UNIVERSITY OF
NEWCASTLE UPON TYNE

THE REGULATION OF CELLULAR SIGNALLING BY THE 5S RNP

Submitted for Doctor of Philosophy

ZHAO ZHAO

September 2018

Supervisor: Dr Nick Watkins

Institute for Cell and Molecular Biosciences

Faculty of Medical Sciences

Newcastle University

Abstract

Ribosome biogenesis is a tightly regulated process that is essential for cell survival. Genetic mutations in genes encoding ribosomal proteins or ribosome biogenesis factors lead to the development of rare genetic disorders, known as ribosomopathies. The 5S RNP is a ribosome assembly intermediate composed of the 5S rRNA, ribosomal protein RPL5 and RPL11. During the 5S RNP assembly, the newly transcribed 5S rRNA is stabilized by RPL5 first, before RPL11 binds. Once it is assembled, the 5S RNP is incorporated into the large ribosomal subunit precursor. Ribosome biogenesis defects, which induce ribosomal stress, cause the accumulation of the 5S RNP. The accumulated non-ribosomal 5S RNP induces tumour suppressor p53, by binding and inhibiting its main negative regulator, MDM2. It has been suggested that any factor involved in the 5S RNP assembly and its integration into the ribosome will affect p53 signalling.

I therefore set out to evaluate factors that have been proposed to be connected to the 5S RNP. Different proteins, including HEXIM1, Mybbp1a, B23, NML and SRSF1, have been suggested to be involved in the 5S RNP-p53 signalling, therefore I aimed to investigate their roles in this signalling pathway. Furthermore, I also aimed to investigate the role of RPL11 phosphorylation in ribosome biogenesis and the 5S RNP-p53 signalling.

In this work, I present evidence supporting the proposed role of splicing factor SRSF1 in 5S RNP assembly. I also demonstrate that SRSF1 could be a part of the 5S RNP complex that is essential for p53 activation during ribosomal stress, while protein HEXIM1, Mybbp1a and NML are not needed for this pathway. I further provide evidence suggesting the threonine 73 phosphorylation in RPL11 could control its binding to the 5S RNP, whereas serine 51 phosphorylation in RPL11 could be important in the 5S RNP incorporation into the large ribosomal subunit. Last but not the least, my results also suggest that serine 140 in RPL11 could be transiently phosphorylated during the ribosomal maturation process.

In conclusion, this work addresses the important role of the 5S RNP, in both ribosome biogenesis and the 5S RNP-p53 signalling.

Declaration

I certify that the work presented here in this PhD thesis is my own, except where acknowledged. There is no part in this work has been previously submitted for any other qualifications at this or any other university.

Acknowledgement

I would like to express my gratitude to my supervisor, Dr. Nick Watkins, for his guidance, patience and endless support. I could also like to thank the rest of the members from the Watkins/Schneider lab for, their support and wonderful cakes – especially Dr. Loren Gibson for her help and training during my beginning in the lab, and Dr. Andria Pelava for her protocols and laughter. I would also like to express my gratitude for my panels, Dr. Ian Cowell and Prof. Zofia Chrzanowska-Lightowlers for their suggestions and kind words during my panel assessment meetings.

Furthermore, I would like to thank all those lovely peoples working on the fourth floor, CBCB. Special thanks to Dr. Richard Daniel and Dr. Ling Juan Wu, for hosting those Christmas parties and lovely foods. I would also like to thank my best friend Tsunho Julian Clement Chan, for his friendship and his post-dinner history expertise.

I am truly grateful to the support came from my parents and all other members from my family. I would finally like to thank Manwai Hazel Chow, for her support and accompany throughout the years.

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Abbreviations

A	Alanine
ActD	Actinomycin D
AKT	Protein Kinase B
AML	Acute Myeloid Leukaemia
Asn	Asparagine
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine triphosphate
C-terminal	Carboxy-terminal
CDK	Cyclin Dependent Kinase
cDNA	complementary DNA
CHK	Checkpoint Kinase
cDNA	Complementary Deoxyribonucleic Acid
D	Aspartic acid
DAPI	4',6-Diamidino-2-Phenylindole
DBA	Diamond-Blackfan
DC	Dyskeratosis
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxy-nucleoside triphosphate
DTT	Dithiothreitol
E.coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
ETS	External Transcribed Spacer
FCS/FBS	Fetal Bovine Serum
FRT	Flippase recognition target
GTP	Guanosine 5'-triphosphate
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid
HRP	Horseradish peroxidase
IF	Immunofluorescence
IP	Immunoprecipitation
ITS	Internal Transcribed Spacer
KCl	Potassium Chloride
kDa	kilo Dalton
LSU	Large ribosomal subunit
M	molar
M. musculus	*Mus musculus*
MDM2	Mouse double minute 2
ml	Millilitre

mM	Milli-molar
mTOR	mammalian target of rapamycin
mTORC	mammalian target of rapamycin complex
N-terminal	Amino-terminal
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NaPO4	Sodium Phosphate
Nut3a	Nutlin-3a
NLS Nuclear	Nuclear Localization Signal
ORF	open reading frame
PAGE	Polyacrylamide gel
PBS	Buffer Saline Phosphate
PCR	Chain Reaction Polymerase
Phe	Phenylanine
Pol	Polymerase
pre-rRNA	ribosomal RNA precursor
R. rattus	*Rattus rattus*
RNA	Ribonucleic acid
RNAi	RNA interference
RNP	Ribonucleoprotein particle
RP	Ribosomal Protein
rRNA	Ribosomal RNA
RT	Reverse Transcription
S-phase	Synthesis phase
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Saccharomyces pombe*
SDS	Sodium Dodecyl Sulphate
siRNA	Small-interfering RNA
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
SRSF1	Serine Argenine Rich Splicing Factor 1
SSU	Small ribosomal subunit
TBE	Tris-Boric Acid EDTA
TC	Treacher Collins
Tet	Tetracycline
TFIIIA	Transcription factor 3A
Thr	Threonine
U.V.	Ultraviolet
X. leavis	*Xenopus leavis*
µg	micro-gram
µl	micro-litre
µM	micro-molar

Chapter 1. Introduction

1.1 The eukaryotic ribosome

The ribosome is the protein synthesis machinery in both prokaryotic and eukaryotic organisms. It translates messenger RNA (mRNA) into polypeptides using amino acids delivered by tRNAs. Even though prokaryotic ribosomes are smaller than those in the eukaryotes, ribosomes across all living organisms are highly conserved in their structures (Moore and Steitz, 2002). A mature eukaryotic ribosome (80S) consists of two subunits, the large ribosomal subunit (60S, LSU) and the small ribosomal subunit (40S, SSU). During protein translation, the SSU associates with the mRNA and scans the sequence for a start codon. After translation initiation, the LSU catalyses condensation reaction joining amino acids to produce polypeptide chains (Gamalinda and Woolford, 2015).

The ribosome is composed of ribosomal proteins (RPs) and ribosomal RNAs (rRNAs). In eukaryotes, the LSU is made up of 46 RPs and 3 rRNAs (the 5S, 5.8S and 28S rRNAs). The SSU consists of about 33 RPs and the 18S rRNAs (Gamalinda and Woolford, 2015). The ribosome is essential for cell survival and the number of ribosomes can dictate the rate of cellular growth.

1.2 Ribosome biogenesis

It is estimated that there are about five to ten million ribosomes in each mammalian cell, which are needed to be synthesized during each round of cell division in an actively proliferating mammalian cell (Kenmochi *et al.*, 1998). Because of their large number and the crucial role ribosomes play in the cell survival, ribosome biogenesis not only consumes substantial amounts of cellular energy, but it is also tightly regulated. For example in yeast, approximately 60% of the cellular energy is used in ribosome production (Warner, 1999). Furthermore in a growing yeast cell, there are about 2,000 ribosomes produced every minute which takes up 60% of the total cellular transcription event (Freed *et al.*, 2010).

RPs are produced in the cytoplasm whereas the rRNAs are transcribed in the nucleolus, and most of the ribosome assembly process takes place in the nucleolus (Kressler *et al.*, 2010). There are more than 200 protein co-factors involved in rRNA processing and ribosomal

subunit precursor maturation (Henras *et al.*, 2008). Ribosome production is upregulated during cell growth, whereas it is supressed during cell differentiation (Warner, 1999; Sulic *et al.*, 2005). In addition, ribosome biogenesis is up regulated during tumourigenesis, and ribosome production defects have been linked to human diseases (Freed *et al.*, 2010).

1.2.1 The nucleolus

Ribosome biogenesis largely takes place in a specific compartment in the nucleus called the nucleolus. Using electron-microscopy (EM) it has been revealed that there are three distinct regions recognised in the nucleolus, namely the fibrillarin centre (FC), the dense fibrillarin component (DFC) and the granular component (GC) (Boisvert *et al.*, 2007; Smirnov *et al.*, 2016). In humans, the formation of the nucleolus requires the nucleolus organizer regions (NORs), chromosomal regions from the short arms of the acrocentric chromosomes (chromosomes 13, 14, 15, 21 and 22) (Kenmochi *et al.*, 1998). NORs contain rDNA genes in the form of tandem repeats and the nucleolus is formed around these NORs (McStay, 2016). rDNA is transcribed by RNA polymerase I (Pol I) into the 47S ribosomal RNA precursor (pre-rRNA), which contains the sequences of 18S, 5.8S and 28S rRNAs (Cheutin *et al.*, 2002). This process takes place at the border which separates the FC and DFC and GC (Boisvert *et al.*, 2007). The first 47S pre-rRNA cleavage step takes place in the DFC (Derenzini *et al.*, 1989), whereas the later rRNA processing events are found happening in the GC, where RPs incorporate to the rRNAs (Gerbi and Borovjagin, 1997; Boisvert *et al.*, 2007).

1.2.2 rDNA transcription and pre-rRNA production

In yeast, the rDNA gene is flanked by an upstream promoter region and a downstream terminator sequence. In humans additional to the promotor, the upstream region of rDNA also contains an upstream control element while multiple termination sequences are located downstream to the rDNA (Russell and Zomerdijk, 2005).

In humans, the upstream binding factor (UBF) binds to the upstream control element of the rDNA gene. UBF subsequently recruits the human selectivity factor complex (SL1) to the promotor region (Tuan *et al.*, 1999). The SL1 complex is composed of a TATA-binding protein (TBP) and three TBP-associated factors (Grummt, 1999). The SL1 complex recruits RNA Pol I to the promotor region (Russell and Zomerdijk, 2005). UBF, SL1 and RNA Pol I form the transcription preinitiation complex, opening the promotor region and allowing rDNA

transcription by RNA Pol I. UBF and SL1 remain on the promotor region in order to recruit multiple RNA Pol I to achieve several rounds of transcription (Panov *et al.*, 2001; Russell and Zomerdijk, 2005). Once RNA Pol I migrates to the termination sequence, it is stopped by the transcription termination factor 1 (TTF-1) which binds on the termination sequence and it is released from the gene by the polymerase I and transcription release factor (PTRF) which is associated with the TTF-1 (Jansa and Grummt, 1999; Russell and Zomerdijk, 2005).

The 5S rRNA is transcribed by RNA Pol III separately in the nucleoplasm (Paule and White, 2000). In human cells, the gene clusters coding for 5S rRNA are located on chromosome 1, with a 5'-end flanking region which is important for the RNA Pol III-mediated transcription process (Sørensen and Frederiksen, 1991).

In some eukaryotes, the 5'-end flanking region consists of several elements, such as A-box at the 5'-end, the intermediate element (IE) and the C-box at the 3'-end (Cloix *et al.*, 2003). This type of upstream promoter region is found in organism such as *S. cerevisiae*, *D. melanogaster* and *X. laevis* (Pieler *et al.*, 1987; Sharp and Garcia, 1988; Lee *et al.*, 1995). RNA Pol III binds to the upstream promoter region with three transcription initiation factors (TF): TFIIIA, TFIIIB and TFIIIC, with TFIIIA regulating 5S rRNA transcription specifically (Pelham and Brown, 1980).

In higher eukaryotes, including humans and mice, the promotor region of the 5S rRNA includes an additional upstream element called the D-box (Hallenberg and Frederiksen, 2001). The D-box is important for the efficient transcription of 5S rRNA. During transcription initiation, TFIIIA first associates with the C-box and subsequently recruits TFIIIC. The binding of TFIIIA and TFIIIC leads to the assembly of TFIIIB and RNA Pol III to the promoter region (Ishiguro *et al.*, 2002). After transcription is initiated, TFIIIC dissociates from the promoter while TFIIIB remains bound to the promotor region, allowing multiple rounds of transcription initiation to happen (Kassavetis *et al.*, 1998; Kumar *et al.*, 1998).

1.2.3 rRNA processing in yeast and humans

Pre-rRNA contains the internal transcribed spacers (ITS1 and ITS2) and the external transcribed spacers (5'-ETS and 3'-ETS), which are removed through a series of endonucleolytic and exonucleolytic cleavage steps. It eventually produces mature 18S rRNA

in the SSU as well as 5.8S and 28S rRNA (25S in yeast) in the LSU. Our current understanding of the rRNA processing pathways is largely based on studies in yeast.

The pre-rRNA maturation starts with the production of pre-rRNA containing the 18S rRNA sequence, which involves in the endonucleolytic processing of the 5'-ETS and ITS1. In yeast, either full-length 35S pre-rRNA is transcribed or, alternatively, co-transcriptional cleavage occurs at the A_0, A_1 and A_2 sites producing the 27S-A_2 pre-rRNA (containing sequences of the 5.8S and 25S) and 20S pre-rRNA (containing sequences of the 18S).

