments at 80S upon glycerol gradient ultracentrifugation. In contrast, late pre-40S particles lacking the U3 snoRNP, migrate at 40-43S (Zemp et al., 2009). This type of analysis has been widely used to examine ribosome subunit formation and previously identified an SSU processome pre-cursor of approximately 50S, observed upon RNA pol. I inhibition (Turner et al., 2009).

HEK293 cells containing the tagged proteins of interest were induced as before, harvested, sonicated and extracts separated by glycerol gradient centrifugation. Protein and RNA was extracted from the individual fractions and analysed by Western and Northern blotting respectively. Western blots were probed using antibodies specifically against hU3-55k, PRP43 (a NOB1 interacting protein in yeast (Pertschy et al., 2009)), and the FLAG-tags of NOB1, PNO1 and DIM1 (Figure 5.13 A). Northern blot hybridisation was performed using probes specific for the U3 and U13 snoRNAs, and the 18S-E pre-rRNA precursor. The migration of U13 snoRNA on a glycerol gradient was also examined, as it has been suggested that U13 snoRNA may form base pairs with the 3' end of the 18S rRNA and is required for efficient processing (Cavaillé and Bachellerie, 1996). As expected, the U3 snoRNA was found to co-migrate with hU3-55k, distributed in a 12S peak; representing the U3 monoparticle containing only the core proteins and hU3-55k (Figure 5.13 A&B). U3 snoRNA was also observed in the 80S peak; representing the SSU processome complex containing the U3 snoRNP with hU3-55k, accessory factors and the pre-rRNA (Granneman et al., 2004; Turner et al., 2009). Upon examination of the Western blots, NOB1 was observed to localise at the top of the gradient, most likely as free protein or in a small complex. This was similar to the migration of PRP43; an RNA helicase that functionally interacts with NOB1 (Combs

et al., 2006; Lebaron et al., 2009; Pertschy et al., 2009), which was also observed towards the top of the gradient. NOB1 also migrated in a peak at approximately 40-50S, believed to represent the pre-40S complex. Similarly, PNO1 was observed in a peak at 40-50S although this also spread into the 80S region. In comparison, DIM1 was located in an approximately 50-80S peak.

These profiles are overlapping but not identical, adding weight to the idea of a dynamic SSU processome, with components associating and dissociating during pre-rRNA processing. Furthermore, this is consistent with our immunoprecipitation and immunofluorescence data which suggest that DIM1 is not associated with NOB1, but that PNO1 may be capable of association with both DIM1 and NOB1.

The U13 snoRNA localised towards the end of the 12S peak, overlapping slightly with the NOB1/PNO1 pre-40S peak. U13 is believed to base-pair at the 3' end of 18S pre-rRNA; overlapping the A1779, A1780 DIM1-methylated loop region, close to the site 3 cleavage loci (Lafontaine et al., 1995; Tyc and Steitz, 1989). Most interestingly, the NOB1/PNO1 peak at approximately 40-50S corresponds precisely to the sedimentation pattern of both the mature 18S and the final precursor 18S-E. This suggests, with evidence from the S1 nuclease protection assay and PNO1's co-migration with the 80S, that PNO1 may associate with pre-rRNA after the initial cleavage step and remain associated in the pre-40S complex. Furthermore, NOB1 most likely associates into the pre-40S complex after many of the initial cleavage steps. In contrast, it would appear that DIM1 is part of a larger SSU processome complex; quite possibly associating with an initial form of the pre-rRNA.

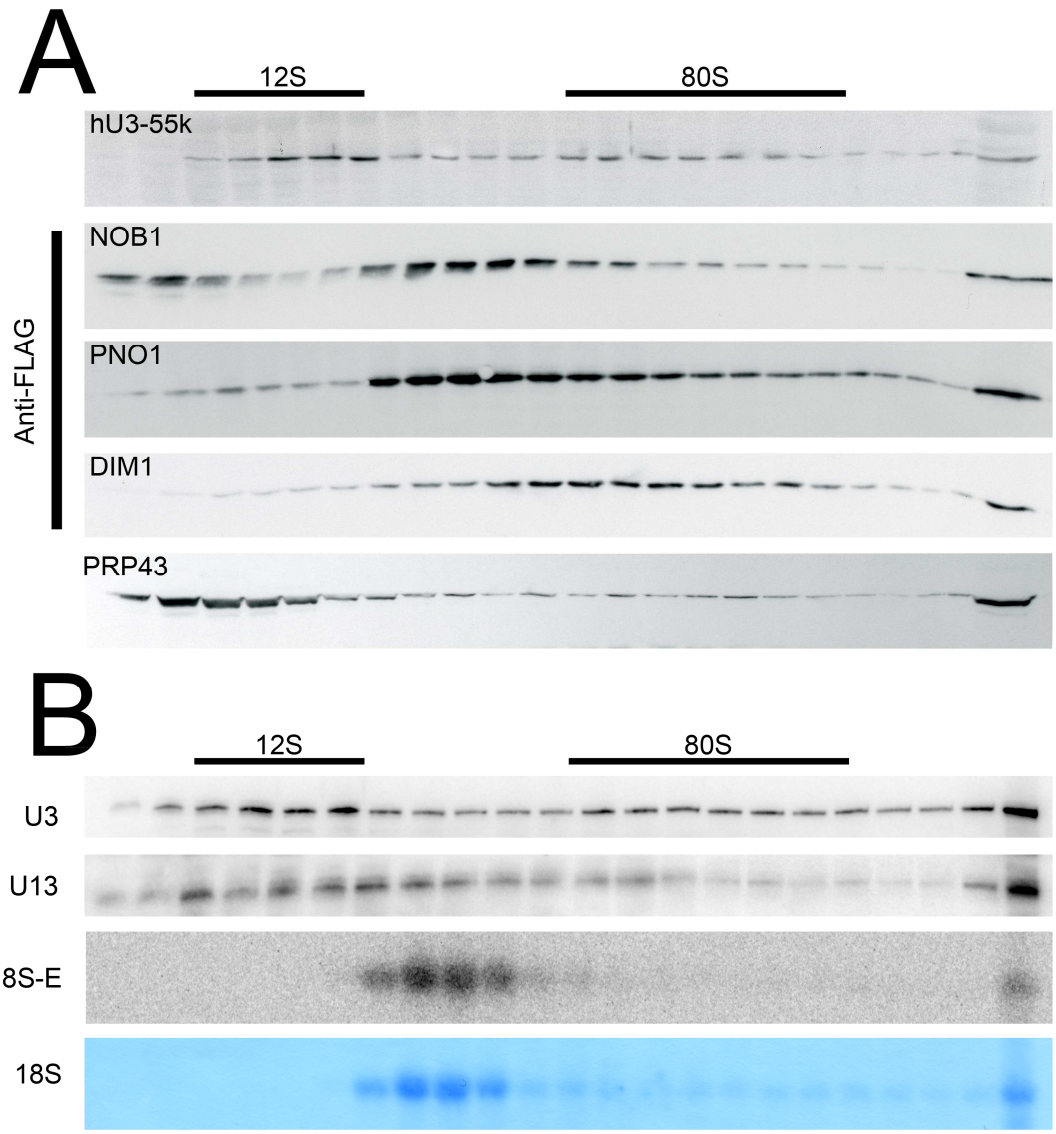


Figure 5.13 Glycerol Gradient Analysis of 3' pre-18S rRNA Processing Factors, the U3 snoRNA and pre-18S rRNA

Extracts were prepared from cells expressing FLAG-tagged proteins at equimolar amounts to the endogenous hU3-55k protein or expressing the FLAG-tag alone and separated by glycerol gradient centrifugation. Protein and RNA was extracted from the individual fractions. **A)** Proteins were separated by SDS PAGE and analysed by Western blotting. Western blots of FLAG-tagged lysate from NOB1, PNO1 or DIM1 expressing cells were probed using antibodies specific to the FLAG motif (left of each panel) whereas FLAG-tag alone cells were probed for hU3-55k or PRP43. FLAG-tagged and endogenous proteins indicated at the top left of each panel. 12S monoparticle and 80S SSU processome indicated above the panels. **B)** Northern blots of RNA from FLAG-tag alone cells, separated by denaturing polyacrylamide gel electrophoresis, were probed for U3 or U13 snoRNA. Similarly, glyoxal agarose gel blots of such RNA were stained using methylene blue to show the mature 18 rRNA and probed for 18S-E rRNA (indicated to the left of each panel). 10% of the total input in each case is loaded on the far right lane of each gradient.

To further investigate the potential sub-complexes of processing factors, the tagged cell lines were treated with the CRM1 inhibitor LMB and RNA Pol. I inhibitor ActD, as previously detailed. Once again, cellular lysates were separated upon glycerol gradients by ultra centrifugation. The resulting fractions were analysed by Western blot analysis, using anti-FLAG or anti-PNO1 antibodies (Figure 5.14) and Northern blot analysis, using a radiolabeled probe to visualise the 18S-E pre-rRNA (Figure 5.15).

Western blot analysis showed that the FLAG-tagged hU3-55k (upper band) closely followed the profile of the endogenous protein (lower band) giving rise to 12S and 80S peaks, as described previously. Upon treatment with LMB however, the 12S peak could no longer be observed whilst the 80S peak appeared reduced in size, overlapping with the pre-40S peak. This suggested that the SSU processome may accumulate on pre-rRNA, potentially due to the block in CRM1 mediated export of the pre-40S particle. This may well represent accumulation on the 26S (A0 - site 2) pre-rRNA previously observed with LMB treatment (Rouquette et al., 2005). Upon ActD treatment, the 80S peak was lost, instead a 50S peak was observed; described previously as an SSU processome precursor (Turner et al., 2009).

When examining NOB1, the protein was observed in a free pool and in the pre-40S complex. This sedimentation pattern did not appear different after CRM1 inhibition with LMB. Treatment with ActD however, resulted in a reduction of the levels of free protein; with the larger pre-40S complex appearing unaltered. Similarly, both endogenous and FLAG-tagged PNO1 migrated at the size of the pre-40S complex and 80S SSU processome, indicating that tagged protein was behaving as the endogenous protein. This migration pattern appeared unaffected by LMB treatment; remaining throughout the 40S-80S peak. The spread of the PNO1 peak was however, reduced by treatment with ActD, causing a more compact and concentrated 40S peak, and a much reduced 80S peak.

In untreated cells, DIM1 was observed in a 40-80S peak however, upon treatment with either ActD or LMB, DIM1 was also observed as free protein. Furthermore, in

both cases DIM1 was not prominently observed in larger 80S complexes but instead appeared in ~50S complexes.

The data, in combination with our previous observations, suggest that in untreated cells, NOB1, PNO1 and DIM1 are part of an overlapping pre-40S complex, with the complex most likely undisrupted by LMB treatment. ActD treatment causes a block in RNA pol. I transcription but also in processing of the 18S-E pre-rRNA. Under this condition, PNO1 and NOB1 were observed to remain associated and co-migrate on the glycerol gradient. This may suggest that PNO1 and NOB1 are associated on this late pre-rRNA precursor whereas DIM1 is not.

It appeared that the various proteins examined were differently affected upon LMB and ActD treatment. The complexes in which they associated were observed to alter their compositions when treated with drugs. It was however, not clear how this affected the 18S-E pre-rRNA. To better understand the requirement of the CRM1 nucleoplasmic shuttling mechanism and RNA pol. I transcription for 18S-E maturation, the pre-rRNA from drug treated cells was also analysed by glycerol gradient analysis. Tagged hU3-55k and NOB1 protein expression was induced from the HEK293 cells as before and subject to Western blot analysis using anti-hU3-55k and ant-FLAG antibodies respectively. FLAG-tag alone expressing cells were also induced, both in the presence and absence of LMB or ActD (no drugs; Figure 5.15). Lysate from the cells were separated upon glycerol gradients, as previous described. RNA fractions were separated upon a glyoxal agarose gel and blotted and the 18S-E was detected by Northern blot analysis.

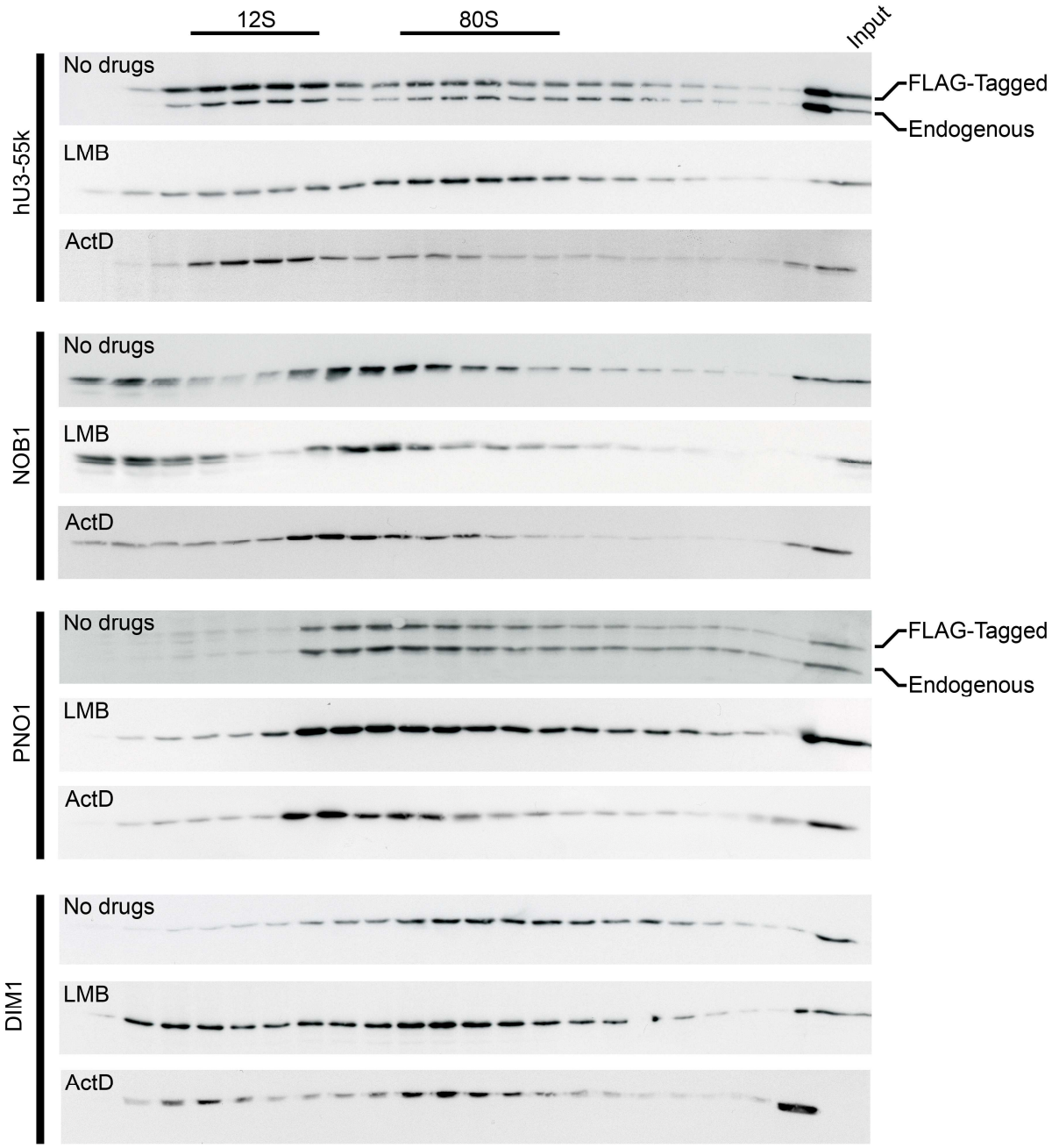


Figure 5.14 Glycerol Gradient Analysis of 3' pre-18S rRNA Processing Factors with CRM1 and RNA Pol. I Inhibitors

HEK293 cells were treated with no drug, LMB or ActD as described in the text. Extracts were prepared from the cells expressing FLAG-tagged proteins at equimolar amounts to the endogenous hU355k protein and separated by glycerol gradient centrifugation. Proteins were extracted from the individual fractions, separated by SDS PAGE and analysed by Western blotting. Western blots of FLAG-tagged lysate from hU3-55k, NOB1, PNO1 or DIM1 expressing cells were probed using antibodies specific to the FLAG motif or with antibodies specific to hU3-55k and DIM1 in the no drug conditions, as appropriate; enabling detection of both endogenous and tagged proteins, as indicated to the right of the panels. FLAG-tagged proteins expressed indicated to the left of each panel, the drug condition at the top left of each panel. 12S monoparticle and 80S SSU processome indicated above the panels. 10% of the total input in each case was loaded on the far right lane of each gradient.

Tagged and endogenous hU3-55k were observed to migrate in the 12S monoparticle and 80S SSU processome peaks (Figure 5.15). NOB1 migrated as a free protein / small complex at the top of the gradient and as a complex at ~40-50S (pre-40S complex), as previously described, co-migrating with the mature 18S-E rRNAs. Upon LMB treatment, 18S-E remained in the pre-40S peak, with some also appearing towards the top of the gradient, although this can not be explained. Upon treatment with ActD, the 18S-E rRNA was also observed over much the same peak as in untreated cells (~40-50S), with a slight tail running into the 80S peak. This further demonstrates that 18S-E accumulates upon ActD treatment, most likely associated with PNO1 and NOB1. This is in contrast to the previous belief that upon inhibition of RNA pol. I, all remaining pre-rRNAs were processed to completion (Shcherbik et al., 2010; Turner et al., 2009).

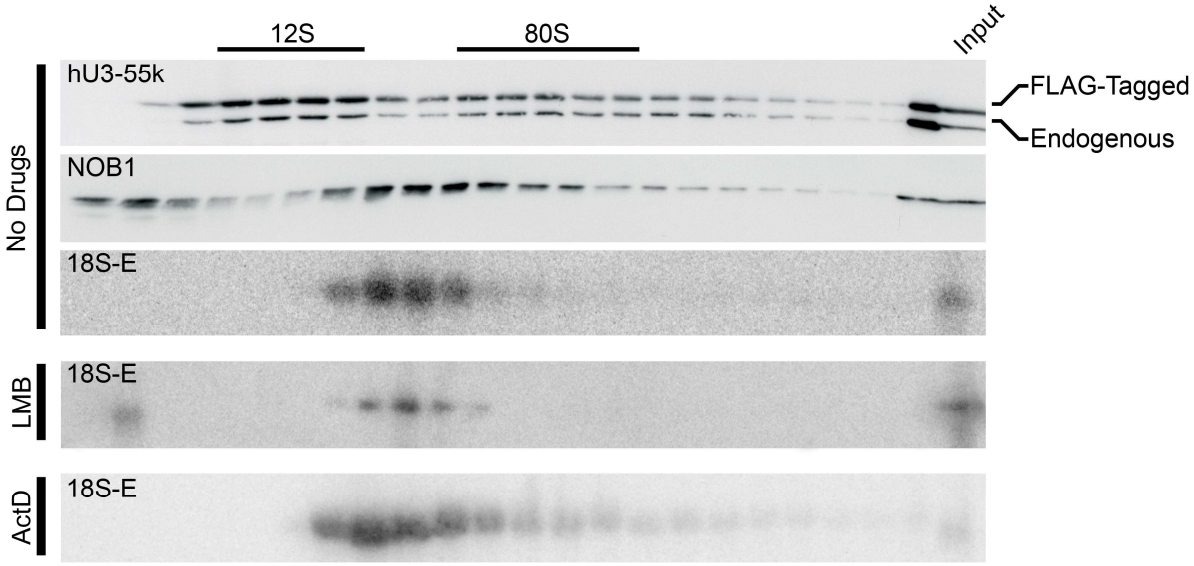


Figure 5.15 Glycerol Gradient Analysis of pre-18S rRNA with CRM1/XPO1 and RNA Pol. I Inhibitors

HEK293 cells were treated with no drug, LMB or ActD as described in the text, indicated to the left of each panel. Extracts were prepared from cells expressing the hU3-55k or NOB1 FLAG-tagged proteins and separated by glycerol gradient centrifugation. Protein and RNA was extracted from the individual fractions. Proteins were separated by SDS PAGE and analysed by Western blotting using antibodies specifically against hU3-55k and the FLAG motif respectively (left of each panel). The tagged protein expressed is indicated at the top left of each panel, with the tagged and endogenous forms indicated to the right of the panel. 12S monoparticle and 80S SSU processome indicated above the panels. Northern blots of RNA from FLAG-tag alone expressing cells, separated by glyoxal gel electrophoresis, were stained using methylene blue to show the mature 18 rRNA and probed for 18S-E RNA (indicated to the left of each panel). 10% of the total input in each case is loaded on the far right lane of each gradient.

5.3 Discussion

5.3.1 Overview

In humans, there is little known about the proteins responsible for pre-rRNA processing, especially with respect to those linked to SSU production. Whilst a number of homologous proteins to those observed in yeast have been identified, relatively little has been done to investigate their roles. Furthermore, considering the importance of this process, very little data is available from either yeast or humans about the association of these proteins with the pre-rRNA. It should however, be noted that this work was carried out prior to the direct crosslinking analysis of proteins on the pre-rRNA in yeast (Granneman et al., 2009; Granneman et al., 2010).

In this chapter a range of tools were successfully created to enable further study of the SSU processome in humans, including stable HEK293 cell lines capable of inducible expression of a range of tagged SSU processome components, with representative proteins of many of the known sub-complexes. Using these tools, we investigated protein-protein and protein-RNA associations, and localisation of the SSU processome components. We also examined the affect on the SSU processome components, of inhibiting the CRM1 protein involved in nuclear – cytoplasmic shuttling, the mTOR pathway, and RNA pol. I mediated rRNA transcription. Unfortunately, due to time constraints, a complete study of all of the SSU processome components chosen was not possible. We have however, demonstrated a comprehensive tool set which will enable in depth study of these proteins, with interesting results thus far for NOB1, PNO1 and DIM1.

5.3.2 Early SSU Processome Proteins

We have demonstrated that the majority of the proteins examined are human orthologs of their yeast counterparts and are associated with the SSU processome; associating with the U3 snoRNP, pre-rRNA, other SSU processome proteins

(PNO1 and KRR1) and localising to the nucleolus. It should be noted however, that the association of these proteins with PNO1, KRR1 and the U3 snoRNA were relatively weak, potentially due to the dynamic nature of the SSU processome.

Unfortunately, we could not detect associations with either PNO1 or KRR1 with the UTP-B protein UTP6. Moreover, only weak interactions were detected with the U3 snoRNA and pre-rRNA. In human cells, UTP-B proteins PWP2 and UTP12 were both shown to associate with U3 snoRNA and with the pre-rRNA before and after A' cleavage (Turner et al., 2009). This raised doubts as to the functionality of the tagged protein and further examination is therefore required, to ascertain if it is reliably behaving like the endogenous protein.

All other proteins were observed to associate with pre-rRNA, as would be expected for SSU processome components. Interestingly, RCL1 and RRP7 appeared to preferentially associate with A' uncleaved pre-rRNA, although were also capable of association with A' cleaved pre-rRNA.

Intriguingly, we previously demonstrated that hU3-55k requires phosphorylation to enable active cleavage at the A' site, potentially mediated through the CKII pathway. The UTP-C complex contains not only RRP7, but also UTP22 and the four CKII subunits CKA1, CKA2, CKB1 and CKB2. We demonstrated a bias for association of RRP7 before the initial cleavage event. If indeed the UTP-C complex associates with the SSU processome at this point, it may well be responsible for hU3-55k phosphorylation which in turn, we speculate, enables A' mediated cleavage of the pre-rRNA. This may then be followed by cleavage at sites 1 and 2, most likely catalysed by UTP24 (Bleichert et al., 2006). Moreover, it is likely that RRP7 bring the UTP-C complex to the pre-rRNA as it is the only component within the complex thought to contain an RNA recognition motif (RRM).