Although the mechanisms behind these cleavage events are not yet fully clear, it is thought that they involve the formation of the SSU processome complex (Phipps *et al.*, 2011) and also a number of enzymes and protein cofactors such as different ribosomal proteins (O'Donohue *et al.*, 2010), snoRNAs (Fayet-Lebaron *et al.*, 2009), nucleolar ribonucleoprotein U14 (Henras *et al.*, 2015) and U3 (Dragon *et al.*, 2002).

Cleavage at the A_2 site located in the ITS1 results in formation of the 20S and 27S-A_2 pre-rRNAs (Henras *et al.*, 2015), and it also leads to the separation of pre-SSU and pre-LSU (Kressler *et al.*, 1999). Furthermore, recent studies also revealed that the endonuclease Utp24 cleaves at the A_2 cleavage site (Wells *et al.*, 2016). After the 5'-ETS removal and A_2 cleavage in ITS1, the 20S pre-SSU rRNA is subsequently exported from the nucleus to the cytoplasm, where it undergoes cleavage at site D to produce the mature 18S rRNA. The endonuclease Nob1p (Pertschy *et al.*, 2009) is required during this D site cleavage process (Kressler *et al.*, 1999).

There are two pathways in the pre-LSU rRNA maturation process which produce alternative versions of the 5.8S rRNA 5' end. In the first pathway, the 27S-A_2 pre-rRNA undergoes cleavage at site $B1_L$, producing a long form of the 5.8S rRNA. In the second pathway, A_3 cleavage takes place in the 27S-A_2 pre-rRNA by the MRP RNase (Lygerou *et al.*, 1996), producing the 27S-A_3 pre-rRNA. A number of proteins are important in the A_3 site cleavage process, such as RNA-binding protein Rrp5p (Venema and Tollervey, 1996) and the nucleolar protein Nop4p (Sun and Woolford, 1994). The 27S-A_3 pre-rRNA is subsequently processed exonucleolyticlly by proteins Rat1p and Rrp17p, producing the short form of the 5.8S rRNA.

Both long and short forms of the 5.8S-sequence-containing 27S pre-LSU rRNA are subsequently cleaved at the C2 site located in the ITS2, producing the 26S (containing the

25S rRNA) and 7S (containing the 5.8S rRNA) pre-rRNAs. Recent work showed that the endonuclease Las1 preforms this cleavage process (Gasse *et al.*, 2015).

The 26S LSU pre-rRNA maturation takes place in the nucleus which involves the 5' end exonucleolytic cleavage mediated by the Rai1p protein (El Hage *et al.*, 2008). It produces the mature 25S rRNA which is then exported to the cytoplasm.

The 7S LSU pre-rRNA undergoes the 3' end exonucleolytic processing in the nucleus, which is mediated by the exonuclease Rrp6p (Briggs *et al.*, 1998) and the RNA helicase Mtr4p (Jia *et al.*, 2012), producing the 6S LSU pre-rRNA. It is subsequently transported to the cytoplasm where the final step of maturation is mediated by the RNA nuclease Ngl2p (Thomson and Tollervey, 2010), resulting in the mature 5.8S rRNA.

In humans the rRNA processing is conserved at a degree to that is seen in yeast, except for a few differences. For example, the human 47S pre-rRNA consists of an extra A' site at the 5' end of its 5' ETS. Secondly, in human the removal of 5' ETS takes place after the ITS1 cleavage (Henras *et al.*, 2015). Finally, there are two distinct pathways involved in separating human LSU and SSU pre-rRNAs by cleavage in ITS1, which are known as the major and the minor pathways (Mullineux and Lafontaine, 2012; Sloan *et al.*, 2013b).

In humans rDNA is transcribed into the 47S pre-rRNA and the rRNA processing starts with the A' site cleavage in the 5' ETS. It is mediated by the UTP-A complex, exonuclease XRN2 and protein factor MTR4, and this cleavage produces the 45S pre-rRNA (Sloan *et al.*, 2014). Subsequently, the 45S pre-rRNA enters either the major or the minor pathway (Henras *et al.*, 2015).

During the major pathway, a single cleavage event at the site 2, located in ITS1, produces the 30S SSU pre-rRNA (containing 18S rRNA sequence) and 32S LSU pre-rRNA (containing 5.8S and 28S rRNA sequences). Although the nuclease mediating this cleavage event remains unclear, it has been reported that the RNA binding protein RRP5 is important for this process (Sloan *et al.*, 2013b). In addition, the RNA binding protein RBM28, the nucleolar protein NOL12 and the ribosome biogenesis protein BOP1 are also important for this event (Sloan *et al.*, 2013b). The 30S SSU pre-rRNA undergoes A1 and exonuclease processing, producing the 18SE pre-rRNA, whereas the 32S LSU pre-rRNA undergoes site 4 cleavage separating the 12S (containing the 5.8S rRNA sequence) and the pre-28S rRNAs (Henras *et al.*, 2015).

During the minor pathway on the other hand, in addition to the site 2 cleavage event, the site 2a endonucleolytic cleavage also takes place. It leads to the production of 18SE SSU pre-rRNA and the 36S LSU pre-rRNA. The endonuclease UTP24 has recently been shown to be essential for the site 2a cleavage event (Wells *et al.*, 2016) and the ribosome biogenesis protein RRP5 is important for both 2 and 2a cleavage events (Sloan *et al.*, 2013b). The 36S LSU pre-rRNA subsequently gives rise to the 12S pre-rRNA and the 28S rRNA (Henras *et al.*, 2015).

The 18SE SSU pre-rRNA, produced by either the major or the minor pathway, is exported from nucleus to the cytoplasm. It undergoes the site 3 cleavage which is mediated by the endonuclease NOB1, and results in the mature 18S rRNA (Henras *et al.*, 2015). For the LSU, the 12S pre-rRNA processing which gives rise to the mature 5.8S rRNA, involves a number of different proteins in the nucleus (Coute *et al.*, 2008), the exosome associated factors MTR4, C1D (Schilders *et al.*, 2007) and the exosome component RRP6 (Tafforeau *et al.*, 2013b). After being exported to the cytoplasm, exonuclease ERI1 is also found to be involved in in the 5.8S maturation process (Ansel *et al.*, 2008).

1.2.4 Ribosomal protein production and regulation

In humans, the ribosomal protein encoding genes are located in all chromosomes (including X and Y) except chromosome 7 (Kenmochi *et al.*, 1998) and they are transcribed by RNA Pol II. Translation of RPs takes place in the cytoplasm, and the produced RPs are imported into the nucleus, and then localize to the nucleolus where they are integrated into the LSU or SSU production.

Ribosomal protein encoding genes are transcribed into 5' TOP mRNAs, a form of mRNAs with a 5' terminal oligopyrimidine sequence tract (5’ TOP) attached at their 5’ end (Gentilella *et al.*, 2015). The 5’ TOP is a translational regulator sequence starting with a cytidine, following by 5 to 15 pyrimidine nucleotides. Other than ribosomal proteins, these regulator sequences are also found in mRNAs encoding for translation factors and other ribosome biogenesis factors. The translation of these mRNAs is regulated by the mTOR pathway, in response to various external stimuli and to ribosomal defects (Levy *et al.*, 1991).

Protein kinase mTOR (also known as the target of rapamycin), is a member of the phosphatidylinositol 3-kinase related kinase family. mTOR is the core protein forming two

types of protein complexes, mTOR complex 1 and 2 (mTORC1 and 2) (Gentilella *et al.*, 2015). Other than mTOR itself, mTORC1 consists of regulatory-associated protein of mTOR (Raptor) (Hara *et al.*, 2002), G protein beta subunit-like (MLST8) (Kim *et al.*, 2003), PRAS40 (Oshiro *et al.*, 2007), DEP domain-containing protein 6 (DEPDC6) and Rheb-GTPase RAG (Groenewoud and Zwartkruis, 2013). Being essential for metabolism and cell growth, mTORC1 regulates protein synthesis in response to various internal and external stimuli, such as nutrient and energy status, growth factors and redox levels (Laplante and Sabatini, 2009). A series of signalling pathways downstream to mTORC1 play important roles in regulating pyrimidine synthesis, which is linked to rDNA transcription, and also mRNA translation initiation and elongation (Gentilella *et al.*, 2015).

1.2.5 The 5S RNP assembly

The 5S ribonucleoprotein (RNP) is a subcomplex of the LSU which is composed of the 5S rRNA and the ribosomal proteins RPL5 and RPL11. The 5S rRNA is 120 nucleotides in length and in eukaryotic cells, the 5S rRNA is transcribed by RNA polymerase III in the nucleoplasm. Once transcribed, 2-3 nucleotides at the 3' end of the 5S rRNA precursor are cleaved in order to produce the mature 5S rRNA in both human (Sloan *et al.*, 2014) and yeast (van Hoof *et al.*, 2000). In human this 3' end processing of the 5S rRNA is dependent on RPL5 binding (Sloan *et al.*, 2013a). In yeast, exonucleases Rex1p, Rex2p and Rex3p are required for the 3' end processing of the 5S rRNA (van Hoof *et al.*, 2000).

The 5S rRNA is the smallest rRNA which the structure is highly conserved throughout most organisms and it can be found in all organisms except in the mitochondrial ribosomes from fungi and animals (Szymanski *et al.*, 2002; Bhat *et al.*, 2004). However, in *Xenopus laevis* oocytes, the 5S rRNA is found associated with transcription factor TFIIIA and together they form the 7S RNP complex (Layat *et al.*, 2013). In humans, after transcription the 5S rRNA is bound and stabilised by RPL5 in the nucleoplasm, forming the pre-5S RNP complex. Studies have shown that RPL5 binding is required for the maturation of the 5S rRNA (Sloan *et al.*, 2013a). The level of the stable 5S rRNA is directly related to RPL5 protein levels, as the unbound 5S rRNA is unstable and is rapidly turned over. RPL11 subsequently binds to the RPL5/5S rRNA complex, forming the mature 5S RNP. Thereafter, the 5S RNP is integrated to the pre-60S large ribosomal subunit precursor (Sloan *et al.*, 2013a; Pelava *et al.*, 2016).

1.2.6 5S RNP incorporation into the large subunit

After the 5S RNP is assembled, it is incorporated into the LSU in the nucleolus. The 5S RNP is important for the structure and function of the LSU and it is also an essential component for the production of a mature ribosome.

Within the human mature LSU, the 5S rRNA is positioned in the central protuberance (CP), closely located to the 28S rRNA (Khatter *et al.*, 2015). During mRNA translation, the 5S rRNA is neither associated with tRNAs from at the P or A site of the ribosome nor with any other translation factors. However, the region in the ribosome that harbours the 5S rRNA was found to be important for associating tRNAs, elongation factors and peptidyltransferase centre factor binding (Dinman, 2005).

In yeast, ribosome biogenesis factors Rrs1 and Rpf2p are important in recruiting the 5S RNP into the pre-LSU (Zhang *et al.*, 2007). Firstly, evidence showed that Rpf2p binds to RPL5 and RPL11 in yeast two-hybrid studies (Miyoshi *et al.*, 2002; Morita *et al.*, 2002). Secondly, structural studies showed that in fungus *A. nidulans*, the Rrs1-Rpf2p complex interacts not only with both the 5S rRNA and RPL5, but also with the ribosome assembly protein 4 (Rsa4) (Kharde *et al.*, 2015). From this structure, only Rpf2p, but not Rrs1, has direct contact with the 5S rRNA (Kharde *et al.*, 2015). In yeast the homologous Rrs1-Rpf2p complex indeed interact with Rpl5, Rsa4 and the 25S rRNA (Madru *et al.*, 2015).

In humans, ribosome biogenesis factors RRS1 and BXDC1 are the human homologues of Rrs1 and Rpf2p, respectively. However, there is no evidence showing they bind to non-ribosomal 5S RNP by pull-down assay, moreover, knockdown of either of them has little effect on 5S RNP incorporation into the LSU (Sloan *et al.*, 2013a). Another ribosome biogenesis factor, PICT1, was found to be essential for the ribosomal incorporation of the 5S RNP (Sloan *et al.*, 2013a). The function of human RRS1 and BXDC1 in 5S RNP integration into the ribosomes is are not fully clear.

1.2.7 The 5S RNP remodelling event in the LSU

In yeast, the 5S RNP undergoes a structural remodelling event during the maturation of pre-LSU. The 5S RNP-mediated remodelling event of the pre-LSU was recently identified by the analysis of a yeast pre-LSU cryo-EM structure (Leidig *et al.*, 2014). Compared to the mature

LSU structure, the 5S RNP was rotated approximately 180° in the pre-LSU (Leidig *et al.*, 2014).

Interestingly there is growing evidence suggesting the Rrs1-Rpf2p complex, which is important for recruiting the 5S RNP into the pre-LSU, together with additional biogenesis factors such as Rsa4 and Nog1, are critical in stabilising the pre-rotated state of the 5S RNP (Leidig *et al.*, 2014; Madru *et al.*, 2015).

Recently ATPase and GTPase activities have been shown to be important for driving and regulating the remodelling event, such as Nug2 GTPase and, especially, the Rea1-Rix1 complex (Matsuo *et al.*, 2014; Barrio-Garcia *et al.*, 2016). Rea1 protein is a member of the AAA (+) family of ATPases and it was previously found to be important for the late rRNA processing, pre-LSU maturation and nuclear export (Galani *et al.*, 2004). In addition, Rsa4 was identified as a co-substrate of Rea1, and the activity of Rea1 was also linked to the removal of ribosome biogenesis factors during pre-LSU processing and maturation (Bassler *et al.*, 2010; Bassler *et al.*, 2014).