The RNA cyclase-like protein (RCL1) in yeast associates with BMS1, which contains a G-domain, believed to act as a molecular switch with SOF1 and DHR1 to initiate pre-rRNA cleavages (Billy et al., 2000; Wegierski et al., 2001). In contrast

to this, BMS1 is known to associate with pre-rRNA mainly after the initial cleavage in human cells (Turner et al., 2009) whereas our data shows RCL1 association mainly prior to the cleavage event. This may suggest that RCL1 may associate first, potentially aiding BMS1 association, with its GTPase activity causing the dissociation of RCL1 from the SSU processome complex after the cleavage event.

We also examined the localisation of the various tagged proteins to further investigate if they were indeed SSU processome components, with the vast majority localising throughout the FC/DFC/GC of the nucleolus in untreated cells (Table 5.2), as was expected.

Table 5.2 SSU Processome Protein Localisation in HEK293 Cells with and without Actinomycin D Treatment

	No Drugs				ActD			
	Nucleolar		Nuclear	Cytoplasmic	Nucleolar		Nuclear	Cytoplasmic
	FC/DFC	GC			Grannular Body	Fibrillar Cap		
fibrillarin	■					■		
hU3-55k	■	■			▒	■	▒	
NOB1				■			▒	■
PNO1	■	■	▒	▒	■	■	■	
DIM1	■	■	▒		■	■	■	
ESF2	■	■			▒	■	▒	
KRR1	■	■			■			
MRD1	■	■				■	■	
RCL1	■	■				■	■	■
RRP7	■	■			■	▒	▒	
tUTP4	■	■				■		
UTP6	■	■				■	■	
UTP23	■	■			■	▒	▒	
UTP24	■	■			■	▒	▒	

Drug treatments are shown at the top as are the sub-cellular compartments of interest for each condition. The protein is shown down the farthest left column. Dark grey indicates where most of the signal was detected, lighter grey indicates that some signal was detected, white indicates no signal detected; indicating protein localisation.

The SSU processome has previously been shown indirectly, to be sensitive to RNA pol. I inhibition, preventing pre-rRNA transcription and therefore 80S SSU processome formation (Turner et al., 2009). It is believed that upon ActD treatment, the granular component remains in the centre of the nucleolus, whereas the fibrillar components (such as fibrillarin) move to the periphery of the nucleoli to form

nucleolar / fibrillar caps (Chen and Jiang, 2004; Leary et al., 2004). Accordingly, when cells were treated with ActD, we observed many of the SSU processome proteins to retain some granular body localisation, with KRR1 solely localised there. The suggestion that the granular components remain in these granular bodies is not always the case however, as MRD1, RCL1 and UTP6 were all observed to be excluded from these structures.

A range of SSU processome proteins displayed varying degrees of fibrillar cap localisation, including RCL1 which was observed in the nucleoplasm and fibrillar caps. This contradicts previous observations in HeLa cells, where ActD treatment resulted in granular body localisation for both RCL1 and MPP10 (Leary et al., 2004). It should however, be noted that the previous study used GFP-tagged proteins and a lower concentration of ActD. Furthermore, it has been demonstrated that endogenous MPP10 in HeLa cells localises in fibrillar cap structures upon treatment with ActD (Turner *et*. *al*., 2010; manuscript in preparation), suggesting that some GFP-tagged nucleolar proteins may not localise correctly.

The tUTP complex has previously been demonstrated to be linked to both pre-rRNA transcription and processing (Prieto and McStay, 2007). It has also been shown that tUTP10 associates mainly before the initial pre-rRNA cleavage step. Moreover, depletion of the protein results in the accumulation of an incomplete SSU processome, similar to that observed upon ActD treatment (Turner et al., 2009), implying that it is required for SSU processome recruitment to the pre-rRNA. Our observations demonstrate that tUTP4 is associated with pre-rRNA both before and after the primary cleavage event, contradicting what was previously observed for tUTP10 (Turner et al., 2009). Interestingly, tUTP4 was the only transcriptionally associated UTP previously found not to be required for pre-rRNA transcription and has been proposed to be responsible for linking the t-UTP complex with other SSU processome components (Prieto and McStay, 2007). Furthermore, tUTP4 showed a greater association with the U3 snoRNA than many of the other SSU processome components examined. Thus, it is possible that the protein may

remain associated with the pre-rRNA after the initial cleavage step to aid subsequent protein recruitment steps or to help maintain U3 snoRNP association.

Upon RNA pol. I inhibition with ActD, tUTP4 exclusively localised in fibrillar cap structures. These have previously been shown to contain UBF associated NORs, tUTP10 and pre-rRNA processing factors including fibrillarin and the U3 snoRNA (Dousset et al., 2000; Kopp et al., 2007; Prieto and McStay, 2007). Moreover, as part of the tUTP complex, tUTP4 is thought to be sequestered by NORs in a UBF-dependent fashion (Prieto and McStay, 2007), suggesting that these factors may remain co-localised upon RNA pol. depletion, in keeping with the belief that they may interact with the rDNA independently of transcription (Prieto and McStay, 2007).

Interestingly, our data indicated that DIM1 and NOB1 proteins may function at temporally and physically separate points in the pre-rRNA processing pathway, in contrast to their closely related processes in yeast (Fatica et al., 2003; Lafontaine et al., 1998b; Schafer et al., 2003). For this reason we decided to focus on these proteins, alongside PNO1 with which NOB1 and DIM1 have been shown to associate in yeast (Tone and Toh, 2002; Vanrobays et al., 2004).

5.3.3 3’ pre-18S rRNA Processing Factors DIM1, PNO1 and NOB1

We examined the association of NOB1, PNO1 and DIM1 with pre-rRNA before and after the initial A’ cleavage step in human cells. This revealed that NOB1 could not be observed to associate with the pre-rRNA. This is most likely because NOB1 is thought to associate with pre-rRNA after the A0 cleavage, whereas the probe used was not capable of detecting pre-rRNA species downstream of site A0 cleavage. Indeed, NOB1 is believed to associate with and cleave the penultimate pre-rRNA species, 20S in yeast (Fatica et al., 2003; Lamanna and Karbstein, 2009; Pertschy et al., 2009). In contrast, although the associations were weak, DIM1 appeared bias towards A’ uncleaved pre-rRNA, and PNO1 towards A’ cleaved pre-rRNA. This indicated that there may be differences in the timing of PNO1 and DIM1

associations with the pre-rRNA. This contradicts observations in yeast, where DIM1 is thought to associate with pre-rRNA in the nucleolus and remain associated to catalyse methylation in the cytoplasm, dependent on PNO1 association (Lafontaine et al., 1998b; Vanrobays et al., 2004).

This was further demonstrated through immunofluorescence analysis of these proteins (Table 5.2). DIM1 was not observed within the cytoplasm, but was within the nucleus and nucleolus. It is within the nucleolus that the protein has been reported to localise and associate with the pre-rRNA in yeast, where it has also been observed to shuttle to the cytoplasm (Lafontaine et al., 1998b; Schafer et al., 2003). We however, saw no evidence of this in human cells. Glycerol gradient analysis further confirmed that NOB1 and DIM1 may function separately as the proteins were observed in different sized complexes; NOB1 mainly in a pre-40S complex (co-migrating with 18S-E; its believed cytoplasmic substrate), and DIM1 co-localising with hU3-55k in the 80S SSU processome.

This is in contrast to data from yeast, whereby NOB1 and DIM1 are observed to co-localise not only in 40S pre-ribosomal particles but also in larger particles, reported to correspond to the early 80S pre-ribosome (Schafer et al., 2003). Moreover, DIM1 is observed within pre-ribosomal particles both in the nucleolus and cytoplasm of yeast cells (Lafontaine et al., 1998b; Schafer et al., 2003), with DIM1 responsible for the cytoplasmic methylation of 20S pre-rRNA (Lafontaine et al., 1998b). Interestingly, also in yeast, DIM1 has been demonstrated to bind rRNA sequences involved in tRNA interactions and mRNA decoding in the mature ribosome, indicating that DIM1 association is incompatible with translation (Granneman et al., 2010).

We however, observe that methylation is unlikely to occur cytoplasmically in human cells. Our data suggests that in humans, DIM1 is not a major component of cytoplasmic / late 40S pre-ribosomes as it is in yeast, but instead that it associates, and most likely functions, in the 80-90S pre-ribosomes in the nucleolus. This is supported by data from HeLa cells, where DIM1 is not observed by mass-

spectrometry in late pre-40S complexes (immunoprecipitated with RIO2; (Zemp et al., 2009)). Furthermore, preliminary primer extension analysis of pre-rRNA indicates that the DIM1 methylation event occurs prior to the loss of ITS1 (data not shown; performed by Nick Watkins), further indicating that this occurs prior to cytoplasmic export in human cells.

In yeast it has been demonstrated that DIM1's enzymatic function can be separated from its involvement in pre-rRNA processing, such that methylation is not a pre-requisite for pre-rRNA processing (Lafontaine et al., 1995; Lafontaine et al., 1998b). It may therefore, be possible that these disparate functions of the protein in yeast occur simultaneously in the nucleolus of human cells.

In contrast to DIM1, we have shown in human cells that PNO1 was mainly associated, albeit weakly, after the initial pre-rRNA cleavage event and that it also associated with the U3 snoRNA. This is in agreement with data from yeast whereby PNO1 is known to be part of a late pre-40S processing complex involved in 3' end processing of the 18S pre-rRNA with NOB1 (Schafer et al., 2003; Vanrobays et al., 2004). In yeast, PNO1 is believed to act as an rRNA binding protein required for intranuclear transport and nucleocytoplasmic export of the pre-40S subunit, potentially recruiting NOB1 to the pre-rRNA (Tone and Toh, 2002; Vanrobays et al., 2004; Vanrobays et al., 2008). It should however, be noted that PNO1 is also required for early processing steps in yeast at sites A_1 and A_2, although not A_0 (Vanrobays et al., 2004).

Interestingly, PNO1 was the only protein observed throughout the cell. This appeared to be in agreement with data from yeast, where PNO1 is thought to shuttle between the nucleolus and cytoplasm (Vanrobays et al., 2004; Vanrobays et al., 2008). In contrast, our observations contradict previous data from HeLa cells whereby PNO1 is mainly observed within the nucleolus, with cytoplasmic localisation only detectable upon depletion of the kinase RIO2 (Zemp et al., 2009). It should however, be noted that in the published data, the effects of RIO2 depletion were largely variable between cells (Zemp et al., 2009).

Upon examination of protein-protein interactions between the 3' pre-rRNA processing factors, it appeared that in contradiction to observations in yeast (Merl et al., 2010), neither DIM1 nor PNO1 associated with KRR1 in human cells. PNO1 was however, observed to be capable of association with both NOB1 and DIM1, although most likely at different points in the processing pathway, due to their different localisation profiles. This was further illustrated by glycerol gradient analysis which indicated that PNO1 was both in the pre-40S complex; most likely containing NOB1, and in the 80S SSU processome; co-localising with hU3-55k and DIM1. This suggests that PNO1 is present throughout processing, associating after the initial A' cleavage event through to the final stages of cytoplasmic maturation.

In contrast, NOB1 was mainly observed within the cytoplasm and not observed to associate with early pre-rRNAs, the U3 snoRNA or KRR1. Furthermore, NOB1 was not observed in 80S SSU processome sized complexes, but rather in complexes believed to represent pre-40S particles, further suggesting that NOB1 and DIM1 function in different cellular compartments, in contradiction to what has been observed previously in yeast (Brand et al., 1977; Ferreira-Cerca et al., 2005; Ferreira-Cerca et al., 2007; Lafontaine et al., 1998b; Schafer et al., 2003). NOB1 in yeast is reported by some, to localise homogenously throughout the cell and is suggested to join the pre-40S in the nucleolus (Fatica et al., 2003; Fatica et al., 2004), whereas other groups suggest that NOB1 is found mainly on late cytoplasmic pre-40S subunits, where it performs the site D cleavage step (Pertschy et al., 2009; Schafer et al., 2003). In HeLa cells NOB1 has been said to be present in the cytoplasm and nucleus of the cell, although the nucleoplasmic signal is not consistent between cells within the published data, appearing only in the cytoplasm of some cells (Zemp et al., 2009). We therefore suspected that NOB1 may be a shuttling protein which is mainly present in the cytoplasm of untreated human cells.

Interestingly, upon ActD treatment (used to block pre-rRNA transcription), NOB1 localisation was observed in both the nucleoplasm and cytoplasm, whilst being excluded from the nucleolus. Conversely, PNO1 appeared to be blocked from cytoplasmic localisation, accumulating in the nucleus and nucleolus. We also

observed PNO1 and NOB1 in the same sized complexes, with the proteins remaining associated with one another under this drug treatment. This indicates that an active nucleolus is required for correct PNO1 and NOB1 localisation into the cytoplasm, and potentially for pre-rRNA export. Furthermore, this demonstrates that NOB1 and PNO1 are both nucleocytoplasmic shuttling proteins that maintain their association in the absence of active RNA pol. transcription.

In contrast to this, PNO1-DIM1 association was lost upon ActD treatment and the proteins were no longer observed in similarly sized complexes, as PNO1 became restricted to the pre-40S complex, co-localising with NOB1 and 18S-E. This may be explained by our observation that ActD treatment abolished all pre-rRNA precursors examined with the exception of 18S-E. This is a pre-rRNA species extending from the mature 5' end of 18S to a 3' extension, 24 nucleotides downstream of site 3 (Rouquette et al., 2005). It is therefore possible that PNO1 and NOB1 accumulate on the 18S-E pre-rRNA, most likely in the nucleus prior to export. In contrast, the ActD block in pre-rRNA transcription prevents PNO1-DIM1 associations, indicating that they may associate indirectly, potentially via an early pre-rRNA.

Low concentrations of ActD are widely documented to selectively block RNA pol. I and therefore pre-rRNA transcription however, upon doing so, it was always assumed that all pre-rRNA precursors were abolished (Perry, 1963; Turner et al., 2009). Similarly, depletion of specific RNA pol. I factors have also been shown to deplete pre-rRNA transcription and all pre-rRNA precursors, although the presence of 18S-E pre-rRNA was never examined (Prieto and McStay, 2007; Turner et al., 2009). Our data therefore, indicate that low concentrations of ActD may have inhibitory effects on site 3 cleavage, although it is not clear if this is direct or indirect. It would therefore be interesting to examine the presence of the 18S-E pre-rRNA upon pol. I subunit depletion, to examine its association with PNO1 and NOB1, and its cellular localisation. Based on our current data, we would expect the rRNA to remain bound to PNO1 and NOB1, and cytoplasmic export of the pre-RNA

to be blocked, in accordance with the block in PNO1 / NOB1 shuttling we observed and their maintained association.

The shuttling of NOB1 may correspond with the protein's previously documented nuclear localisation in yeast, and association with the pre-40S ribosomal complex. This complex contains ribosomal proteins and late-assembly factors, some of which are involved in pre-ribosomal CRM1-mediated shuttling between the nucleus and cytoplasm, such as PNO1 (Ferreira-Cerca et al., 2005; Ferreira-Cerca et al., 2007; Pertschy et al., 2009; Schafer et al., 2003; Vanrobays et al., 2008; Zemp et al., 2009). Late-stage pre-ribosomal particles are known to move from the granular component of nucleoli to the nucleoplasm and are then exported to the cytoplasm via a CRM1-mediated pathway (Henras et al., 2008; Moy and Silver, 2002). Usually this translocation relies on the NES of the protein itself, or an adapter protein, the movement of which can be disrupted by LMB (Askjaer et al., 1998; Fukuda et al., 1997; Ohno et al., 2000; Paraskeva et al., 1999).

Upon CRM1 inhibition with LMB, NOB1 was again observed in the nucleus of the cell, although excluded from the nucleolus, as when treated with ActD. Similarly, PNO1 again appeared blocked from cytoplasmic localisation. In contrast, DIM1 remained in the nucleolus and nucleoplasm. The data indicate that CRM1 is required for both PNO1 and NOB1 shuttling between the cytoplasm and nucleus, as previously demonstrated in HeLa cells for NOB1 (Zemp et al., 2009).

Immunoprecipitation, localisation and glycerol gradient data suggest that NOB1 and PNO1 are capable of association in the absence of active pre-rRNA transcription, U3 snoRNA association or CRM1-mediated export, indicating a potential direct interaction. This has recently been demonstrated *in vitro* by Katherine Sloan (personal communication, data not shown). In contrast, the association between PNO1 and DIM1 appears to depend on active pre-rRNA transcription but not CRM1-mediated export, potentially indicating an indirect association of the proteins through an early pre-rRNA. It should be noted however, that whilst PNO1 and NOB1 may directly interact, they may also become stably

associated on 18S-E pre-rRNA, most likely requiring cytoplasmic RIO2 kinase activity for their removal (Zemp et al., 2009).

Neither ActD treatment, nor LMB treatment however, was observed to result in a solely nucleoplasmic NOB1 signal, with much of the protein remaining in the cytoplasm. NOB1 was however, observed by glycerol gradient analysis in not only a pre-40S peak but also a smaller peak; co-localising with PRP43. In yeast, it is known that these proteins must interact to achieve efficient site D endonucleolytic cleavage of pre-rRNA (Pertschy et al., 2009) and it is possible that this may represent the portion of the cytoplasmic NOB1 protein not observed to shuttle. Alternatively, this may merely represent free protein, not present in a complex.

In yeast it has previously been demonstrated that rapamycin blocks PNO1 shuttling. We therefore used this drug to similarly inhibit the mTOR pathway and further examine PNO1 / NOB1 shuttling. We demonstrate in human cells that, as in yeast (Vanrobays et al., 2008), PNO1 is regulated by the mTOR pathway; known to act as a nutrient sensor (Dowling et al., 2009; Rohde et al., 2001), with rapamycin specifically targeting the mTORC1 complex. The inhibition of this pathway results in nuclear retention of the PNO1 protein and therefore, presumably, the pre-40S ribosomal subunit. This was further supported by the detection of a weak signal for NOB1 localisation in the nucleus upon rapamycin treatment, once again indicating that the proteins may be directly associated with one another.

The regulation of PNO1 localisation by mTOR may not however, be unique. In yeast, the conserved GTP-binding protein NOG1, associated with early and late stages of 60S ribosome maturation, is usually localised throughout the nucleus. Upon treatment with rapamycin and nutrient starvation, the NOG1-containing complex becomes tethered to the nucleolus and intranuclear transport of pre-60S complexes is consequently inhibited (Honma et al., 2006). How mTOR controls the CRM1 and non CRM1-mediated translocation of proteins has not however, been demonstrated. It is possible that the effects observed are due to an upstream block

in pre-rRNA transcription as it is known that mTOR coordinates transcription by all three classes of nuclear RNA polymerases (Mayer and Grummt, 2006). Nonetheless, it is worth noting that PNO1 is thought to contain a range of phosphorylatable residues, providing potential targets for mTOR regulation (Gnad et al., 2007; Olsen et al., 2006; Wang et al., 2008) either directly, or through the kinase RIO2 (Zemp et al., 2009). Similarly, NOB1 is believed to contain a number of post-translational modifications (Choudhary et al., 2009; Dephoure et al., 2008; Olsen et al., 2006) flanking the metal binding site; believed to be required as a co-factor to the nuclease. These may potentially aid in controlling the activity of the endonuclease.

We observed that in human cells, PNO1 was capable of association with both DIM1 and NOB1 proteins, and with the U3 snoRNA and pre-rRNA. We however, also noted that PNO1 displayed a bias for association with pre-rRNA most likely after DIM1 association. In contrast, NOB1 was not observed to associate with early pre-rRNA. It is therefore likely that PNO1 associates after the initial cleavage step and associates into a complex already containing DIM1. This appears to be dependent on active transcription but not nuclear export. DIM1 may then dissociate from the complex and subsequent processing may occur, with PNO1 potentially bringing in other factors and acting as an adapter protein to facilitate translocation of the pre-40S complex through the nucleolus, nucleus and to the cytoplasm, where PNO1 and NOB1 dissociate and are recycled to the nucleus.

Similarly to PNO1, LTV1 has been suggested as an NES adapter protein in yeast, required for nucleocytoplasmic transport of the small pre-ribosomal subunit (Seiser et al., 2006). Depletion of LTV1 however, is not thought to completely block pre-40S export as it is not an essential protein, thus suggesting that it plays a non-essential role (Zemp and Kutay, 2007). Data from HeLa cells indicates that the NES domain in RIO2 also plays a non-essential role in pre-40S export but is required for the release of trans-acting factors such as PNO1, LTV1, and NOB1, and for 18S-E pre-rRNA cleavage (Zemp et al., 2009). In contradiction to our data however, PNO1 was not observed cytoplasmically unless RIO2 was depleted in

HeLa cells (Zemp et al., 2009), although this may merely represent different efficiencies at which the protein is recycled in different cell types. Nonetheless, our data indicate that PNO1 may act as an NES adapter protein, influencing NOB1 localisation through the cell, potentially via a direct interaction. Furthermore, based on data from yeast, this may also regulate the shuttling of late pre-40S particles from the nucleus to the cytoplasm (Vanrobays et al., 2008). Unlike in yeast however, pre-rRNA methylation by DIM1 may occur before export to the cytoplasm.

5.3.4 Future Work and Conclusions

In this chapter we have described a range of tools developed to aid in the dissection of 18S pre-rRNA processing. We have demonstrated the ability to purify a range of recombinant SSU processome components. Due to time constraints however, we were not able to carry out *in vitro* protein-RNA interaction assays. In the future we hope to investigate the roles of these proteins further by examining additional protein-protein and protein-rRNA associations through *in vitro* pull-down experiments, with the aim of attaining a higher resolution of detail about the timing of their interactions and the dynamics of the SSU processome. Furthermore, we would like to investigate the RNA-binding ability of proteins such as PNO1, KRR1 and RRP7, and also the potential cleavage activities of NOB1 and UTP24 directly on radiolabeled rRNA substrates, to verify their roles indicated in yeast but not demonstrated in human cells. Similarly, we would like to investigate which proteins are responsible for recruitment of the U3 snoRNP to the pre-rRNA through siRNA depletion and immunoprecipitation experiments, with previous data suggesting that the tUTP proteins may play a crucial role in this recruitment process (Prieto and McStay, 2007; Turner et al., 2009).

In relation to work in previous chapters, we would also like to further investigate the UTP-C complex and its potential role as a protein kinase. We demonstrated here the preferential association of the UTP-C component RRP7 with uncleaved pre-rRNA. The complex also contains all four CKII subunits, therefore it may act to

phosphorylate hU3-55k, thus facilitating the initial A' cleavage step. To investigate this intriguing possibility, it would be interesting to examine the effects of UTP-C subunit depletion on hU3-55k phosphorylation and pre-rRNA cleavage efficiencies. We would also like to verify if RRP7 is indeed responsible for UTP-C recruitment to the pre-rRNA, as suspected.