Furthermore, mutation of the MIDAS domain (or the ATP-binding domain) of Rea1 was found to block the release of Ras4 and Nug2 from the pre-LSU processing complex (Matsuo *et al.*, 2014), suggesting its ATPase activity is critical for the pre-LSU maturation. Finally, the interaction between Rea1 and Rix1 were found to be essential for pre-LSU maturation.

In summary, it was proposed that the Rrs1-Rpf2p complex, together with other biogenesis factors such as Ras4 and Nog1, provide an interaction platform recruiting and stabilising the pre-LSU incorporation of the 5S RNP for the subsequent remodelling event. The activity of Rea1-Rix1 complex is proposed to drive the rotation movement of the 5S RNP and the conformational change of the pre-LSU, and it also facilitates the release of those associated biogenesis factors, enabling the pre-LSU maturation process.

In human, it was recently reported that AAA ATPase MDN1, the human homologue of Rea1, interacts with the pre-LSU processing particle. The MDN1/pre-LSU association was regulated by sumolation of PELP1, a MDN1 associated protein (Raman *et al.*, 2016). However, there is still lack of evidence explaining ether the mechanics or the functional role of the 5S RNP-mediated pre-LSU remodelling event in human cells. It is possible that the remodelling event

takes place to provide a binding interface for the ribosome biogenesis factors during the LSU maturation process.

1.2.8 Nuclear export of the ribosome

After pre-LSU and pre-SSU are assembled in the nucleolus, they are exported from nucleus to the cytoplasm through the nuclear pore complexes (NPCs). Subsequently, they undergo a series of final maturation processes and they gain translation functions in the cytoplasm.

In yeast although pre-LSU and pre-SSU are translocated independently to the cytoplasm, they both require the Crm1/Ran GTPase system (Zemp and Kutay, 2007). Crm1 (chromosomal maintenance 1, Exportin 1 or XPO1) is involved in the leucine rich nuclear export signal (NES)-dependent protein export, and it is found to be essential for the cytoplasmic export for both pre-LSU and pre-SSU (Johnson *et al.*, 2002). Apart from Crm1, exportin 5 (Exp5) also mediates the export of pre-LSU (Wild *et al.*, 2010) The GTP-binding nuclear protein Ran is a small GTPase which is able to hydrolyse GTP. Functions of Crm1 and Exp5 are GTPase-dependent and during the nuclear export process, the small GTPase Ran hydrolyses GTP to GDP providing the required energy (Johnson *et al.*, 2002).

In yeast, the nuclear export of pre-LSU is mediated by Crm1, Nmd3p and other factors recognising NES (Hutten and Kehlenbach, 2007). Nmd3p is a shuttling factor associated with the late pre-LSU particles, and it acts as an adaptor between the pre-LSU and Crm1 (Zemp and Kutay, 2007; Sengupta *et al.*, 2010). Furthermore, ribosome biogenesis factor Arx1 is also found to be crucial for the pre-LSU export. It is thought to associate with Nmd3, Crm1 and the NPC, and depletion of Arx1 results in the accumulation of nuclear pre-LSU (Hung *et al.*, 2008).

In addition, there are other biogenesis factors which are less-well characterized, such as the HEAT-repeat protein Rrp12p. This protein interacts with premature ribosomal particles and it is involved in their nuclear export (Schafer *et al.*, 2003). Depletion of Rrp12p leads to impaired nuclear export of premature ribosomal particles (Oeffinger *et al.*, 2004). Furthermore, the protein Mtr2p, known to be important for mRNA export, is also implicated as a protein cofactor mediating pre-LSU export. Although it is not involved in the RanGTP-associated exportin system, Mtr2p is found associated with the late pre-LSU particles in proteomic analysis (Nissan *et al.*, 2002).

In yeast, Crm1 also mediates nuclear export of the pre-SSU. In contrast to the pre-LSU that requires Nmd3p, biogenesis factors Rio2p, Dim2p and Ltv1p are involved in the pre-SSU export in yeast, since inhibiting Crm1 leads to the nuclear accumulation of these factors (Schafer *et al.*, 2003). However their role in the export process is not yet fully clear since there are no clear export defects observed when deleting these proteins (Zemp and Kutay, 2007). Rrp12p is also thought to mediate pre-SSU export as it is found associated with pre-SSU particles (Oeffinger *et al.*, 2004).

In humans, the protein kinase Rio2 (hRio2) has been shown to associate with late pre-SSU particles, and its depletion leads to recycling defects of other biogenesis factors and slows down pre-SSU maturation (Zemp *et al.*, 2009). Furthermore, depletion of hTsr1 leads to nuclear accumulation of pre-SSU particles (Carron *et al.*, 2011).

1.2.9 Ribosome production quality control

Ribosome biogenesis is an energy costly process and ribosome biogenesis defects lead to serious diseases called ribosomopathies (see Section 1.4). Hence ribosome production is tightly regulated and under constant surveillance by a series of quality control systems, during or after ribosome production.

Firstly, when ribosome biogenesis defects occur, pre-rRNAs are not found accumulating significantly, suggesting without binding to the correct ribosomal protein or biogenesis factor, pre-rRNAs are unstable and being turned over rapidly (Houseley and Tollervey, 2009).

Secondly, cytoplasmic maturation of pre-SSU is important for the ribosome to acquire translation competency and the maturation process needs binding of LSU to complete (Lebaron *et al.*, 2012), adding additional quality control mechanisms for ribosome biogenesis. In addition, the joining of the LSU and SSU is suppressed by a number of ribosome biogenesis factors. For example, the yeast biogenesis factor Rei1 and the adaptor protein Nmd3 are found to prevent the two subunits from joining (Greber *et al.*, 2016). Furthermore, in yeast, biogenesis factor Tif6p binds to LSU to prevent the LSU association with the SSU (Klinge *et al.*, 2011). In humans, the translation initiation factor 6 (eIF6, human homologue of Tif6p) has also been found to prevent the joining of pre-LSU and pre-SSU (Finch *et al.*, 2011).

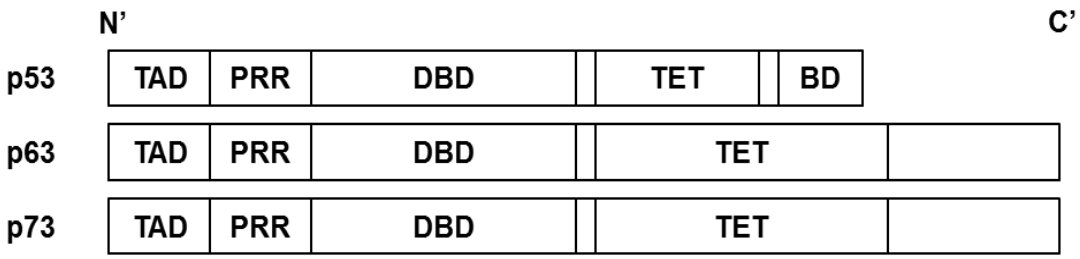

Figure 1.1. Schematic representation of the domain organization in p53, p63 and p73.

The N-terminus transactivational domain (TAD), the proline rich region (PRR), the central DNA-binding domain (DBD), the tetramerization domain (TET) and the basic C-terminus domain (BD) are indicated. The N-terminus (N') and C-terminus (C') are indicated above the representation.

Finally, for those defective ribosomes escaping the nuclear degradation mechanism and reaching the cytoplasm, there are also quality control systems in the cytoplasm. For example, cytoplasmic ribosomes carrying critical mutations affecting functionally essential structures, such as in the decoding centre of the SSU or the peptidyl transfer centre of the LSU, are targeted for the non-functional rRNA decay (NRD) (LaRiviere *et al.*, 2006; Cole *et al.*, 2009).

1.3 The tumour suppressor p53

1.3.1 The p53 protein family

p53 protein was first described as an antigen involved in cell transformation in multiple publications in 1979 (DeLeo *et al.*, 1979; Linzer and Levine, 1979). Ten years later the *TP53* gene which encodes p53 protein was classed as a tumour suppressor gene (Levine, 1989). The *TP53* gene consists of 11 exons and p53 protein contains five domains, including the N-terminus transactivational domain (TAD), the proline rich region (PRR), the central DNA-binding domain (DBD), the tetramerization domain (TET) and the basic C-terminus domain (BD) (Figure 1.1) (Joerger and Fersht, 2008).

The N-terminus transactivational domain (TAD) enables p53 to activate transcription factors and allows it to interact with other proteins (Kamada *et al.*, 2016). The proline rich region is important for apoptotic activity and is important for the stability of the protein (Green and Kroemer, 2009). The central DNA-binding domain is responsible for p53 binding to the DNA consensus sequence for p53 binding (McLure and Lee, 1998). The tetramerization domain

allows p53 proteins to form tetramers and is important for DNA binding and interactions with other proteins (McLure and Lee, 1998; Kamada *et al.*, 2016). The C-terminus regulates DBD-DNA interaction (Joerger and Fersht, 2008; Kamada *et al.*, 2016).

Transcription factors p63 and p73 are members of p53 protein family encoded by individual genes, and they share the same structural organization as p53 (Levrero *et al.*, 2000). They share similar structures and domains, except p63 and p73 contain a longer C-terminus in their tetramerization domains and lack the basic C-terminus domain (Figure 1.1) (Joerger and Fersht, 2008). The DNA-binding motifs of p53, p63 and p73 are largely conserved and they are able to form heterotetramer complexes, which are involved in gene transcription and cell cycle regulation (Brandt *et al.*, 2009). However, p63 and p73 have also been shown to regulate non-p53-targed genes and they have non-p53 roles in neuronal or epithelial development (Harms *et al.*, 2004).

1.3.2 p53 regulation: MDM2 and MDMX

In unstressed cells p53 is normally maintained at a low level. One key negative regulator of p53 is the murine double minute 2 protein (MDM2 or HDM2 in human) (Wade *et al.*, 2013). MDM2 has domains such as p53 binding domain, acidic domain, zinc finger domain and RING (Really Interesting New Gene) -finger domain (Figure 1.2).

The p53 binding domain of MDM2 recognises the N-terminus transactivational domain (TAD) on p53 (Kussie *et al.*, 1996), and its binding to TAD inhibits p53 activity (Momand *et al.*, 1992). MDM2 mainly functions as an E3 ubiquitin ligase which targets itself and p53 for ubiquitination. After the lysine residues located at the C-terminus of p53 are ubiquitinated by MDM2, p53 is degraded by the 26S proteasome (Honda and Yasuda, 1999; Michael and Oren, 2003). Apart from being involved in proteasome-mediated p53 degradation, MDM2 also directly inhibits the transcriptional activity of p53. The acidic domain of MDM2 interacts and inhibits the DNA binding domain of p53 (Cross *et al.*, 2011).

It has been shown that multiple forms of MDM2-p53 interactions exist, and that their nature is important for MDM2 regulation of p53 stability and activity. Firstly, the central domain of MDM2 also interacts with p53 and has shown to be essential for ubiquitination of p53 (Ma *et al.*, 2006). Furthermore, the N-terminus of MDM2 also binds to the C terminus of p53 (Poyurovsky *et al.*, 2010).

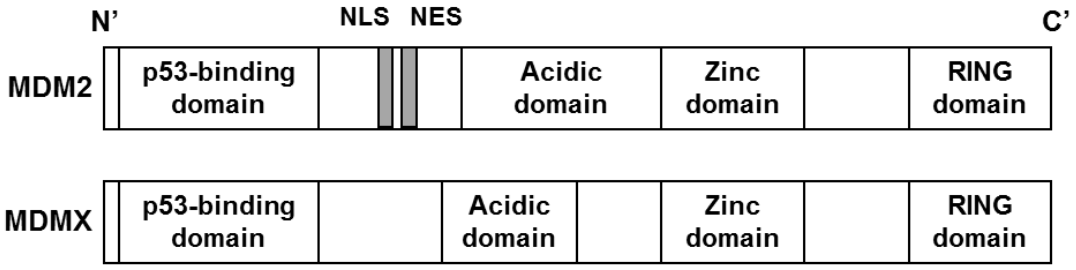

Figure 1.2. Schematic representation of the domain organization in MDM2 and MDMX.

Protein domains are indicated. The nuclear localization signal (NLS) and nuclear export signal (NES) in MDM2 are indicated and coloured in grey. The N-terminus (N') and C-terminus (C') are indicated above the representation. (Adapted from (Marine *et al.*, 2007))

The interaction between MDM2 and p53 is inhibited by a group of compounds called Nutlins. They are *cis*-imidazoline antagonists discovered in a chemical screen (Vassilev *et al.*, 2004). Although the molecular mechanism is not fully understood, it has been shown that nutlin treatment changes the modification status at the C-terminus of p53 and may inhibit MDM-p53 interaction (Poyurovsky *et al.*, 2010).

In addition, the second intron of MDM2 contains a p53 responsive element, therefore MDM2 is also in turn transcriptionally regulated by p53. This creates a negative feedback loop between MDM2 and p53 (Wu *et al.*, 1993). Finally, other than p53, MDM2 has also been found to suppress transactivation activity of p73 and p63 (Zeng *et al.*, 1999; Kadakia *et al.*, 2001).

Murine double minute X, also known as MDMX or MDM4, also plays an important role in negatively regulating p53. MDMX shares 90% homology with MDM2, mainly in its p53-binding and RING domains (Figure 1.2) (Shvarts *et al.*, 1996). However, in contrast to MDM2, MDMX lacks E3 ligase activity, therefore it is not able to target p53 for proteasome-mediated degradation (Jackson and Berberich, 2000). Furthermore, MDMX lacks the nuclear localization signal (NLS) or the nuclear export signal (NES) (Figure 1.2) (Migliorini *et al.*, 2002). MDMX is also a polyubiquitination target of MDM2 and its levels are regulated by MDM2 (Shadfan *et al.*, 2012).