In yeast, NOB1, PNO1 and DIM1 proteins have been proposed to interact as part of a complex involved in 3' end processing of the 18S pre-rRNA (Granneman et al., 2010; Rouquette et al., 2005; Schafer et al., 2003; Vanrobays et al., 2004) whereby, PNO1 acts as an rRNA binding protein required for pre-40S intranuclear transport and nucleocytoplasmic export of the pre-ribosomal subunit, potentially recruiting DIM1 and NOB1 to the pre-rRNA (Tone and Toh, 2002; Vanrobays et al., 2008). DIM1 is responsible for cytoplasmic methylation of the 20S pre-rRNA (Lafontaine et al., 1998b) which occurs prior to the NOB1 catalysed site D cleavage event in yeast (Lafontaine et al., 1995; Pertschy et al., 2009). We present evidence that suggests in humans, DIM1 associates early in the rRNA processing pathway, binding uncleaved pre-rRNA which it may then methylate. It has been suggested in yeast that this methylation step may play a regulatory role in ribosome biogenesis (Lafontaine et al., 1998b). If this is also the case in human cells, the spatial and temporal segregation of the regulatory steps discussed (DIM1 catalysed methylation and PNO1-mediated export) may act to allow a greater resolution of control over the processing of pre-rRNA and ribosome production in human cells. Alternatively, placing the check point at an earlier point of the pathway may prevent the cell wasting energy or may reduce the risk of producing an aberrant product.

Furthermore, we demonstrate that PNO1 associates after the first cleavage event and shuttles, most likely with the pre-rRNA, to the cytoplasm. The shuttling of this protein, its association with NOB1 and analysis of its sequence suggests that PNO1 may act as an NES adapter protein for the pre-40S particle, to facilitate CRM1-mediated nucleocytoplasmic shuttling. This is also regulated through the

mTOR pathway, indicating that it may be one of the many ways, in which ribosome biogenesis is controlled in concert with cell growth and division.

Further experiments are however required to more fully characterise these proteins. It would, for example, be intriguing to compare the effect of specific RNA pol. I inhibition (through subunit depletion), with ActD treatment on 18S-E pre-rRNA - PNO1 accumulation, association and localisation. Moreover, it would be interesting to examine if inhibition of PNO1 shuttling leads to inhibition of pre-rRNA export, and the affect of this on DIM1 catalysed pre-rRNA methylation. Similarly, mutations of PNO1's NES motif may provide further information on the protein's potential role as an export adapter protein. Accordingly, further investigation of mTORC1's role in controlling this export pathway requires further study. We also aim to investigate the rRNA binding properties of these proteins and would like to examine if, as in yeast, NOB1 is the site 3 endonuclease in human cells.

Chapter Six

Discussion

6.1 Overview

Ribosome biogenesis is known to be a highly complex process requiring many snoRNP and protein complexes. A key step in the production of the small ribosomal subunit in eukaryotes is the sequential processing of the pre-rRNA. Crucial to the SSU processome, and the initial cleavage steps of the pre-rRNA, is the U3 snoRNP which is associated with the U3-specific protein hU3-55k. This is the only non-Box C/D snoRNP protein associated with the U3 snoRNP monoparticle when not in the SSU processome. hU3-55k is believed to be crucial for association of U3 snoRNA with the pre-rRNA, yet how this is controlled is not clear. Moreover, U3 snoRNA levels have previously been suggested to fluctuate independently of other snoRNAs, although how this is achieved was also unknown.

Much of the work to date regarding the SSU processome has focused upon the model organism *S. cerevisiae*, with proteomic screens offering novel insights into the components involved in the complex. However, little is known about the exact role of many of these proteins with even less known about the homologous proteins in human cells. SSU processome assembly is believed to be hierarchical, requiring the successive assembly of various sub-complexes. It is however, not clear which protein(s) directly bind the pre-rRNA to recruit these sub-complexes into the processome, or which proteins perform the pre-rRNA cleavage steps.

In this study, we therefore set about producing a range of tools to enable the examination of the human SSU processome with a particular focus on both hU3-55k and potential rRNA-binding / rRNA-modifying proteins thought to be components of the complex in yeast. These tools enabled us to determine how hU3-55k was able to control U3 snoRNA levels within the cell, independently of

other snoRNAs, and how this may be important in cellular differentiation and potentially tumourogenesis. We were also able to verify a host of proteins as SSU processome components capable of U3 snoRNA association. Importantly however, clear differences were observed between what is known in yeast about 3' 18S rRNA processing proteins and our observations in human cells.

6.2 The Role of hU3-55k in U3 snoRNP formation, Cellular Differentiation and the SSU Processome

In both higher eukaryotes and yeast the U3 snoRNA is required for the pre-rRNA processing steps surrounding the pre-18S rRNA (Beltrame and Tollervey, 1995; Borovjagin and Gerbi, 2000; Borovjagin and Gerbi, 2001; Granneman et al., 2004; Hughes, 1996). Furthermore, it is the only snoRNA found to be crucial for production of the 18S rRNA across all species examined. It is therefore important to understand its function in ribosome biogenesis.

The 3' region of the snoRNA is the protein-binding domain and contains the Box C/D motif and the associated core proteins NOP58, NOP56, 15.5K and fibrillarin (Granneman et al., 2009; Lubben et al., 1993; Watkins et al., 2002; Watkins et al., 2004; Watkins et al., 2000). The U3 snoRNA also contains a U3-specific box B/C motif to which an additional 15.5K protein and the U3-specific hU3-55k (Rrp9 in yeast) are associated (Granneman et al., 2002; Lubben et al., 1993; Pluk et al., 1998; Venema et al., 2000). Whilst U3 snoRNA – pre-rRNA base-pairing is crucial for pre-rRNA cleavage, it is the Box B/C motif that is required for the incorporation of the U3 snoRNP into the SSU processome (Granneman et al., 2004). This suggests that hU3-55k may play a crucial role in defining the U3 snoRNP and facilitating SSU processome assembly.

As previously demonstrated, we show that the WD repeats in the C-terminus of the hU3-55k protein are required for association with the U3 snoRNA (Lukowiak et al., 2000). Interestingly, we also demonstrate that the U3 snoRNP may be under tight control via the N-terminus of the protein, and therefore most likely acts to regulate

the formation and activity of the SSU processome complex. Specifically, we show that hU3-55k is likely to modulate the A' cleavage event, thus controlling the amount of "active" SSU processome and therefore, 18S rRNA production.

Our data suggests that this is may be achieved through the phosphorylation of highly conserved N-terminal serine residues, at positions 50, 51 and 53 in the human protein. Sequence alignments show that similar residues are present across all species examined and sit adjacent to a stretch of negatively charged glutamic acid residues (E-stretch), previously hypothesized to facilitate protein-protein interactions (Pluk et al., 1998). The serine residues 51 and 53 are potentially phosphorylated by casein kinase 2 (CK2), based on the amino acid motif (Gnad et al., 2007; Olsen et al., 2010). If this is the case, the upstream binding factor (UBF) would provide an intriguing parallel to hU3-55k.

The C-terminal domain of UBF contains blocks of aspartic and glutamic acid residues, each terminating in serine residues. This domain binds and recruits SL1, with binding enhanced by phosphorylation of the serine residues, probably by CK2 (Moss et al., 2007; Tuan et al., 1999). This is important in formation of the pre-initiation complex on the DNA, and is required for transcription of the pre-rRNA in eukaryotes (Lin et al., 2002; Moss et al., 2007). This suggests that hU3-55k phosphorylation may aid protein recruitment, potentially by altering the folding of hU3-55k to provide binding sites for the factors required in A' pre-rRNA processing, or to alter how the U3 snoRNP is associated with the pre-rRNA, potentially exposing the residues to be cleaved.

In yeast, it is known that the UTP-C complex contains the proteins RRP7, UTP22 and the four subunits of casein kinase 2; CKA1, CKA2, CKB1, CKB2 (Krogan et al., 2004). It has also been shown in yeast that the UTP-C complex associates with the pre-rRNA at a similar time point to the U3 snoRNP, after tUTP association (Krogan et al., 2004; Perez-Fernandez et al., 2007). In human cells, we demonstrate that RRP7 is also an SSU processome component. Moreover, it displays a bias for association with pre-rRNA prior to the A' cleavage event. This is

the only protein of the UTP-C complex thought to contain an RNA-binding motif, suggesting that it may act to recruit UTP22 (known in yeast to contain a series of phosphoserine / threonine residues (Albuquerque et al., 2008; Chi et al., 2007)) and the four CK2 subunits to the pre-rRNA. The data therefore, suggests that the UTP-C complex is recruited to the pre-rRNA prior to the A' cleavage. This may allow the CK2 components to phosphorylate hU3-55k which in turn facilitates the cleavage event by an unidentified endonuclease. It should however, be noted that whilst these proteins are conserved in yeast, there is no A' cleavage event. Nonetheless, both human and yeast pre-rRNAs possess an A0 cleavage site (Rouquette et al., 2005). It may therefore be possible that A0 cleavage is also facilitated by the phosphorylation of hU3-55k, although this is yet to be examined.

Presumably of equal importance in controlling ribosome biogenesis are the phosphatase(s) responsible for de-phosphorylation of hU3-55k. Both serine / threonine specific phosphatases and dual specificity phosphatases are capable of targeting phosphoserine residues (Camps et al., 2000; Mumby and Walter, 1993), with these phosphatases crucial in regulating cell growth and division (Camps et al., 2000; Mumby and Walter, 1993). It is however, unclear which may be responsible for de-phosphorylating hU3-55k, or when this occurs.

We also examined the role hU3-55k may play within the U3 snoRNP monoparticle. It has previously been demonstrated in yeast that U3 snoRNA accumulation is not dependent on the hU3-55k ortholog Rrp9 (Venema et al., 2000). However, we observed a significant difference in the C' motif between that of humans and yeast. Humans, and indeed most vertebrates, display a "weak" C' box motif which differs from the consensus sequence observed in lower eukaryotes, and from C boxes of all other box C/D snoRNAs. We demonstrate that this sub-optimal C' motif prevents NOP58 association in the absence of hU3-55k, therefore allowing hU3-55k levels to directly modulate U3 snoRNA accumulation. We also demonstrate *in vitro* that hU3-55k is capable of directly binding NOP56, NOP58 and the biogenesis factor NOP17. This is in agreement with previous data indicating that hU3-55k associates early in the biogenesis of the U3 snoRNP (Lukowiak et al., 2000;

Watkins et al., 2004). Collectively, this demonstrates that hU3-55k is a key U3 snoRNP biogenesis factor which allows U3 snoRNP levels to be modulated independently of other snoRNPs.

To investigate if this regulation was used *in vivo*, we examined the differentiation of adenocarcinoma cell lines CaCo-2 and CaLu-3. Upon differentiation, the CaCo-2 cells lose their tumourigenic phenotype, so represent a useful tool for examining tumour progression (Stierum, 2003). Moreover, proliferation and differentiation are mutually exclusive events, therefore many malignancies appear as immature cells (Heath et al., 2000), *i.e.* undifferentiated cells. This would suggest, in agreement with many studies, that ribosome biogenesis may be up-regulated during tumourogenesis, as cell cycle progression is typically dependent upon cell growth. This in turn is dependent on ribosome biogenesis (Jorgensen et al., 2004; Rudra and Warner, 2004), with the two processes coordinately controlled (Neufeld et al., 1998; Neufeld and Edgar, 1998; Oskarsson and Trumpp, 2005).

This appears to be the case upon epithelial cell differentiation, as shown in CaCo-2 and CaLu-3 cells, where we show U3 snoRNA levels to be specifically down-regulated whilst all other RNAs examined remained constant, relative to total cellular RNA. In agreement with our model, hU3-55k levels were also observed to decrease, independently of the core Box C/D snoRNP proteins. This suggests that hU3-55k is down-regulated upon cellular differentiation in order to limit the cellular content of U3 snoRNP, most likely to regulate pre-rRNA processing. In support of this, the expression of many SSU processome components was also down regulated upon differentiation, indicating that ribosome biogenesis is not only controlled at the level of pre-rRNA transcription but also at the level of pre-rRNA processing. This may be important not only in cellular differentiation, but also in tumourogenesis.