Through its RING-finger domains, MDMX is able to bind to MDM2 (Tanimura *et al.*, 1999). MDM2 and MDMX are able to form heterodimers or homodimers. Despite a lack of E3 ligase

function, MDMX is still able to inhibit p53 activity by binding to the TAD of p53 (Danovi *et al.*, 2004). The heterodimer of MDM2-MDMX enhances the Mdm2-mediated ubiquitination and degradation of p53 (Gu *et al.*, 2002; Linares *et al.*, 2003; Uldrijan *et al.*, 2007; Wade *et al.*, 2013). Moreover, MDM2-MDMX interaction is important for the nuclear localization of MDMX since the latter lacks a nuclear localization signalling peptide (Gu *et al.*, 2002).

MDM2 and MDMX are both involved in regulating p53, individually or cooperatively. A study using a mouse model revealed that MDMX deletion leads to p53-dependent cell cycle arrest whereas MDM2 deletion causes apoptosis (Chavez-Reyes *et al.*, 2003). Evidence also shows that Nutlin specifically inhibits MDM2-p53 binding but not the MDMX-p53 interaction (Joseph *et al.*, 2010).

1.3.3 p53 post-translational modification and activation

p53 is induced and activated by several upstream signalling pathways, in response to various types of cellular stress, such as DNA damage (induced by UV or ionizing radiation) and oncogene expression (Brown *et al.*, 2009; Meek and Anderson, 2009).

From mass spectrometry studies, p53 was found to be modified extensively at about 50 residues. These modifications include ubiquitination, phosphorylation, acetylation, methylation, glycosylation, neddylation and sumolation (Jimenez *et al.*, 1999; Meek and Anderson, 2009). In respond to cellular stresses, p53 is not only stabilized with its half-life increasing significantly, but it is also transcriptionally activated, leading to the expression of p53-regulated genes. The post-translational modifications on p53, especially phosphorylation and acetylation, are important in both its stabilisation and its activation (Meek and Anderson, 2009).

There are several well-studied upstream pathways regulating p53 through its modification. In the case of DNA damage, p53 is phosphorylated and activated by two major protein kinases: Ataxia telangiectasia mutated (ATM) is a serine/threonine kinase recruited and activated in response to DNA double strand break. Serine/threonine kinase ATR, also known

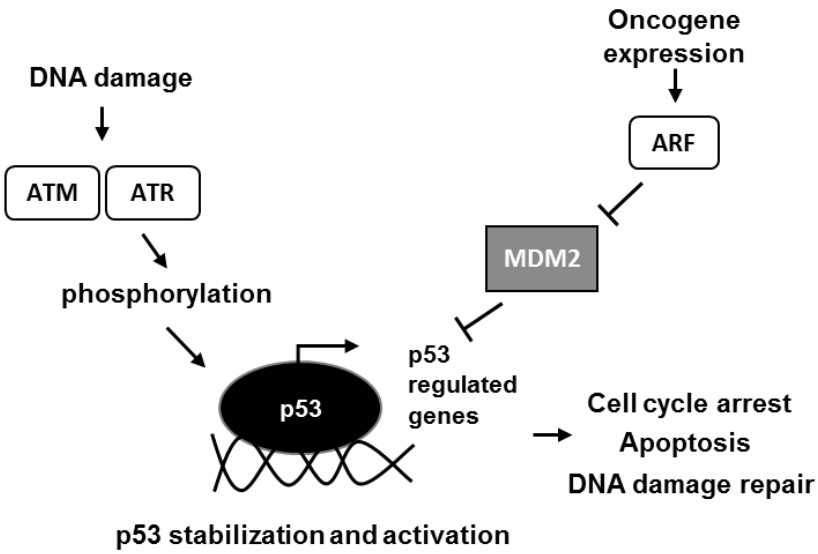

Figure 1.3. p53 is activated in response to DNA damage and oncogene expression.

as ataxia telangiectasia and Rad3-related protein (ATR) or FRAP-related protein 1 (FRP1), is also responsible for phosphorylating serine 15 and serine 37 (Lakin and Jackson, 1999). ATM and ATR are the primary kinases responsible for the phosphorylation of serine 15 (Ser15) of p53 (Meek and Anderson, 2009), whereas ATM is also thought to modify serines 9, 20 and 46 (Saito *et al.*, 2002; Loughery *et al.*, 2014).

Interestingly, ATM-mediated phosphorylation was found to recruit histone/lysine acetyltransferases (HAT) such as p300/CBP (Meek and Anderson, 2009). p300/CBP are two closely related transcription coactivator proteins which interact with and activate a number of transcription factors, including p53. p300/CBP is responsible for the acetylation of seven C-terminus lysine residues (Meek and Anderson, 2009), and six of these lysine residues are also the targets of MDM2-mediated ubiquitination (Rodriguez *et al.*, 2000). Lysine acetylation of p53 is induced in response to several of stresses and leads to p53 stabilisation. Because acetylation and ubiquitination of lysine residues are mutually exclusive, acetylation helps displacing p53 from MDM2 and interferes with its negative regulation (Tang *et al.*, 2008; Meek and Anderson, 2009).

p53 is also activated by the ectopic oncogene expression, such as c-Myc. $p14^{ARF}$ is a tumour suppressor protein translated from an alternate reading frame of the *CDKN2A* locus. $p14^{ARF}$ is activated by c-Myc expression and it functions as a negative regulator of MDM2. It binds and inhibits MDM2 resulting in stabilisation and activation of p53. Although there is a lack of

evidence showing the $p14^{ARF}$ pathway promotes p53 phosphorylation, acetylation of p53 has been reported (Meek and Anderson, 2009).

1.3.4 p53 transcriptional activities and the p53-regulated genes

In response to cellular stress signals, activated p53 drives the expression of a number of important genes, promoting cellular responses including DNA damage repair, apoptosis, cell cycle arrest and senescence (Purvis *et al.*, 2012).

1.3.4.1 p53 transcriptional functions in DNA damage repair

DNA damage is caused by either endogenous sources, such as reactive oxygen species (ROS) and replication errors, or exogenous sources, such as UV, x-rays or gamma radiations, toxins, mutagenic chemicals and viruses.

There are different types of DNA damage, such as oxidized base formation and single strand breaks which are repaired by the base excision repair (BER) mechanism, or DNA double strand breaks which are fixed by homologous recombination (HR) or non-homologous end joining (NHEJ). Other types of damage such as insertions, deletions or mismatches induced during DNA replication, are repaired by mismatch repair (MMR) mechanism, while the bulk of DNA adducts as a result of UV radiation are removed by the nucleotide excision repair (NER) mechanism (Sancar *et al.*, 2004; Williams and Schumacher, 2016).

During NER, activated p53 upregulates the expression of DNA damage binding protein 2 (DDB2) (Hwang *et al.*, 1999) and XPC protein (Adimoolam and Ford, 2002; Hastak *et al.*, 2012). DDB2 recruits XPC to the site of DNA damage, enhancing the cell's ability to target DNA damage in response to UV radiation (Williams and Schumacher, 2016). Furthermore, p53 also binds to XPC during NER (Wang *et al.*, 1995).

In addition to NER, the transcriptional activity of p53 is also involved in HR and BER. The proliferating cell nuclear antigen protein (PCNA) functions as a DNA clamp which binds to DNA forming a complex with GADD45α (Growth arrest and DNA-damage-inducible protein alpha) (Hall *et al.*, 1995; Jung *et al.*, 2013). This complex assembles other protein factors during homologous recombination (Pfander *et al.*, 2005) and base excision repair (Shivji *et al.*, 1992; Jung *et al.*, 2013). Activated p53 also induces the expression of the gene *GADD45A*, which encodes for Gadd45α (Carrier *et al.*, 1999).

1.3.4.2 p53 mediated apoptosis pathways

Apoptosis is a genetically programmed cell death procedure and an important process in multicellular organisms. There are two distinctive pathways which p53 transcriptionally regulates, namely the extrinsic and intrinsic pathways (Ouyang *et al.*, 2012).

The extrinsic pathway involves the activation of the tumour necrosis factor receptor (TNFR) family and the formation of a death-inducing-signalling-complex (DISC) (Locksley *et al.*, 2001). This subsequently causes caspase-induced apoptosis. One of the key death receptors involved in extrinsic pathway is the cell surface receptor Fas (cell surface first apoptosis signal receptor) encoded by gene *CD95/Apo-1*. In T-cells, Fas interacts with its ligand FasL to become activated on the cell surface. Activated p53 functions as a transcription regulator and upregulates the transcription of Fas (Muller *et al.*, 1998). The second membrane receptor protein being upregulated by p53 is the death receptor-5 (DR5). DR5 levels are induced by p53 in response to DNA damage and further promotes cell death via Caspase 8 (Wu *et al.*, 1997).

The intrinsic apoptotic pathway mainly involves activation of genes of the Bcl2 family and the release of cytochrome-C (CytoC) from the mitochondria (Tsujimoto, 1998). Bcl2 family members can be further classed into pro-survival and pro-apoptotic homologs which are structurally similar. Cell survival/apoptosis is maintained by the balance between pro-survival and pro-apoptotic signalling (Ouyang *et al.*, 2012). A subset of Bcl2 genes are regulated by transcriptional activation of p53.

The first member of the Bcl2 family identified to be induced by p53 was BAX (Miyashita and Reed, 1995). Activated BAX proteins form a homodimer which leads to the release of CytoC from mitochondria. CytoC, apoptotic protease-activating factor-1 (APAF1) and procaspase 9 subsequently form the apoptosome complex, resulting in cellular apoptosis through a cascade of caspase signalling.

There are other Bcl2 family members induced by the p53, including the p53-upregulated modulator of apoptosis (PUMA) and Noxa. In response to DNA damage, the PUMA gene is induced directly by p53 and PUMA is essential for the BAX-mediated apoptosis pathway (Nakano and Vousden, 2001). Noxa is upregulated by p53 in response to X-ray radiation (Oda *et al.*, 2000). Hence in response to DNA damage, there are at least three p53-mediated Bcl2 family genes induced, shifting the balance towards pro-apoptotic signalling.

1.3.4.3 p53 in cell cycle regulation

In eukaryotic cells, the cell cycle is comprised of interphase, mitotic phase and cytokinesis. Interphase is divided into three stages, including G_1, S and G_2 phase, while the mitotic phase contains prophase, metaphase, anaphase and telophase. The cell cycle is essentially regulated by the cyclin-dependent serine/thronine kinases (CDKs) and their regulatory subunit, the cyclins (Dulic *et al.*, 1992; Schafer, 1998). They form CDK-cyclin complexes which activate proteins required for cell cycle progression. For example, Cdk2-cyclin E is required for the G_1 –S phase transition (Dulic *et al.*, 1992) whereas Cdk4 or the Cdk6-cyclin D complex is required for G_1 phase progression (Bates *et al.*, 1994).

Cyclin-dependent kinase inhibitor 1 (p21), encoded by *CDKN1A* gene, is able to bind to and inhibit multiple types of CDK-cyclin complexes, however it primarily inhibits Cdk2 (Harper *et al.*, 1993; Xiong *et al.*, 1993). Activated p53 promotes p21 expression which inhibits Cdk2 and triggers G_1 phase arrest (Harper *et al.*, 1993). p21 expression is regulated by p53-mediated transcriptional activation, therefore p21 expression levels are widely used to monitor p53 transcriptional activity. Furthermore, activated p53 promotes expression of scaffold protein 14-3-3 sigma which inhibits Cdk1-cyclin B complex and leads to G_2 phase cell cycle arrest (Laronga *et al.*, 2000).

1.3.5 The role of ribosomal proteins in p53 and other signalling pathways

As an energy costly process, ribosome biogenesis is crucial for many cellular activities, including cell proliferation. It is important to maintain the integrity of ribosome production for cell survival and many studies have linked ribosomal proteins to the regulation of the tumour suppressor p53. In the context of ribosome biogenesis defects, ribosomal proteins are involved in p53 stabilisation and activation by blocking the p53 regulator, MDM2. Misregulated p53 signalling has been found in numerous genetic diseases caused by aberrant ribosome biogenesis, which are also known as ribosompopathies.

A number of different ribosomal proteins interact directly with MDM2, such as RPL5, RPL11, RPL23, RPL26, RPL37 from the LSU and RPS3, RPS7, RPS14 from the SSU (Kim *et al.*, 2014) and the RP-MDM2 interaction have been linked to p53 activity, such as RPL5, RPL11 (Sloan *et al.*, 2013a; Pelava *et al.*, 2016), RPL23 (Dai *et al.*, 2004; Jin *et al.*, 2004), RPL26 (Takagi *et al.*, 2005; Ofir-Rosenfeld *et al.*, 2008; Chen *et al.*, 2012), RPS7 (Chen *et al.*, 2007; Zhu *et al.*, 2009)

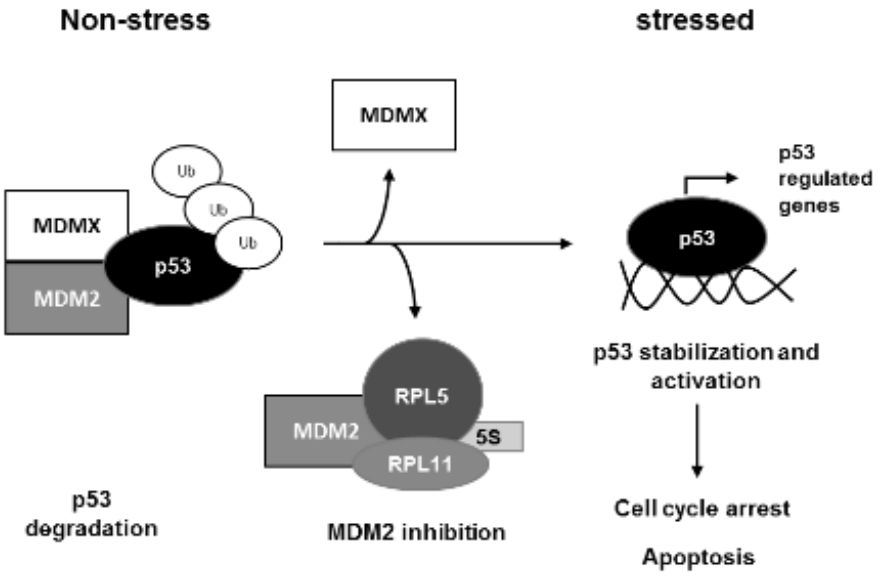

Figure 1.4. Schematic representation of the 5S RNP-MDM2 mediated p53 activation.

and RPS3 (Yadavilli *et al.*, 2009). These reports imply that p53 regulation via ribosomal proteins can be influenced by multiple pathways.

Most interestingly, it has been demonstrated that Actinomycin D (ActD)-induced p53 activation is neither dependent on RPL23, RPL26 nor RPS7 whilst it is dependent on RPL5 and RPL11 (Bursać *et al.*, 2012). ActD is a drug which has been used as an anti-cancer chemotherapeutic. A low dose of ActD inhibits transcription elongation of RNA polymerase I which abolishes pre-rRNA production and results in ribosomal stress-induced p53 activation (Sobell, 1985). This suggests that RPL5, RPL11 and the 5S RNP complex activate p53 via a signalling pathway different from other ribosomal proteins.

1.3.6 The 5S RNP-MDM2 involvement in p53 regulation

The 5S RNP, which is composed of RPL5, RPL11 and the 5S rRNA, is assembled separately from the rest of the LSU. Normally the 5S RNP is integrated into the pre-LSU before the pre-LSU is processed and becomes mature LSU (Zhang *et al.*, 2007; Sloan *et al.*, 2013a; Pelava *et al.*, 2016). Ribosome biogenesis defects cause accumulation of free (non-ribosomal) 5S RNP in the nucleoplasm, where it interacts with and inhibits MDM2 mediated ubiquitination of p53. This subsequently results in the stabilisation and activation of p53 (Figure 1.4) (Sloan *et al.*, 2013a; Pelava *et al.*, 2016). It has been recently identified that all three component of the 5S RNP are needed for the 5S RNP-MDM2 interaction, and that MDM2 binds RPL11,

RPL5 and the 5S rRNA (Donati *et al.*, 2013; Sloan *et al.*, 2013a). Structural studies has been shown that the RPL11-MDM2 interface largely overlaps with the RPL11-28S rRNA interface after 5S RNP integration into the pre-LSU (Khatter *et al.*, 2015; Zheng *et al.*, 2015).

Large ribosomal subunit production defects cause p53 activation through inhibition of MDM2 function by non-ribosomal 5S RNP (Horn and Vousden, 2008; Donati *et al.*, 2013; Sloan *et al.*, 2013a). In addition to large ribosomal subunit biogenesis defects, small ribosomal subunit production also causes p53 stabilisation and activation through the MDM2-5S RNP signalling pathway (Fumagalli *et al.*, 2009; Dutt *et al.*, 2011; Fumagalli *et al.*, 2012). Fumagalli et al. showed that small ribosomal subunit production defects upregulate ribosomal protein production, which causes increased 5S RNP production leading to p53 stabilisation (Fumagalli *et al.*, 2012).

The 5S RNP is also involved in other important cellular signalling pathways. For example, 5S RNP is important for $p14^{ARF}$-mediated p53 activation (Sloan *et al.*, 2013a). Furthermore, Liao *et al.* reported that RPL11 and RPL5 recruit the RNA-induced silencing complex (RISC) leading to c-Myc inactivation (Liao *et al.*, 2014). Finally, both RPL5 and RPL11 bind to and inhibit MDM2 function, causing the activation of p73 (Zhou *et al.*, 2015).

1.4 Ribosomopathies

A number of rare genetic diseases arise due to mutations in the genes encoding either ribosomal proteins or ribosome biogenesis factors, and they are also known as ribosomopathies (Table 1.1) (Freed *et al.*, 2010; Narla and Ebert, 2010). Some well characterized ribosomopathies include Diamond Blackfan anaemia (DBA), Treacher-Collins (TC) syndrome, and 5q- syndrome, Dyskeratosis Congenita (DSC), and Shwachman-Diamond syndrome (Table 1.1). Ribosomopathies are caused by mutations of different genes, including genes encoding for large or small ribosomal proteins (Cmejla *et al.*, 2009; Lipton and Ellis, 2010), and genes encoding for different ribosome biogenesis factors, including TCOF1 (Gonzales *et al.*, 2005) and DKC1 (Angrisani *et al.*, 2014).

Although the causes of different ribosomopathies vary at the genetic level and the mutations affect ribosome biogenesis at different stages, patients with ribosomopathies share some similar phenotypes. These phenotypes include anaemia, skeletal development

Ribosomopathy	Gene Defects	Clinical features	Cancer risk	p53 condition
Diamond Blackfan Anemia (DBA)	Ribosomal proteins	Macrocytic anemia Short stature Craniofacial defects Thumb abnormalities	osteosarcoma MDS AML	Increased levels (symptoms dependent)
Treacher Collins syndrome	*TCOF1* *POLR1C* *POLR1D*	Craniofacial abnormality	None reported	Increased levels
5q-syndrome	*RPS14*	Macrocytic anemia Hypolobulated micro-megakatyocytes	AML (Low rate); MDS	Increased levels deregulated
Dyskeratosis Congenita (DSC)	*DKC1* *NOP10* *NHP2* *TERT* *TERC* *TINF2*	Cytopenias Skin hyperpigmentation Nail dystrophy Oral leukoplakia	AML Head and neck tumours	Regulation affected
Shwachman-Diamond syndrome	*SBDS*	Neutropenia/ infections Pancreatic insufficiency Short stature	MDS and AML	Increased levels
Alopecia, neurological defects, and endocrinopathy syndrome	RBM28 (Nop4)	Absence of mature hair follicles Tooth malplacement Mental retardation	None reported	Symptoms dependent

Table 1.1. Summary of ribosomopathies and other diseases in related to gene defects and p53 conditions. (Adapted from (Narla and Ebert, 2010)).

defects and predisposition to cancers, especially to acute myeloid leukaemia (AML) (Table 1.1) (Narla and Ebert, 2010).

Although efficient ribosome biogenesis is crucial for all tissue development, ribosome biogenesis defects have been shown to cause more severe defects in hematopoietic cells during embryogenesis in both ribosomopathies patients and animal models (Yelick and Trainor, 2015). In addition, there is increasing evidence from animal models indicating that the tumour suppressor p53 is involved in the phenotype development (Amsterdam *et al.*, 2004; Jones *et al.*, 2008; Pellagatti *et al.*, 2010; Jaako *et al.*, 2015). Since ribosome biogenesis defects are directly linked to p53 regulation, investigating the ribosomal stress-induced p53 signalling pathway could provide greater understanding of the p53 involvement in these diseases.

1.4.1 Diamond Blackfan Anemia

One of the best characterized ribosomopathies is Diamond Blackfan Anemia (DBA). DBA is a rare congenital erythroid hypoplasia and it occurs in 1 : 100,000 to 200,000 births (Narla and Ebert, 2010). It is characterized by severe erythroid precursor cell reduction in the bone marrow (Narla and Ebert, 2010). DBA patients are also found to be predisposed to cancers, such as osteocarcoma, myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML) (Table 1.1) (Lipton *et al.*, 2001; Narla and Ebert, 2010). Genetically, most of DBA cases are caused by mutations in large or small ribosomal subunit proteins (Ellis, 2014). The current treatment strategy for DBA involves steroids, blood transfusion or bone marrow transplantation (Vlachos *et al.*, 2001; Khanna-Gupta, 2013).

The most frequently reported gene mutation in DBA is *RPS19* which encodes the ribosomal protein S19 (Lipton and Ellis, 2010; Horos and von Lindern, 2012). In addition, mutations in other ribosomal proteins, such as RPS7, RPS10, RPS17, RPS26, RPS27, RPS28 and RPS29 from the small ribosomal subunit, or RPL5, RPL11, RPL15, RPL26 and RPL35A from the large ribosomal subunit, have also been identified (Cmejla *et al.*, 2009; Lipton and Ellis, 2010; Narla and Ebert, 2010; Delaporta *et al.*, 2014). Interestingly, clinical manifestations vary dependent on the genetic defect. For example, patients affected by mutated RPL11 protein show thumb abnormalities whereas mutated RPL5 is linked to cleft palate (Gazda *et al.*, 2008).

Tumour suppressor p53 has been identified to be involved in DBA pathogenesis using a mouse model for DBA, which indicated that certain DBA symptoms are linked to cellular p53 levels (Jaako *et al.*, 2015). The study further showed that the development of anaemia is dependent of the 5S RNP-MDM2 interaction (Jaako *et al.*, 2015).

1.4.2 Treacher Collins syndrome

Treacher Collins (TC) syndrome is an autosomal dominant congenital disorder that affects approximately 1 in 50,000 births (Posnick and Ruiz, 2000). It was characterized by craniofacial defects which can affect breathing, seeing, hearing and brain development of the patients (Sakai and Trainor, 2009; Narla and Ebert, 2010).

Mutations in the gene Treacher-Collins-Franceschetti syndrome 1 (*TCOF1*) were linked to the pathogenesis of the disorder (Dixon *et al.*, 1996). *TCOF1* encodes for the nucleolar

phosphoprotein treacle, which is involved in rDNA transcription and methylation of the pre-rRNA (Valdez *et al.*, 2004; Gonzales *et al.*, 2005). Treacle protein associates with the upstream binding factor (UPF) and RNA Pol I in the nucleolus (Valdez *et al.*, 2004; Gonzales *et al.*, 2005). Furthermore, mutation in the two genes, *POLR1C* and *POLR1D*, which encode for DNA-directed RNA Pol I and III subunit proteins RPAC1 and RPAC2, were also identified in TC patients (Yelick and Trainor, 2015).

Tumour suppressor p53 was shown to be involved in the development of TC syndrome. Studies using mouse embryos showing a TC phenotype revealed that mutated *TCOF1* is linked to p53 stabilisation and apoptosis of the neuronal crest cells (NCC) which are responsible for facial tissue development during embryogenesis (Jones *et al.*, 2008). Furthermore, deleting p53 in these embryos inhibits the cyclin G1-driven apoptosis of the NCC and rescues the craniofacial abnormality (Jones *et al.*, 2008).

1.4.3 5q- syndrome

5q- syndrome, also known as the chromosome 5q deletion syndrome, is an acquired hematological disorder which has been classed as a subtype of the myelodysplastic syndrome (MDS) (Vardiman *et al.*, 2002; Narla and Ebert, 2010). It is caused by the loss of a long arm of chromosome 5 in bone marrow myelocytes (Van den Berghe *et al.*, 1974). This syndrome is characterized by macrocytic anemia and compares to patients with other MDSs, though 5q- syndrome patients have a lower progression rate to AML (Vardiman *et al.*, 2002; Narla and Ebert, 2010). Lenalidomide is able to treat symptoms of the 5q- syndrome and it is used as treatment for MDS as it promotes erythroid differentiation (List *et al.*, 2006).

As a result of the long arm deletion of chromosome 5, haploinsufficiency of the *RPS14* gene has been identified in 5q- syndrome patients (Ebert *et al.*, 2008; Narla and Ebert, 2010). The *RPS14* gene encodes the small ribosomal protein RPS14, and the loss of this protein causes defects in SSU biogenesis and impaired erythropoiesis (Ebert *et al.*, 2008). Similar to DBA, the tumour suppressor p53 is involved in the 5q- syndrome. p53 was reported to be activated and deregulated in erythroid progenitor cells in patients with 5q- syndrome (Pellagatti *et al.*, 2010; Dutt *et al.*, 2011). Furthermore, it has been reported that Lenalidomide leads to p53 degradation by restoring the ubiquitination function of MDM2 (Wei *et al.*, 2013).

1.4.4 Ribosome and cancer

Since ribosome biogenesis and ribosomal proteins are involved in p53 regulatory signalling, it is fair to speculate that misregulation of p53 could play a role in cancer development, with or without the involvement of ribosompopathies. Most patients with ribosomopathies exhibit an increased risk of developing different types of cancers. For example, DBA, 5q- syndrome, Dyskeriatosis Congenita or Shwachman-Diamond syndrome especially result in a predisposition for AML (Alter *et al.*, 2009; Narla and Ebert, 2010; Vlachos *et al.*, 2012; Khanna-Gupta, 2013). In turn, ribosomal protein mutations are also reported in cancers. For example, exome sequencing identified RPL5 and RPL11 mutations in T-cell Acute Lymphoblastic Leukaemia (T-ALL) (De Keersmaecker *et al.*, 2013). RPL22 mutations have also been reported in T-ALL (Rao *et al.*, 2012). RPS27a has been identified to be linked to leukemia pathogenesis (Wang *et al.*, 2014).