Although it is not clear what controls hU3-55k levels within the cells, signaling pathways such as Ras/PKA and TOR (Guertin et al., 2006; Jorgensen et al., 2004; Soulard et al., 2009; Yang et al., 2008), c-Myc (Dai and Lu, 2008; Li et al., 2010;

Shiue et al., 2009; Teleman et al., 2008), p53, pRB and ARF (Larminie et al., 1998; Oskarsson and Trumpp, 2005; Sugimoto et al., 2003; White, 1997; White, 2005) all appear to be key regulators in both ribosome biogenesis and cell cycle control in eukaryotes and may potentially regulate expression of the protein.

It has been well documented that rDNA transcription is closely regulated throughout the cell cycle and differentiation (Arabi et al., 2005; Grandori et al., 2005; Poortinga et al., 2004) although it is believed that ribosome biogenesis is regulated at both the level of rRNA transcription and rRNA processing (Schlosser, 2003; Schlosser et al., 2005). This may be illustrated by the murine homolog of the SSU processome protein ESF2 (mABT1). In *S. cerevisiae*, ESF2 has been demonstrated to be an SSU processome component (Granneman et al., 2006b; Hoang et al., 2005). In mice however, it has been shown to associate with a component of the transcription factor TFIID complex; responsible for binding the TATA box and associating with factors such as c-Fos, c-Myc and p53 (Oda et al., 2000). Moreover, expression of the protein was observed to promote basal transcription levels, suggesting it may aid cellular growth (Oda et al., 2000). Unfortunately, due to time constraints we were unable to examine ESF2 levels in differentiated cells or the effect of ESF2 over expression. However, this is something we would hope to do in the near future.

In Burkitt's lymphoma derived cells, driven by c-Myc activation, processing of the 47S rRNA precursor but not pre-rRNA transcription, is dependent on the presence of c-Myc (Schlosser et al., 2005) which has been shown to control mRNA levels of pre-rRNA processing factors (Boon et al., 2001; Coller et al., 2000; Schlosser, 2003). Similarly, during myoblast differentiation (Bowman and Emerson, 1977) and the regeneration of rat livers, ribosome biogenesis is also controlled post-transcriptionally (Dudov and Dabeva, 1983). However, Adele Traynor (personal communication) has demonstrated that c-Myc levels are not altered upon the differentiation of either CaCo-2 or CaLu-3 cells. Furthermore, siRNA depletion of the protein resulted in decreased levels of many SSU processome and snoRNP proteins. This indicates that c-Myc regulation alone may not be responsible for

modulating expression levels of the specific sub-set of SSU processome proteins we observed to be down-regulated upon epithelial cell differentiation. Interestingly, the Myc co-factor Max (Myc-associated factor X), was observed to be downregulated in differentiated epithelial cells, suggesting that it may either be co-regulated or that it may itself act as the regulator for expression of the processing factors. Indeed, Max is known to be a member of the basic helix-loop-helix leucine zipper family of transcription factors and is capable of forming both homo- and hetero-dimers with a range of proteins, believed to modulate the specificity and activity of the transcription factor (Grandori et al., 2000).

Together with our data, it would therefore appear that not only pre-rRNA transcription is down-regulated upon differentiation and up-regulated upon tumourogenesis, but so too is the machinery required for pre-rRNA processing. This is intriguing as these proteins may offer novel therapeutic targets. Indeed, it has been demonstrated that it is possible to force malignant cancer cells to differentiate by treating them with transcription factor agonists (such as those to peroxisome proliferator-activated receptors (PPAR)), causing a reversal of the malignant phenotype (Mueller et al., 1998; Mueller et al., 2000; Sarraf et al., 1998; Spira and Carducci, 2003; Tontonoz et al., 1997). Thus, “differentiation therapy” may offer a novel approach to controlling previously difficult to treat cancers (Beug, 2009; Spira and Carducci, 2003; Waxman, 2000).

6.3 The Human SSU Processome Contains Orthologous Components to those Found in *S. cerevisiae*

Research in *S. cerevisiae* has identified a host of SSU processome components, although many of their roles are unknown. Furthermore, there is a severe lack of information in the literature regarding which proteins are responsible for binding and modifying the rRNA. This is surprising considering the processing complex is so RNA-rich. It should also be stressed that this work was started before UV cross-linking and cDNA analysis (CRAC) data was available from yeast (Granneman et al., 2009; Granneman et al., 2010), meaning that the binding sites for almost all of

the SSU processome proteins on the rRNA were unknown. Nonetheless, only a few protein binding sites have been identified in this manner and there is still much to learn about the SSU processome proteins' rRNA binding / modifying capabilities, and their roles in pre-rRNA processing, particularly in humans.

We therefore set about making a range of tools to enable the study of potentially orthologous factors in human cells. We successfully expressed and purified a range of proteins and produced polyclonal antibodies capable of specifically detecting the proteins of interest. A range of tagged, inducible stable cell lines were also created. Together with other tools, we demonstrate that many of the components found in the yeast SSU processome are also present in the human complex. Furthermore, they appear to be largely orthologous to their yeast counterparts and are capable of U3 snoRNA and pre-rRNA associations.

Interestingly, the UTP-C protein, RRP7, and the cyclase-like protein, RCL1, displayed preferential association prior to the A' cleavage of pre-rRNA. This suggests that they may function early in the processing pathway and dissociate from the SSU processome complex shortly afterwards. This is consistent with data from yeast regarding UTP-C association early in processome formation (Perez-Fernandez et al., 2007) and with our hypothesis that the UTP-C complex may phosphorylate hU3-55k, thus promoting the A' cleavage event.

In yeast, RCL1 associates with BMS1, which contains a G-domain, believed to act as a molecular switch with SOF1 and DHR1 to initiate pre-rRNA cleavages (Billy et al., 2000; Wegierski et al., 2001). It has been proposed that the BMS1/RCL1 sub-complex associates with pre-ribosomes via the U3 snoRNP, with RCL1 thought to be deposited on the pre-ribosome whilst BMS1-GDP dissociates (Karbstein and Doudna, 2006; Karbstein et al., 2005). This model is however, speculative and based upon *in vitro* observations.

In contrast to this, BMS1 is known to associate with pre-rRNA mainly after the initial cleavage in human cells (Turner et al., 2009) whereas our data shows RCL1

association mainly prior to the cleavage event. This may therefore suggest that RCL1 associates first, potentially aiding BMS1 association. Subsequent BMS1 GTPase activity may then cause the dissociation of RCL1 from the SSU processome complex after the cleavage event. This would then place RCL1 as a potential pre-rRNA binding protein to enable to association of the RCL1/BMS1 sub-complex. Unfortunately, due to time constraints I was unable to utilise the purified proteins to perform direct protein-protein and protein-RNA interaction studies *in vitro* although this is something we would hope to complete in the near future.

6.4 The Human SSU Processome and 3' 18S rRNA Processing

NOB1 is the only endonuclease so far demonstrated to cleave pre-18S rRNA, with the protein being responsible for the cytoplasmic cleavage at site D in yeast (Lamanna and Karbstein, 2009; Pertschy et al., 2009). Some reports state that the protein is observed both in the nucleus and nucleolus (Fatica et al., 2003; Pertschy et al., 2009), suggesting that the protein associates early in the processing pathway and that its activity is suppressed until it reaches the cytoplasm. However, others report that NOB1 is mainly associated with later-stage, cytoplasmic pre-40S particles (Schafer et al., 2003). Prior to this cleavage step in yeast, DIM1 di-methylates two adenosine residues towards the 3' end of the 18S pre-rRNA, with both PNO1 and DIM1 required for this event (Lafontaine et al., 1994; Vanrobays et al., 2004). In yeast this occurs cytoplasmically (Brand et al., 1977; Lafontaine et al., 1998b) although the protein has been found to localise to both the nucleolus and cytoplasm (Lafontaine et al., 1998b; Schafer et al., 2003). Accordingly, DIM1 has been found to associate with both early and intermediate 40S pre-ribosomes, indicating that in yeast, DIM1 plays a role in both early and late 18S rRNA processing (Lafontaine et al., 1998b; Schafer et al., 2003).

In yeast, the PNO1 protein may act as a NES-adapter protein as it is one of few SSU processome proteins known to contain an NES motif and is observed to behave similarly to another potential CRM1 adapter protein LTV1; shuttling between the nucleolus and cytoplasm (Seiser et al., 2006; Vanrobays et al., 2008).

Interestingly, this appears to occur under the control of the TOR signaling pathway (Vanrobays et al., 2004; Vanrobays et al., 2008). Moreover, the protein is thought to directly bind the pre-rRNA through its conserved KH domain (Vanrobays et al., 2008) with the protein required for pre-40S intranuclear transport and nucleocytoplasmic export (Vanrobays et al., 2008). In addition, NOB1 and PNO1 have been observed together in an RNA-free module separate to DIM1, RIO2, ENP1, LTV1 and KRR1 (Merl et al., 2010). Collectively, this suggests that in *S. cerevisiae*, PNO1 may directly bind both the pre-rRNA and NOB1 to facilitate CRM1 mediated export from the nucleus.

We demonstrate that, in contrast to yeast, DIM1 mainly associates with pre-rRNA prior to the initial pre-rRNA cleavage step, with the protein not detected in the cytoplasm. Moreover, we have preliminary evidence that the DIM1 methylation event occurs on the pre-rRNA prior to 18S-E formation (data not shown; performed by Nick Watkins), indicating that this occurs prior to cytoplasmic export in human cells. In contrast, PNO1 was observed to associate mainly after the initial cleavage with evidence suggesting it may shuttle between the nucleolus and cytoplasm; playing a role in pre-ribosomal export as in yeast. In comparison, NOB1 appeared similar to previous observations in both yeast and humans, localising to the cytoplasm in a U3 snoRNA-free complex (Rouquette et al., 2005; Schafer et al., 2003; Zemp et al., 2009).

In many instances, NOB1 and PNO1 were observed to co-localise, were found in similarly sized complexes and maintained their association with one another, suggesting that they are capable of directly interacting. In contrast, PNO1-DIM1 interactions were dependent on pre-rRNA transcription, indicating that they may only associate via the rRNA precursor. Indeed, this has recently been demonstrated *in vitro* that PNO1 and NOB1 are capable of directly interacting, whereas DIM1 does not significantly bind either protein (Katherine Sloan; personal communication). Furthermore, NOB1, PNO1 and DIM1 were all capable of directly binding the pre-rRNA within the 3' end of 18S rRNA sequence (Katherine Sloan; personal communication). This is in agreement with recent crosslinking data from

yeast for DIM1 and NOB1, indicating sites of direct binding within the same region of pre-rRNA (Granneman et al., 2010). This suggests conservation of rRNA binding motifs for these proteins between yeast and humans.

We also demonstrate that both PNO1 and, to a lesser extent NOB1, rely upon CRM1-mediated export for translocation to the cytoplasm and site 3 cleavage (orthologous to site D in yeast). Interestingly, inhibition of this pathway also prevented upstream pre-rRNA processing, as previously reported (Rouquette et al., 2005), including a block in A0 processing. This further demonstrates that this site also exists in human pre-rRNA. The accumulation of other pre-rRNA precursors suggests that there may be some form of feedback mechanism in place. It is possible to envisage that due to the lack of export and site 3 processing, crucial rRNA binding factors such as PNO1 accumulate on partially processed precursors such as 18S-E and so are unable to be recycled. Therefore, they are unable to associate with nascent pre-rRNA transcripts, thus the occurrence of larger aberrant precursors is observed.