Furthermore, being the subcellular compartment responsible for ribosome biogenesis, evidence has also shown that the nucleolar proteins are involved in disease development. For example, mutations of *NPM1* gene, encoding protein nucleophosmin, a multifunctional protein involved in ribosome biogenesis, was identified in patients affected by AML (Federici and Falini, 2013) and MDS (Grisendi *et al.*, 2005). The *NPM1* gene mutation occurs in 27% of the AML cases (Federici and Falini, 2013). In addition, transcription factor Runx1, which is important for ribosome biogenesis, was found involved in AML, MDS and breast cancer development (Browne *et al.*, 2015; Behrens *et al.*, 2016). All these reported defects confirm that genetic defects in ribosomal proteins and their related biogenesis factors play an important role in a broad range of diseases.

1.5 p53 regulator proteins linked to ribosome

When ribosome biogenesis defects occur, non-ribosomal 5S RNP accumulates in the nucleoplasm where it activates p53. However, the regulation of this 5S RNP-p53 signalling pathway, and whether other proteins are involved in this pathway remains unclear. It has been suggested that proteins involved in 5S RNP assembly, localization and incorporation into the ribosome could be important in regulating p53 through the 5S RNP (Sloan *et al.*, 2013a).

The 5S RNP assembly and its subsequent incorporation into ribosomes is a complicated process which has been shown to require the coordination from other protein co-factors. Some proteins interacting with RPL11 or RPL5 affect p53 induction, presumably via 5S RNP (Donati *et al.*, 2013; Fregoso *et al.*, 2013; Sloan *et al.*, 2013a). Several proteins that are involved in either ribosome production (Mybbp1a, NML and B23), mRNA transcription (HEXIM1) or splicing (SRSF1) have recently been linked to regulating p53 activity (see below).

1.5.1 SFSF1

Serine/Arginine rich Splicing Factor 1 (SRSF1), known as Alternative Splicing Factor 1 (ASF/SF2) was first identified as an alternative splicing factor protein, which is also involved in post-splicing events such as translation initiation (Michlewski *et al.*, 2008), nuclear export of mature mRNA and nonsense mediated decay (Long and Caceres, 2009). The SRSF1 gene is up regulated in breast cancer and its overexpression was shown to promote transformation of mammalian cells (Anczukow *et al.*, 2012; Anczukow *et al.*, 2015).

Recently it has been reported that SRSF1 interacts with RPL5 (Fregoso *et al.*, 2013). Interestingly they also showed that in BJ-TT cells, SRSF1 depletion abolishes ActD-induced p53 activation. This suggests that SRSF1 is involved in the 5S RNP-p53 regulatory signalling pathway (Fregoso *et al.*, 2013).

In addition, Loren Gibson, a former member of the Watkins lab, confirmed that SRSF1 not only interacts with RPL5, but also the 5S RNP in U2OS cells (unpublished data). Unpublished results further demonstrated that the overexpression of SRSF1 stabilises p53, whereas depletion of SRSF1 reduces p53 basal activity and counteracts ActD-induced p53 activation in U2OS cells. SRSF1 depletion was also shown to reduce 5S RNP recruitment into the ribosome (Loren Gibson, unpublished data). Taken together, it was proposed that in normal conditions, SRSF1 is involved in the 5S RNP integration into the large subunit. In response to nucleolar stress, SRSF1 assists the 5S RNP in inhibiting MDM2.

Moreover, SRSF1 is highly phosphorylated and its function and cellular localization is regulated by its phosphorylation (Ma *et al.*, 2008). One of the kinases involved is the serine/arginine-rich protein-specific kinase (SRPK). SRPK phosphorylates specifically multiple serine residues located in the arginine/serine rich region (also known as the RS domain)

(Giannakouros *et al.*, 2011; Gammons *et al.*, 2013). Therefore, SRSF1 could be an important player in both ribosome biogenesis and p53 signalling.

1.5.2 HEXIM1

HEXIM1 protein has been well characterized as a positive transcription elongation factor b (P-TEFb) inhibitor which associates with RNA Pol II (Yik *et al.*, 2003; Michels *et al.*, 2004). Lew *et al.* recently reported that HEXIM1 positively regulates p53. They first identified the interaction between p53 and HEXIM1. They further reported evidence showing that overexpression of HEXIM1 leads to stabilised and more active p53, whereas depleting HEXIM1 inhibits p53 activation in response to DNA damage and translation blockage stress in MCF7 cells (Lew *et al.*, 2012). Since HEXIM1 is a substrate for ubiquitination by MDM2 (Lau *et al.*, 2009), it was proposed that HEXIM1 stabilises p53 by antagonizing the MDM2 ubiquitination function upon p53 (Lew *et al.*, 2012; Lew *et al.*, 2013).

Lew *et al.* also showed that ActD treatment, which blocks ribosome biogenesis, increased association between HEXIM1 and p53 in MCF7 cells (Lew *et al.*, 2012), suggesting HEXIM1 could be a potential protein factor involved in the 5S RNP-p53 regulatory signalling pathway. Nevertheless, how HEXIM1 is related to ribosomal stress-inducing p53 activation remains unclear.

1.5.3 Mybbp1a

Myb-binding protein 1a (Mybbp1a) was originally identified as a binding protein for the proto-oncogene c-Myb and since then, increasing evidence connects its involvement in rRNA production and in p53 acetylation and regulation, suggesting its association with ribosome biogenesis and p53 regulation.

Mybbp1a is a nucleolar protein that is associated with RNA Pol I in HeLa cells, and the expression of Mybbp1a represses rRNA transcription (Hochstatter *et al.*, 2012). They also showed that Mybbp1a is linked to rRNA processing. Furthermore, they demonstrated that Mybbp1a depletion delays the proliferation of HeLa cells, suggesting that it is also needed for cell cycle regulation (Hochstatter *et al.*, 2012).

Under ribosomal stress, Mybbp1a is translocated from the nucleolus to the nucleoplasm and induces p53 acetylation via the transcription coactivator P300, which leads to the

tetramerization of p53 (Kuroda *et al.*, 2011; Ono *et al.*, 2013). However, more intriguingly, ActD treatment promotes p53-K382 acetylation and increases protein levels of p21 in MCF7 cells, whereas depleting Mybbp1a counteracts such effects caused by the ActD treatment (Kuroda *et al.*, 2011).

1.5.4 NML

Nucleomethylin (NML; also known as RRP8) was identified as a component of the energy-dependent nucleolar silencing complex (eNoSC). This complex includes two other protein components, sirtuin 1 (SIRT1) and Histone-lysine N-methyltransferase (SUV39H1). NML helps to recruit SIRT1 and SUV39H1, resulting in methylation and deacetylation of histone H3K9 which inhibits rDNA transcription when cells are in low glucose conditions (Vaquero *et al.*, 2007; Murayama *et al.*, 2008; Yang *et al.*, 2013).

NML is an RNA binding protein associating with 5S, 5.8S and 28S rRNA. The NML-SIRT1 protein interaction is inhibited by the rRNA binding. During nutrient starvation, reduced rRNA production which promotes eNoSC recruitment via NML, results in a positive feedback loop further inhibiting the production of rRNAs. It was proposed that the NML-SIRT1 interaction couples nutrient levels to ribosome biogenesis (Yang *et al.*, 2013).

NML is also a methyltransferase which is essential for the N^1-methyladenosine (m^1A) modification in the human and mouse 28S rRNA (Waku *et al.*, 2016). In addition, Oie e*t al.* reported evidence showing NML protein is also linked to fatty acid metabolism in a mouse model. NML-null mice showed reduced lipid accumulation in liver cells and after feeding these mice with a high fat diet, their weight gain and fat accumulation were lower than their wild type counterparts fed with the same diet (Oie *et al.*, 2014). Kumazawa et al. provided evidence showing that in response to the glucose starvation Mybbp1a translocates to the nucleoplasm where it promotes p53 acetylation and activation. They also showed that Mybbp1a depletion reduces p53 activation in response to glucose starvation (Kumazawa *et al.*, 2011).

1.5.5 B23

Nucleophosmin, also known as phosphoprotein B23, NPM1 or numatrin, is a multifunctional protein which is involved in multiple stages of ribosome biogenesis (Lindström, 2011). Wild

type B23 is predominantly localized in the nucleolus, whereas the NPMc+, a mutated form of B23 caused by chromosomal aberrations, is found mislocated in the cytoplasm in acute myeloid leukaemia (AML) cells (Wang *et al.*, 1993). There are approximately 35% of AML patients that have NPMc+ mutations (Federici and Falini, 2013).

The interaction between B23 and MDM2 was confirmed biochemically in SaOS-2 cells and UV radiation increases B23/MDM2 complex formation (Kurki *et al.*, 2004). Knockdown of B23 protein reduces p53 stability and activity in response to radiation treatment (Colombo *et al.*, 2002; Kurki *et al.*, 2004). It was proposed that B23 inactivates MDM2 and subsequently stabilising p53 in response to DNA damage stress.

Furthermore, evidence also suggests that B23 itself is able to associate with p53 (Kurki *et al.*, 2004; Lambert and Buckle, 2006). In addition, B23 interacts with both RPL5 and the 5S rRNA (Yu *et al.*, 2006). B23 translocating from nucleoli to the nucleoplasm was shown to be caused by ActD treatment (Huang *et al.*, 2005; Yao *et al.*, 2010; Brodská *et al.*, 2016) and UV radiation (Colombo *et al.*, 2002; Kurki *et al.*, 2004).

1.6 Aims and objectives

Ribosome biogenesis is a tightly regulated process that is essential for cell survival. Mutations in genes encoding ribosomal proteins or ribosome biogenesis factors lead to a group of rare genetic disorders called ribosomopathies, and upregulated ribosome biogenesis is often found in various cancers. Large ribosomal subunit component 5S RNP is composed of the 5S rRNA, ribosomal protein RPL5 and RPL11. This small ribonucleoprotein complex is assembled independently, later being incorporated into the large subunit precursor. When ribosome biogenesis defects occur, non-ribosomal 5S RNP accumulates and inhibits the ubiquitination function of MDM2, leading to stabilisation and activation of p53. The 5S RNP-MDM2 pathway is essential for p53 homeostasis in the cell for responding to oncogene overexpression and to defects in ribosome biogenesis. Ribosome biogenesis is also targeted for anti-cancer therapeutic treatment, showing its significance in cancer development and treatment.

A number of proteins linked to either translation regulation (HEXIM1), mRNA splicing (SRSF1) or ribosome biogenesis (Mybbp1a, B23 and NML) have recently been identified as p53 regulators. Evidence from the literature suggests that they regulate p53 via the 5S RNP-MDM2 signalling pathway. However, their role in relation to the 5S RNP remains unclear. The Splicing factor SRSF1 has recently been established as a p53 regulator and interacts with RPL5 (Fregoso *et al.*, 2013). Loren Gibson, a past member from the Watkins lab proposed that SRSF1 functions as a chaperone during 5S RNP assembly. Nonetheless, whether the function of SRSF1 is directly related to 5S RNP-p53 signalling is not yet clear.

Ribosomal protein RPL11 is important not only for 5S RNP assembly but also the 5S RNP incorporation into the large ribosomal subunit precursor. On the other hand, RPL11 binds to MDM2 and recent structural analyses highlight that the RPL11-MDM2 interface significantly colocalizes with the RPL11-28S rRNA interaction in the mature ribosome (Khatter *et al.*, 2015; Zheng *et al.*, 2015), suggesting that the RPL11 interaction is likely to be important in both ribosome biogenesis and p53 signalling. Since proteomic study revealed that RPL11 is phosphorylated at multiple residues, it is possible that the phosphorylation of RPL11 is involved in both regulating ribosome biogenesis and MDM2-p53 signalling.

Therefore, the aims and objectives in this PhD research project are,

Evaluate the role of protein cofactors HEXIM1, Mybbp1a, NML, B23 and SRSF1 in the 5S RNP-p53 regulatory signalling pathway.

Investigate the role of RPL11 phosphorylation in both ribosomal biogenesis and the 5S RNP-mediated p53 regulatory signalling.

Chapter 2. Materials and Methods

2.1 Molecular cloning and DNA analysis

2.1.1 Plasmid DNA amplification using E. coli

E. coli DH5α ultra-competent cells were prepared according to the Inoue Method (Sambrook and Russell, 2006). Approximately 100 ng of plasmid DNA was added to 100 µl of DH5α cells followed by a 30 minutes incubation on ice. Thereafter, a 90 second heat shock at 42 °C was given to the mixture, followed by an immediate return of the mixture back on ice. Then, 1 ml of Luria Broth (LB) medium was added to the mixture and incubated at 37 °C shaking for one hour. The cells were inoculated on a selective LB agar plate and incubated overnight at 37 °C for transformants selection.

2.1.2 Plasmid DNA extraction from E. coli

A single colony of *E. coli* cells acquired the plasmid DNA transformant was inoculated in 6 ml LB medium containing suitable antibiotic (100 µg/ml of Ampicillin and/or 10 µg/ml of Chloramphenicol dependent on the plasmid backbone) and incubated overnight at 37 °C. The plasmid DNA was extracted the next day from the overnight culture using the GeneJet Plasmid Miniprep Kit (ThermoFisher Scientific) according to the protocol provided.

2.1.3 DNA sequencing

Plasmid DNA sequencing was performed using Sanger sequencing service provided by Source Biosciences to analyze the plasmid construct. Primers (provided by Source Biosciences) targeted DNA sequences either upstream or downstream of the open reading frame.