We believe our data indicates that DIM1 may associate with the pre-rRNA at an early stage, independently of PNO1 (Figure 6.1). Furthermore, it may well methylate the pre-18S rRNA prior to, or around the time of the A' cleavage event. It is then likely that PNO1 associates followed by DIM1 dissociation. It is said KRR1 (another KH-domain containing protein) may be a common ancestor of PNO1, representing a gene duplication event (Vanrobays et al., 2004). Furthermore, it has been suggested that other such couples of related, non-redundant processing factors display a possible handover of pre-ribosomes from one partner to the next (Vanrobays et al., 2004) *e.g.*, BMS1 and TSR1, and RIO1 and RIO2 (Gelperin et al., 2001; Vanrobays et al., 2003; Vanrobays et al., 2001; Wegierski et al., 2001). In agreement with this, we observed KRR1 to associate with the pre-rRNA both before and after the initial cleavage event, suggesting it may act as a primary rRNA binding protein prior to PNO1. The PNO1 protein may then act to provide the NES required for the pre-40S complex to be exported in a CRM1 dependent manner, directly binding both the pre-rRNA and NOB1 to facilitate their translocation to the

cytoplasm. It should be noted that RIO2 also contains an NES and is capable of CRM1 association, as is LTV1. However, neither are thought to be essential for pre-40S export (Zemp and Kutay, 2007; Zemp et al., 2009). In contrast, PNO1 appears to be crucial, as upon depletion of the protein in yeast, accumulation of a pre-rRNA product (A_0-A_3) is reported (Peng et al., 2003), similar to the 26S (A0-2) pre-rRNA species observed by ourselves and others in human cells after blocking CRM1-mediated export (Rouquette et al., 2005). The pre-rRNA is then most likely cleaved by NOB1, as in yeast (Pertschy et al., 2009). Evidence from other groups suggests that the kinase RIO2 is then essential for the release and recycling of ENP1, PNO1, LTV1, and NOB1 as well as for 18S-E pre-rRNA processing (Zemp et al., 2009).

It may be questioned as to why NOB1 is cycled between the nucleus and cytoplasm, if its site of action is in the cytoplasm. It is possible this acts as a form of regulation over the cleavage of the pre-rRNA. Interestingly, we demonstrate that of all the SSU processome components examined, only PNO1 and NOB1 were affected by treatment with the mTOR inhibitor rapamycin; causing nuclear accumulation of the proteins. This is in agreement with data from yeast whereby more than 40 SSU processome components were examined, with pronounced mis-localisation only observed for PNO1 ((Vanrobays et al., 2008) and personal communication with Dr. Lafontaine). This indicates that PNO1 shuttling may be under the control of the mTORC1 pathway. TOR / mTOR has been shown to also control various steps of ribosome biogenesis in both yeast and humans, including the transcription of rRNA and ribosomal proteins, and processing of the 35S rRNA precursor (Powers and Walter, 1999; Rohde et al., 2001; Xiao and Grove, 2009). This may suggest that the affects we observed may be indirect, although no nucleolar disruption was observed and the effects appeared specific. Unfortunately, due to time constraints we were not able to examine this further. It is, nonetheless, possible that humans may control ribosome biogenesis either directly or indirectly through the mTORC1 complex. Therefore, one potential method for late-stage regulation may be to regulate pre-rRNA export to the cytoplasm, via a CRM1-mediated pathway, utilising PNO1 as the export factor.

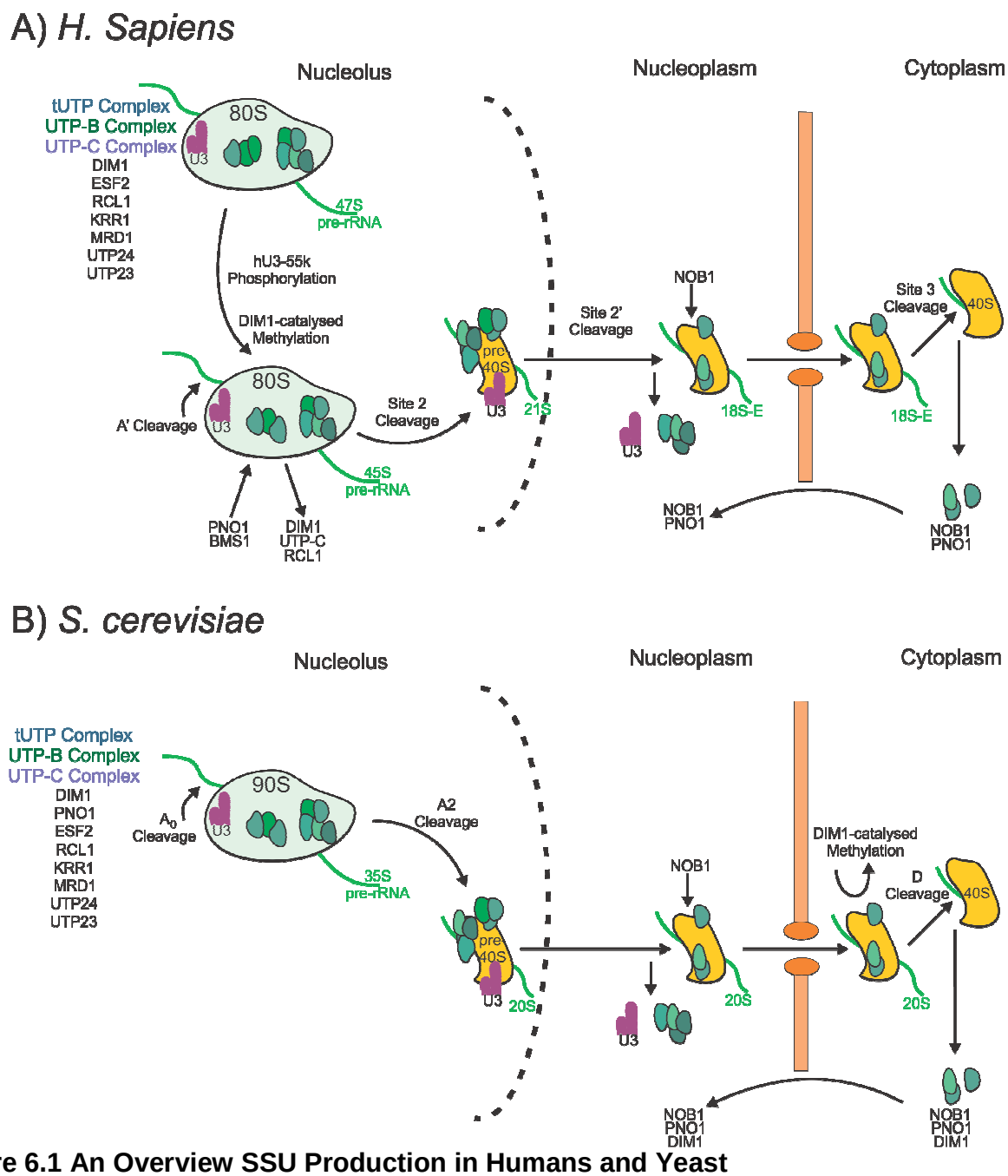


Figure 6.1 An Overview SSU Production in Humans and Yeast

A) Human 47S pre-rRNA (green) is incorporated into the 80S SSU processome, containing the U3 snoRNP (purple) with a host of processing factors, with relevant factors indicated adjacent to the complex. hU3-55k is phosphorylated which facilitates the A' cleavage event. DIM1-catalysed methylation may occur around a similar time. Subsequently, PNO1 associates and site 2 cleavage separates the 40S and 60S precursors and the pre-40S complex is exported to the nucleus where U3 snoRNP is believed to dissociate. A 21S pre-rRNA is subsequently processed to 18S-E around which time NOB1 is thought to associate into the complex prior to cytoplasmic export. This is mediated at least in part, by the NES-containing PNO1 and the export factor CRM1. The 18S-E pre-rRNA is subsequently cleaved to the mature form and PNO1 and NOB1 are recycled to the nucleus. **B)** The yeast 35S pre-rRNA undergoes much the same processing as in human cells. Notable differences however, are that DIM1 and PNO1 both associate early as part of the 90S SSU processome complex, with DIM1-catalysed methylation occurring cytoplasmically. Furthermore, the nuclear 18S pre-rRNA (20S) is not cleaved until the cytoplasm, indicating an additional step in human pre-rRNA processing. Some cleavage steps not shown for simplicity, model based on the data and previous work discussed in this thesis.

6.5 Future Work

6.5.1 hU3-55k

Whilst we have shown that hU3-55k is capable of modulating U3 snoRNA levels, and most likely plays a key role in regulating U3 snoRNA accumulation during cellular differentiation, it is not clear what is responsible for regulating hU3-55k levels. We would like to examine the mRNA levels of hU3-55k and other snoRNP proteins in differentiated and undifferentiated cells to determine if transcription or translation of the gene is regulated. We also aim to investigate if hU3-55k overexpression affects the protein levels of other SSU processome components. Potentially of more importance would be to demonstrate a link between hU3-55k levels and those of a potential regulator such as c-Fos, c-Myc, pRB, p53 or ARF although as with many regulators, post-transcriptional modification and protein-protein interactions may be as important as their expression levels. Moreover, such regulators are often crucial to a multitude of cellular processes (Oskarsson and Trumpp, 2005), making them difficult to directly study. In the longer term, it would be interesting to examine the effects of overexpression and depletion of hU3-55k, and potentially other SSU processome components such as ESF2, with respect to cellular differentiation and proliferation. Moreover, we would like to demonstrate further functional relevance of our findings by examining the potential for specific hU3-55k and U3 snoRNA expression differences between pre-cancerous and cancerous cells.

We have also demonstrated that phosphorylation is most likely important in pre-rRNA processing. However, we would like to demonstrate this more directly by examining if either overexpression of unphosphorylatable hU3-55k, or depletion of the endogenous protein with expression of unphosphorylatable hU3-55k, blocks pre-rRNA processing at the A' site, thus causing an accumulation of 47S pre-rRNA. Furthermore, we aim to examine if the hU3-55k phospho-mimic and unphosphorylatable proteins associate with different forms of the SSU processome. For example, it may be envisaged that the unphosphorylatable form

of the protein preferentially associates with early processing factors such as the tUTP and UTP-C proteins whereas, the constitutively phosphorylated mimic may associate with later processing factors such as BMS1. In the longer term, it would be interesting to investigate the effects of various kinase / phosphatase inhibitors to discern which proteins modulate the post-translational modifications of hU3-55k. Unfortunately however, due to the importance and wide-ranging role of many kinases / phosphatases, it may not be possible to discern specific effects relating to hU3-55k phosphorylation. Nonetheless, an *in vitro* pre-rRNA processing assay in the presence and absence of kinase inhibitors may at least indicate which kinases are important in the process. Furthermore, we would also like to examine the phosphorylation of hU3-55k in differentiated and undifferentiated cells, as it may be expected that this may be one of potentially many ways used to modulate mature rRNA levels within the cell. As we suspect the UTP-C complex may play an important role however, we may direct our focus towards examining the action of the kinase CK2 and the RNA binding properties of RRP7. It should be noted though, that depletion of RRP7 may not be possible, as in yeast it is essential for cellular viability (Baudin-Baillieu et al., 1997).

Whilst the yeast ortholog of hU3-55k (Rrp9) is suggested not to be important for U3 snoRNP accumulation, it is known to be required for its function (Venema et al., 2000). Furthermore, the highly charged E-stretch is conserved in yeast, which also contains phosphorylatable serine/threonine residues at the locus equivalent to that phosphorylated in the human protein. Yeast pre-rRNA however, is not known to contain an A' cleavage site. Instead, the primary cleavage occurs at the A_0 site, also known to be present in human pre-rRNA (Rouquette et al., 2005). It is therefore possible, and would be interesting to investigate if phosphorylation also modulates the A_0 cleavage in both yeast and humans.