2.1.4 Site-directed mutagenesis PCR

PCR with designed primers (listed in Table 2.1) was performed to introduce mutations into the FLAG-tagged RPL11 cDNA sequence cloned within a pcDNA5/FRT/TO plasmid backbone, a plasmid which was previously constructed in the Watkins lab. Approximately 200 ng of plasmid DNA was subjected to the PCR reaction, with 2.5

units of Pfu Turbo DNA polymerase (Agilent Technologies 600254), 1x Pfu Turbo buffer, 100 ng of each primer (mixture of forward and reverse, Table 2.1) and 200 nM dNTPs. PCR cycle conditions were used according protocols provided by the manufacturer.

The PCR products were treated with DpnI restriction enzyme (Promega) at 37 °C for 60 minutes to remove the methylated template, before transformation of DH5α *E. coli* competent cells. Site-directed mutations were confirmed by Sanger sequencing (methods are described in previous sections).

Gene	Mutation	Primer direction	5' to 3' Sequence
RPL11	S29A	Forward	CATCTGTGTTGGGGAGGCTGGAGACAGACTGACGC
		Reverse	GCGTCAGTCTGTCTCCAGCCTCCCCAACACAGATG
	S29D	Forward	CATCTGTGTTGGGGAGGATGGAGACAGACTGACGC
		Reverse	GCGTCAGTCTGTCTCCATCCTCCCCAACACAGATG
	T47V	Forward	GCTCACAGGGCAGGTCCCTGTGTTTTCCAAAGC
		Reverse	GCTTTGGAAAACACAGGGACCTGCCCTGTGAGC
	T47E	Forward	GCTCACAGGGCAGGAGCCTGTGTTTTCCAAAGC
		Reverse	GCTTTGGAAAACACAGGCTCCTGCCCTGTGAGC
	S51A	Forward	GGCAGACCCCTGTGTTTGCCAAAGCTAGATACACTG
		Reverse	CAGTGTATCTAGCTTTGGCAAACACAGGGGTCTGCC
	S51D	Forward	GGCAGACCCCTGTGTTTGACAAAGCTAGATACACTG
		Reverse	CAGTGTATCTAGCTTTGTCAAACACAGGGGTCTGCC
	T73V	Forward	GCTGTCCACTGCGTAGTTCGAGGGGCC
		Reverse	GGCCCCTCGAACTACGCAGTGGACAGC
	T73E	Forward	GCTGTCCACTGCGAAGTTCGAGGGGCC
		Reverse	GGCCCCTCGAACTTCGCAGTGGACAGC
	S140A	Forward	GGTAGGCCAGGTTTCGCCATCGCAGACAAGAAGC
		Reverse	GCTTCTTGTCTGCGATGGCGAAACCTGGCCTACC
	S140D	Forward	GGTAGGCCAGGTTTCGACATCGCAGACAAGAAGC
		Reverse	GCTTCTTGTCTGCGATGTCGAAACCTGGCCTACC

Table 2.1. Primers for RPL11 site-directed mutagenesis PCR.

2.1.5 E. coli transformation and plasmids for recombinant protein expression

recombinant protein	plasmid backbone	Source
GST-RPL5	pGEX6P1	Dr. Loren Gibson
GST-RPL11		
GST-Tip48		
His-SRS1	unknown	gift from Prof. Reinhard Lührmann

Table 2.2. List of plasmid construct used in recombinant protein expression.

Plasmids were transformed into *E. coli* Strain: BL21 Rosetta™ (Novagen). Expressed proteins were purified by Glutathione or Nickel resin. To examine protein yields and qualities, purified product was separated by SDS-PAGE and visualized by coomassie blue staining.

Freshly plated *E. coli* cells were resuspended thoroughly to 120 µl of 50mM $CaCl_2$. Then, 1 µl of recombinant plasmid was added to the cells and the mixture was incubated on ice for 15 minutes. This was followed by a heat shock for 90 seconds at 42 °C, before incubation on ice for another 5 minutes. 700 µl of LB media was then added and the cells were incubated at 37 °C for 30 minutes. The cells were retrieved by centrifugation and plated on ampicillin chloramphenicol double selective plates and incubated at 37 °C overnight for transformant selection.

2.2 RNA extraction, detection and analysis

2.2.1 RNA extraction

RNA was extracted from cells harvested after experimental treatment using Tri-Reagent (Ambion) according to the protocol provided by the manufacturer.

2.2.2 Agarose-glyoxal gel electrophoresis and Northern blotting

The extracted RNA was dissolved in 1x glyoxal loading buffer (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 ethidium bromide) and incubated at 55 °C for 60 minutes. RNA samples were separated on a glyoxal-agarose gel (1.2 %

Agarose (Melford) dissolved in 1x BPTE (30 mM Bis-Tris free acid pH 7.0, 10 mM PIPES free acid, 1 mM EDTA) by electrophoresis at 185V in 1x BPTE buffer. The gel was washed with 75 mM NaOH for 20 minutes (room temperature), then washed twice with Tris-Salt pH 7.4 (0.5 mM Tris-HCl pH 7.4, 1.5 M NaCl) for 15 minutes at room temperature, followed by washing the gel with 6x SSC buffer (1 M NaCl, 0.1 M Sodium citrate) for 20 minutes at room temperature. The separated RNA was transferred to a Hybond-N nylon membrane (GE Healthcare) using the capillary action method, before UV cross-linking using a Stratalinker UV Crosslinker (Stratagene).

For detecting RNA using the 5' ^{32}P-labelled oligo RNA probes, the Hybond-N nylon membrane was pre-hybridized with SESI buffer (0.5 M sodium phosphate pH 7.2, 7 % SDS (w/v), 1 mM EDTA) for 30 minutes at 37 °C. Then, it was incubated with the probe for overnight to 2 days at 37 °C. After the probing, the membrane was washed twice with 1x SSC/0.1 % SDS solution for 20 minutes at 37 °C. The membrane was exposed to a phosphorimager screen and the radioactive signal was scanned by a Typhoon Phosphorimager (Life Technologies).

For detecting RNA using the random-prime ^{32}P-labelled probes, the membrane was incubated with the hybridization (Pre-Hyb) buffer (25 mM $NaPO_4$ pH 6.5, 6x SSC, 5x Denhardts, 0.5 % SDS (w/v), 50 % deionised formamide, 100 µg/ml denatured salmon sperm DNA) for two hours at 42 °C. Then, it was incubated with the probe for overnight to 2 days at 37 °C. After the probing, the membrane was washed twice with the 2x SSC/0.5 %SDS for 5 minutes at 42 °C, before being washed with 2x SSC/0.1 % SDS for 20 minutes at 50 °C. Detection was performed as above using a Typhoon Phosphorimager.

2.2.3 Nucleic Acid labelling using ^{32}P isotope for Northern blotting

Oligo probes (Table 2.3) were purchased from Sigma Aldrich and subsequently labelled with ^{32}P γ-ATP (Perkin Elmer) in the lab. 1 µl of 10 µM oligo probe was used in a 20 µl reaction containing 1 µl of T4 Polynucleotide Kinase (1 U/1 µl, NEB), 2 µl of 10x Polynucleotide Kinase Buffer (NEB) and 4 µl of ^{32}P labelled γ-ATP and incubated at 37 °C for 45 minutes.

At the end of the reaction, reaction volume was adjusted to 50 µl followed by G50 column purification (GE Healthcare). The unbound isotope was removed by centrifugation of the column at 0.9 g for 2 minutes. The labelled probes were retrieved from the column according to the manufacturer's instructions. Probes were incubated at 95 °C for 2 minutes and then added to SES1 buffer for the hybridization process.

Target	Sequence 5' to 3'
5S rRNA	CCGAGATCAGACGAGATCGGGCGCGTTCAGGGTGGTATGG
5.8S rRNA	CAATGTGTCCTGCAATTCAC

Table 2.3. A list of ^{32}P-labelled probes used in northern blotting.

2.3 Protein related assays

2.3.1 Recombinant protein expression

A single colony of the transformed *E. coli* BL21 Rosetta cells was incubated overnight at 37 °C in a 10 ml starter culture. The next day it was diluted 1 : 100 in 1 L of 2x YT medium (Sigma Aldrich) containing 100 µg/ml of Ampicillin and 10 µg/ml of Chloramphenicol. The culture was incubated at 37 °C until the beginning of exponential growth, then 1 mM of IPTG was added to the culture to induce protein expression. The culture was then incubated at 18 °C overnight, before the cells were harvested for recombinant proteins purification.

2.3.2 His-tagged and GST-tagged protein purification

At the end of incubation, the culture was centrifuged in a Beckman J6-HC rotor at 4,000 rpm for 30 minutes at 4 °C to pellet the cells. Cells were resuspended in ice cold GST buffer (20 mM Tris-HCl at pH8, 5 mM $MgCl_2$, 300 mM KCl, 0.1% Tween and 10%

Glycerol). After repeated washing with the GST buffer, the cells were lysed by sonication (3 minutes/5 cycles/90% power). Insoluble cellular material was removed by high speed centrifugation in the JA20 rotor at 18,500 rpm for 45 minutes at 4 °C while the supernatant was recovered.

Glutathione Sepharose beads (Glutathione Sepharose 4 Fast Flow, GE Healthcare Life Sciences) were added to the supernatant to capture the GST-tagged proteins according to the manufacturer's protocol. The beads were recovered, washed and the bound GST-tagged protein was eluted using the GST buffer containing 50mM of glutathione.

For His-tagged proteins, Ni Sepharose beads (Ni Sepharose 6 Fast Flow, GE Healthcare Life Sciences) were used to capture the recombinant protein. Beads were washed with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 to 40 mM imidazole, pH 7.4) and the bound protein was eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4).

2.3.3 Protein dialysis and concentration

The purified recombinant proteins were dialyzed in order to remove glutathione or imidazole. Protein solutions were placed into the size exclusion semipermeable membrane, the Snakeskin Dialysis Tube (Thermofisher Scientific). The sealed membrane was then placed into a large volume of PBS buffer for sufficient time to let buffers exchange. If necessary, the protein solution was concentrated in 500 ml PBS containing 30% PEG8000 (Sigma-Aldrich) at 4 °C for 5-10 hours until the protein solution was reduced to the desired volume.

2.3.4 Protein separation by SDS-PAGE and Coomassie staining

Protein separation was performed using 12% SDS-PAGE, casted using liquid polyacrylamide solution and the Mini-PROTEAN® Tetra Handcast Systems (Bio-Rad). The separation section of the SDS-PAGE consisted of 12% polyacrylamide, 375 mM Tris/HCl pH 8.8 and 2% SDS in water, and the mixture was solidified by 0.1% ammonium persulfate (APS) and 0.2% TEMED.

Gel electrophoresis was run in a Bio-Rad Western Electrophoresis tank, filled with 1x Protein Running Buffer (25 mM Tris-HCl pH 8.3, 250 mM glycine, 0.1 % SDS (w/v)) at

200 V for 40 minutes. The separated proteins were then analyzed by Coomassie staining and/or for Western blotting dependent on the experiment design.

For Coomassie staining a ten-fold dilution of Coomassie stain in 1.0 % in ethanol in a fixation solution (10 % methanol, 10 % acetic acid, 10 % isopropanol and 5 % glycerol) was prepared. After soaking the gel in the stain for approximately one hour, the gel was destained by washes in the fixation solution until the protein signals were visible and the background colour stain was minimised. The image of the stained protein gel was then digitalized as 300dpi images using a HP professional high definition scanner.

2.3.5 Western blotting and antibodies

For Western blotting, protein samples were first separated on SDS-PAGE using conditions stated in the previous section, before being transferred onto a nitrocellulose membrane (Amersham Protran Premium 0.2 NC, GE Healthcare), which was achieved using a Bio-Rad Western Transfer tank containing the Western Transfer buffer (25 mM Tris-HCl pH 8.3, 150 mM glycine, 10 % methanol).

The protein transfer was performed at 65 V for 1.5 hour at room temperature, or at 16V overnight at 4 °C. The transfer quality was examined using a quick one to five minute staining with Ponceau S Solution (Sigma).

The stained membrane was washed by PBS (Phosphate Buffered Saline) containing 0.1 % Triton (PBST), and then blocked in 2 % skimmed milk (Marvel) dissolved in PBST at room temperature for one to two hours.

Primary antibody solution was prepared as indicated in and added to the membrane for an overnight incubation at 4 °C. Membranes were then washed for three 5 - 10 minutes in PBST followed by a secondary antibody solution incubation at room temperature for one to two hours. The secondary antibody solution was prepared according to . The membranes probed with secondary antibody were washed again for 3 times 5 - 10 minutes in PBST.

2.3.6 Visualization and digitalization of Western blots

This work involved two types of signals for visualizing proteins, chemiluminescence (HRP congregated secondary antibody, ECL substrate) or fluorescence (fluorophore congregated secondary antibody) and three different detection methods were used, including X-ray films, chemiluminescence biomolecular imager and fluorescence scanner.

For blots probed with secondary antibodies conjugated with Horseradish Peroxidase (HRP), the membranes were developed by adding ECL substrate (Clarity™ ECL Western Blotting Substrate, Bio-Rad) and the signals were captured on X-ray films (Amersham Hyperfilm ECL, GE Healthcare). The film was processed using an automatic film developer. The developed X-ray films were scanned and digitalized at 300dpi resolution using a HP professional high definition scanner. Alternatively, the chemiluminescence signal was captured and digitalized by an ImageQuant™ LAS 4000 mini biomolecular imager (GE Healthcare Life Sciences).

For those membranes blotted with fluorescently labelled secondary antibodies, the antibody signals were visualised by using the Odyssey-LICOR system (LICOR). Digitalized western blot images generated using either methods were analysed using the ImageQuant™ TL software (GE Healthcare Life Sciences). The same software was used to quantify unsaturated protein signals.