6.5.2 SSU Processome

Due to time limitations, we were not able to proceed beyond the initial analysis of the SSU processome proteins. We were however able to demonstrate that the

human SSU processome contains many of the same components found in yeast. We would therefore, like to examine the association and dissociation of these factors in more detail both temporally and in terms of their physical interactions. In particular, we aim to utilise the purified proteins to investigate which proteins are capable of directly binding specific rRNA sequences with the hope of setting up an *in vitro* processing system to identify potential pre-rRNA endonucleases, similar to that used in yeast (Pertschy et al., 2009). Moreover, we aim to utilise a vector containing a pre-rRNA sequence to demonstrate rRNA binding by our FLAG-tagged SSU processome proteins *in vivo*. This is important as the rRNA binding capability of many of the SSU processome proteins is unknown, particularly in human cells. Furthermore, various factors must be responsible for the recruitment of protein sub-complexes to the pre-rRNA, with these factors most likely playing an important role in the efficiency of each processing step. In the longer term, we would like to demonstrate what it is that recruits the U3 snoRNP to the pre-rRNA. It has been hypothesised that tUTP4 may play a role in this (Prieto and McStay, 2007), although this is yet to be demonstrated experimentally. RNAi depletion of the various pre-rRNA binding proteins may stall pre-rRNA processing at defined points. Analysis of these stalled complexes would provide information about the requirement of each factor for both specific processing steps and subsequent protein recruitment. Furthermore, nuclease protection assays may allow us to determine around which processing step each protein associates / dissociates.

6.5.3 Processing at the 3' end of 18S rRNA

The processing factors DIM1, PNO1 and NOB1 have been demonstrated to function at different, but sequentially overlapping points of the pre-rRNA processing pathway in human cells. Interestingly, it appears that DIM1-catalysed methylation occurs prior to nucleocytoplasmic export, associating with pre-rRNA before PNO1. This is in contrast to yeast whereby PNO1 is required for DIM1-catalysed cytoplasmic methylation of pre-rRNA (Vanrobays et al., 2004). We would therefore like to verify the localisation of the proteins and methylated pre-rRNA in human cells by performing cellular dissection assays. Moreover, it would be interesting to

examine if PNO1 is required for DIM1-mediated methylation in human cells as it is in yeast. KRR1 is said to be a PNO1 paralog. We have demonstrated that it associates with pre-rRNA prior to PNO1 although if this is a direct interaction is not yet known. We would therefore like to investigate the direct rRNA-binding ability of KRR1 *in vitro*. Similarly, examining its potential for protein-protein interactions, such as with DIM1, may provide further data for a model whereby KRR1 passes the pre-rRNA complex to PNO1, as suggested for other paralogous processing proteins (Vanrobays et al., 2004).

We demonstrated that upon blocking pre-rRNA transcription, accumulation of an 18S-E product was observed. This was a novel observation which we believe represents a pre-rRNA bound by NOB1 and PNO1 but unable to be exported for processing to the cytoplasm. We would however, like to demonstrate experimentally that these proteins were bound. We also aim to investigate the endonuclease activity of NOB1 *in vitro* to ascertain that, if in human cells, as in yeast, it is the site 3 endonuclease which cleaves 18S-E.

In the longer term, we would like to further investigate the role of PNO1 in mediating pre-40S export to the cytoplasm with the aim of better defining its role as an NES-adapter protein. It would be interesting to also investigate the role of the mTORC1 complex in regulating PNO1, pre-40S export and pre-rRNA processing. It may be possible to further dissect this by RNAi depletion of downstream effectors in the mTORC1 pathway in order to more precisely block PNO1 export, reducing the possibility of indirect affects influencing the observations.

6.6 Conclusions

The objective of this study was to gain a better understanding of the human SSU processome, with respect to the U3 snoRNP, hU3-55k and proteins involved in binding and modifying the pre-rRNA. In particular, we aimed to understand how U3 snoRNA levels were controlled and the importance of the U3-specific hU3-55k protein. We also wanted to find out if proteins present in the yeast SSU

processome were also components of the human complex, and if any of these were able to directly bind pre-rRNA sequences or catalyse pre-rRNA processing. Lastly, we were interested in the processing of the 3' end of 18S rRNA as an additional processing step is known to be required in humans, compared to its yeast counterpart.

In this study we have demonstrated that, as predicted, hU3-55k is key in defining the U3 snoRNP as different to other Box C/D snoRNPs. It is able to modulate U3 snoRNA levels through aiding the recruitment of core factors to the sub-optimal C' motif, most likely stabilising the complex. We went on to show that this, along with a sub-set of SSU processome proteins, may be important in the differentiation of epithelial cells with potentially interesting implications for tumourogenesis.

Importantly, we demonstrate that the expression of hU3-55k may control ribosome biogenesis by modulating U3 snoRNA levels. We also show however, that hU3-55k may regulate the rate at which the complex processes pre-rRNA, dependent on hU3-55k's phosphorylation state. We show hU3-55k to most likely require phosphorylated N-terminal residues in order to enable the A' cleavage event and therefore, maintain incorporation into an active processome. This demonstrates another level of control exerted by hU3-55k over pre-rRNA processing. We believe that this phosphorylation may be achieved, in part, via the CK2 subunits of UTP-C, although this is yet to be shown experimentally. Furthermore, other phosphorylatable residues may be important in the process, with the regulation of dephosphorylation most likely equally important.

The human SSU processome has been shown to contain many of the potential RNA-binding and processing factors associated with its counterpart in *S. cerevisiae*. Furthermore, RRP7 of the UTP-C complex, and RCL1, show preferential association with uncleaved pre-rRNA, indicating they associate and function within an early SSU processome complex. In contrast, we observed that many of the factors remained after the initial processing step, including the transcriptionally-associated tUTP4, which is in contrast to what has been observed

for tUTP10 (Turner et al., 2009). We were unable however, to perform direct protein - RNA binding, or cleavage assays due to time constraints, although the tools are in place to do so.

We were however, able to demonstrate that DIM1 acts at a different point in the pre-rRNA processing pathway to what has previously been observed in *S. cerevisiae*. Our data indicate that DIM1-directed methylation occurs prior to pre-rRNA export, potentially occurring around the time of the A' cleavage event (Figure 6.1). Moreover, in the light of recent data (personal communication with Katherine Sloan), we believe that DIM1 directly binds the pre-rRNA prior to PNO1, in contrast to the believed mechanism in yeast, of PNO1-mediated DIM1 recruitment. Our data also suggest that PNO1 association may aid transit of NOB1, and therefore the pre-40S complex, through the nuclear pore complex via CRM1. Furthermore, we postulate that PNO1 may be targeted, either directly or indirectly, by the growth regulatory pathway of mTORC1. This would suggest another potential point of ribosome biogenesis regulation linked to cellular growth, through control of the SSU processome and pre-rRNA processing, although further work is required to confirm this.

This thesis has provided important insights into the human SSU processome, with the demonstration that various points of regulation exist within the processing pathway. We were also able to demonstrate important differences and similarities between the human and yeast SSU processome. Unfortunately, a more in-depth analysis was not possible due to time constraints, although a range of tools have been created to enable this analysis. Furthermore, although more comprehensively studied in yeast, factors responsible for pre-rRNA binding and modification are yet to be fully characterised in any species. Moreover, in humans, various aspects of the SSU processome complex may be regulated during cellular growth and differentiation, potentially through pathways not present in yeast. Therefore, whilst *S. cerevisiae* is a useful model organism, further study of the SSU processome in human cells is clearly warranted.

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Publications and Presentations

Publications

Knox, A.A., Sloan, K. E., Watkins, N.J. (2010) Dynamic recruitment of factors involved in the 3' maturation of human 18S rRNA. (Manuscript in preparation)

Knox, A.A., McKeegan, K.S., Debieux, C.M., Traynor, A., Richardson, H. and Watkins, N.J. (2010) Ribosome biogenesis is controlled by hU3-55K phosphorylation state and U3 snoRNP abundance. (Manuscript in preparation)

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Presentations

Poster presentation: Knox, A.A., McKeegan, K.S., Debieux, C.M., Traynor, A., Richardson, H. and Watkins, N.J. (2010) “Ribosome biogenesis is controlled by hU3-55K phosphorylation state and U3 snoRNP abundance”. 15th Annual Meeting of the RNA Society, Seattle, Washington, USA, June 22-26

Poster presentation: Knox, A.A., Sloan, K. E. and Watkins, N.J. (2010) “The 3' maturation of human 18S rRNA”. 15th Annual Meeting of the RNA Society, Seattle, Washington, USA, June 22-26

Oral presentation: Knox, A.A., McKeegan, K.S., Debieux, C.M., Richardson, H. and Watkins, N.J. (2010) “Specific regulation of U3 snoRNP levels during cellular differentiation and tumourogenesis” RNA UK Conference, Lake District, UK, January 22-24

Poster presentation: Knox, A.A., McKeegan, K.S., Debieux, C.M., Richardson, H. and Watkins, N.J. (2010) “hU3-55k Mediates Common Core Box C/D Protein Recruitment to the U3 snoRNP” 8th International Conference on Ribosome Synthesis, Regensburg, Germany, August 26-30

Awards

Prize for Best Oral Presentation: Knox, A.A., McKeegan, K.S., Debieux, C.M., Richardson, H. and Watkins, N.J. (2010) “Specific regulation of U3 snoRNP levels during cellular differentiation and tumourogenesis” RNA UK Conference, Lake District, UK, January 22-24

Abstracts from Manuscripts in Preparation

Ribosome biogenesis is controlled by hU3-55K phosphorylation state and U3 snoRNP abundance

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The rate of ribosome biogenesis, which dictates protein synthesis capacity in the cell, regulates the growth rate and is linked to the cell's proliferative potential. Ribosome production is down regulated in terminally differentiated cells and up-regulated in most cancers. This is achieved through regulating the transcription and processing of ribosomal RNA (rRNA). Much remains unclear, however, about the regulation of rRNA processing. The U3 box C/D small nucleolar (sno)RNP is involved in the initial recognition of the nascent transcript and essential for 18S rRNA processing. Here we report that U3 snoRNP accumulation and function is regulated through the U3-specific phosphoprotein hU3-55K. We show that U3 snoRNP levels are specifically downregulated during human lung (CaCo-2) and colon (CaLu-3) epithelial cell differentiation and that this is likely mediated post-transcriptionally through regulating hU3-55K levels. Furthermore, we show that hU3-55K is required for U3 snoRNP assembly. CaCo-2 are lung adenocarcinoma epithelial cells that are believed to revert to their pre-cancerous state during differentiation suggesting that U3 snoRNP levels are increased during tumourogenesis. The proto-oncogene c-Myc is believed to control the expression of snoRNP proteins. Surprisingly, we found that c-Myc levels are not reduced in the differentiated epithelial cells suggesting another level of regulation controls U3 snoRNP abundance. Finally, we show that phosphorylation of hU3-55K is essential for U3 snoRNP function, being required for the initial cleavage of the rRNA precursor. We therefore show two independent mechanisms by which U3 snoRNP, and therefore ribosome biogenesis, can be regulated.

Nucleolar disruption leads to the spatial separation of key 18S rRNA processing factors

Amy Jane Turner, Andrew Alexander Knox and Nicholas James Watkins

Stress, such as DNA damage, results in the down-regulation of ribosome production and the disruption of normal nucleolar function. This leads to either cell cycle arrest or apoptosis through p53 stabilisation. It is not clear, however, how stress leads to the dramatic changes in nucleolar structure and function. We have investigated the effect of the nucleolar disruption agents Actinomycin D (ActD), camptothecin (CPT) and 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) on 18S rRNA maturation and small subunit (SSU) assembly. We found that all three agents affected RNA polymerase I transcription, primarily at the level of elongation. ActD-treatment resulted in a complete loss of the SSU processome, the complex responsible for small subunit formation. In contrast, the SSU processome was still present in CPT- and DRB-treated cells although the migration behavior of the complex in glycerol gradients was altered. ActD- and DRB treatment resulted in the majority of U3 small nucleolar RNP (snoRNP) localising separately to other key components of the SSU processome. We propose that nucleolar disruption results in the reduction in the local concentration of key SSU processome components at the active sites of pre-rRNA formation thereby compromising the rate of SSU processome formation and pre-rRNA processing.