	Antibody	Animal origin	Dilution	Source
Primary antibody	α-HEXIM1	Rabbit	1 in 5,000	Bethyl A303-113A
	α-Mybbp1a	Rabbit	1 in 1,000	Bethyl A301-327A
	α-NML (RRP8)	Rabbit	1 in 2,000	Bethyl A304-201A
	α-B23 (NPM1)	Mouse	1 in 1,000	Santa Cruz sc-32256
	α-MDMX	Rabbit	1 in 2,000	Bethyl A300-287A
	α-p53	Rabbit	1 in 1,000	Santa Cruz- (FL-393)
	α-p21	Rabbit	1 in 1,000	Santa Cruz- sc-397
	α-FLAG	Rabbit	1 in 10,000	Sigma Aldrich-F725
	α-RPL7	Rabbit	1 in 2,000	Abcam- ab72550
	α-Poly His	Mouse	1 in 2,000	Santa Cruz H-3
Loading control	α-Fibrillarin	Mouse	1 in 200	Santa Cruz G2808
	α-Karyopherin	Rabbit	1 in 500	Santa Cruz (H-300)
	α-Tubulin	Mouse	1 in 10,000	Cell Signalling (DM1A)
Secondary antibody	α-Mouse HRP conjugated	Donkey	1 in 10,000	Santa Cruz- sc-2314
	α-Rabbit HRP conjugated	Donkey	1 in 10,000	Santa Cruz- sc-2313
	α-Rabbit IRDye® 800CW	Donkey	1 in 10,000	LICOR 926-60073

Table 2.4. List of antibodies used in western blotting. Antibodies were diluted in PBST or TBST containing blocking agent recommended by the manufacturer.

2.3.7 Luciferase assay

U2OS cells stably expressing firefly luciferase controlled by the p53-regulated promoter sequence were cultured in 24-well plates (conditions as stated in section Cell culturing, the cells were kindly provided by Professor Neil Perkins, Newcastle University). After experimental treatments the cells were washed with PBS and harvested using 1x Passive Lysis Buffer (Promega) according to manufacturer's protocol. Luciferase reaction substrate (Luciferase Assay System, Promega) was prepared according to manufacturer's protocol. The luciferase assay was performed

according to the protocol provided by Promega and the luciferase intensity was measured using a Lumat 100 luminometer (Berthold).

Cell lysate samples were also subjected to a Bradford assay in order to determine the total protein concentration (Bio-Rad) according to the manufacturer's protocol. Luciferase intensity was normalized against the protein centration.

2.3.8 Bradford assay

Protein concentrations were measured using the Bradford protein assay kit (Bio-Rad) according to the protocol provided by the manufacturer. The optical density (OD) of the samples were measured at 595 nm wavelength using a spectrophotometer (Ultrospec 2000, Pharmacia Biotech).

2.4 In vitro assays

2.4.1 Protein pull-down assay

Purified GST-tagged proteins were incubated with His-SRSF1 for 1 hour at 4 °C (incubation buffer: 20 mM Tris-HCl at pH 8, 5 mM $MgCl_2$, 150 mM KCl, 0.1% Tween and 10% Glycerol). Glutathione sepharose beads were subsequently used to extract GST-tagged protein by one hour incubation and retrieved by centrifugation. Beads were washed with incubation buffer followed by analysis of the bound material using western blotting with anti-His antibody.

To investigate the interaction between SRSF1 and the 5S RNP *in vitro*, equal amount of GST-tagged RPL5, RPL11 or Tip48 were incubated individually with same amount of His-SRSF1 at 4 °C for 5 hours before purification with glutathione sepharose resin. The resins was subsequently washed and the bound material was denatured and separated by SDS-PAGE. To confirm the interaction, an anti-His tag antibody was used in western blotting. 50% of His-SRSF1 input were processed in parallel and used as a positive control.

2.5 Cell culturing and in vivo assays

2.5.1 Cell culturing and Human tissue cell line

U2OS human osteosarcoma cells were maintained in DMEM (Dulbecco's Modified Eagle Medium, Sigma) with 10% fetal bovine serum and 2% penicillin-streptomycin solution. Cells were incubated at 37 °C with 5% CO_2.

2.5.2 RNA interference

7 µl of 20 mM of siRNA stock were used to transfect cells at 50% confluence with 5 µl of Lipofectamine RNAiMAX (Invitrogen) in 2 ml of optiMEM solution. Cells were incubated in antibiotic-free DMEM. The cells were harvested and prepared into samples 48 hours after the siRNA transfection. The siRNA effect of protein knockdown was subsequently determined by Western Blotting.

siRNA	Sequence 5' to 3'	Source	Reference
GL2	CGTACGCGGAATACTTCGATT	Eurofins MWG	(Sloan *et al.*, 2013a)
HEXIM1	SMARTpool	ThermoFisher SCIENTIFIC	(Lew *et al.*, 2013)
Mybbp1a	UGGAUCAUCUUUCGAUUGG	Eurofins MWG	(Hochstatter *et al.*, 2012)
NML	UUCUCCGAACGUGUCACGUTT	Eurofins MWG	(Grummt and Ladurner, 2008)
B23	UGAUGAAAAUGAGCACCAG	Eurofins MWG	(Colombo *et al.*, 2002)
MDMX	AGAUUCAGCUGGUUAUUAAATT	Eurofins MWG	(Gilkes *et al.*, 2006)

Table 2.5. List of siRNAs used in this work.

2.5.3 Stable inducible cell lines creation

Stable inducible cell lines were created using the Flp-In™ system. pcDNA5/FRT/TO plasmid carrying the target gene insert and the pOG44 plasmid were co-transfected into U2OS Flp-In cells grown in 6-well plates using FuGene 6 Transfection Reagent (Promega) according to the protocol provided by the manufacturer. Cells were transferred to T75 flask from 6-well plates and left to grow for 72 hours before selection of the stable transformants using 100 µg/ml hygromycin B and 10 µ g/ml Blasticidin S.

2.5.4 Drug treatments

Human cells were subjected to chemical or drug treatments listed in Table 2.6, before harvested and analyzed as described.

Drug	Dose	Solvent	Treatment length
ActD	5 nM	ethanol	18 hours
Nutlin 3a	5 µM	DMSO	18 hours
SRPIN340	10 µM	DMSO	12 hours

Table 2.6. List of drugs or chemicals used in cell culture.

2.5.5 Immunofluorescence and microscopy

Cells were seeded and grown on glass coverslips in 24-well plates. At the end of the experimental treatment, the coverslips were washed with PBS, before incubation in 200 µl of PBS/0.1 % Triton for 15 minutes, followed by three PBS washes.

Antibody	Animal origin	Dilution in PBS/10% FBS/0.2% Triton	Source
ANTI-FLAG® antibody, polyclonal	Rabbit	1/500	SIGMA-ALDRICH (MERCK)
anti-Rabbit IgG Secondary Antibody, Alexa Fluor 555	Donkey	1/500	ThermoFisher SCIENTIFIC

Table 2.7. List of antibodies used in immunofluorescence.

The blocking reagent was made up of 0.1 % Triton and 10 % Fetal Calf Serum (FCS) in PBS. After the PBS washes the coverslips were treated with the blocking reagent for approximately one to two hours at room temperature, before transferring the cells to a fresh 24-well plate where they were probed with primary antibody for one to two hours at room temperature. The primary antibody solution was prepared by diluting the antibody in the blocking reagent.

Then, the coverslips were washed with PBS for three times, followed by a 10 minutes PBS soaking step. This was followed by a one to two hours secondary antibody incubation in the dark. The secondary antibody solution was prepared as the primary antibody solution. At the end, the coverslips were washed with PBS in the dark three times, followed by a 10 minutes PBS soaking step. Then, PBS containing 0.01 ng/ml DAPI (Sigma) was used to wash the coverslips for three times, following by an additional 10 minutes PBS soaking step in the dark.

Finally, the samples were soaked in deionized water followed by ethanol for five times. Coverslips were air-dried and mounted on glass slides with Moviol. Samples were visualized using a Zeiss Axiovert 200M inverted microscope with a Plan-Apochromat 100x / 1.40 oil DIC x / 0.17 objective lens. The digital images were captured with an AxioCam HRm camera, and they were analyzed using the AxioVision software (Zeiss).

2.5.6 Glycerol gradient for ribosomal material separation

4 ml 10-40 % glycerol gradients(v/v) were prepared by combining 2 ml of 40% glycerol solution (bottom; 40 % Glycerol, 0.15 M KCl, 20 mM HEPES pH 8, 1.5 mM $MgCl_2$, 1 mM DTT, 0.2 % Triton X-100) and 2 ml of 10% glycerol solution (top; 10 % Glycerol, 0.15 M

KCl, 20 mM HEPES pH 8, 1.5 mM $MgCl_2$, 1 mM DTT, 0.2 % Triton X-100) in a thin wall Ultra-Clear Centrifuge Tube (Beckman Coulter), mixed with a BioComp Gradient master (BioComp) rotating at a 83° angle for 70 seconds at 22rpm, before storing at 4 °C for 1 hour.

Gradient samples were prepared from 5 - 7x10^6 cells. They were first sonicated in 0.5 ml of ice cold Gradient Buffer E (20 mM HEPES pH 8, 150 mM KCl, 0.5 mM EDTA, 0.1 mM DTT, 5 % Glycerol) with twice a 15 second pulse, with a 30 second interval at 20 % /0.3 second amplitude. 0.2 % Triton-X 100 was added to the sample and the insoluble material was removed by centrifugation at 13,000 rpm for 10 minutes at 4 °C.

0.4 ml of solution was removed from the top of the gradient and replaced with the same volume of sample, before centrifuging in a SWTi60 rotor (Beckman L7-80) at 52,000rpm for 90 minutes at 4 °C with no brakes and slow acceleration. 40 µl of the remaining sample were stored and used later as 10% total input control during sample analysis. Each gradient was fractionated into 20 200 µl fractions from top to bottom and snap frozen in liquid nitrogen prior to storage at -80 °C.

2.5.7 Pulse trace labeling experiments for ribosome biogenesis study

Cells were seeded and grown in a 6-well plate for the experimental treatment. Prior to the labelling, cellular phosphate was depleted by incubating the cells in phosphate-free medium (Life Technologies) at 37 °C/5 % CO_2 for one hour. Then, the cells were incubated with phosphate-free medium containing inorganic ^{32}P-orthophosphate (0.5 µl/ml) at 37 °C/5 % CO_2 for another hour. After that cells were cultured with normal DMEM media (Sigma Aldrich) for an additional three hours. Cellular total RNA was extracted using Tri-reagent and separated on a glyoxal-agarose gel, before the being transferred onto a Hybond-N nylon membrane (GE Healthcare). A phosphorimager screen was used to expose the radioactivity and the radiolabeled RNA signals were visualized using a Typhoon Phosphorimager (Life Technologies).

2.6 Software and statistical analysis

Pymol software was used to present protein-RNA molecular structures. Software ImageQuant™ TL (GE Healthcare Life Sciences) are used to perform signal quantitation

from images acquired from western blotting or northern blotting analysis. AxioVision software (Zeiss) was used to analyse the immunofluorescence images.

Statistical analysis was performed in Microsoft Excel, using the 't-test: Two-Sample Assuming Equal Variances' function from the data analysis toolkit.

Chapter 3. Characterisation of Co-factors Involved in the 5S RNP-p53 signaling

3.1 Introduction

Ribosome biogenesis is an energy-consuming process. It involves over 200 proteins and an equivalent number of non-coding RNAs in human cells. This complicated process requires coordinated expression of genes, transcription by RNA Pol I (for 18S, 5.8S and 28S rRNAs), RNA Pol II (for ribosomal proteins and biogenesis factors) and RNA Pol III (for the 5S rRNA). Ribosome biogenesis starts within a specific region in the nucleus called the nucleolus, where 18S, 5.8S and 28S rRNAs are transcribed by RNA Pol I as a single precursor transcript, called the 47S pre-rRNA. The 47S pre-rRNA is subsequently processed to become the three mature rRNAs through a series of endonucleolytic and exonucleolytic cleavage events. During these events, pre-rRNAs incorporate ribosomal proteins, forming ribosomal precursors throughout the biogenesis process. During the early stage of the process, the 90S pre-ribosome is formed around the 47S pre-rRNA, which later splits into pre-60S and pre-40S ribosomal subunits. While the majority of these processing steps take place in the nucleolus, some late ribosome maturation events take place in the cytoplasm where pre-ribosomes become mature ribosomal subunits.

In contrast to other rRNAs, the 5S rRNA is transcribed by RNA polymerase III which takes place in the nucleoplasm. The 5S rRNA is 120 nucleotides in length and it is found in all organisms, except in the mitochondrial ribosome from fungi and animals. The 5S rRNA, together with ribosomal proteins RPL5 and RPL11, forms a subcomplex of the large ribosomal subunit called the 5S RNP. In human cells, RPL5 binds and stabilises the 5S rRNA in the nucleoplasm, which is required for the maturation of 5S rRNA. After that the RPL5/5S rRNA complex is transported into the nucleolus, where it recruits RPL11. The 5S RNP is subsequently integrated into the pre-LSU.

Misregulated ribosomal biogenesis, also known as nucleolar stress, causes accumulation of non-ribosomal (free) 5S RNP which promotes p53 activation (Figure 3.1). The tumour suppressor p53, also known as the ‘guardian of the genome’, monitors genome integrity and regulates the cell cycle. It is activated by different types of cellular stress such as DNA

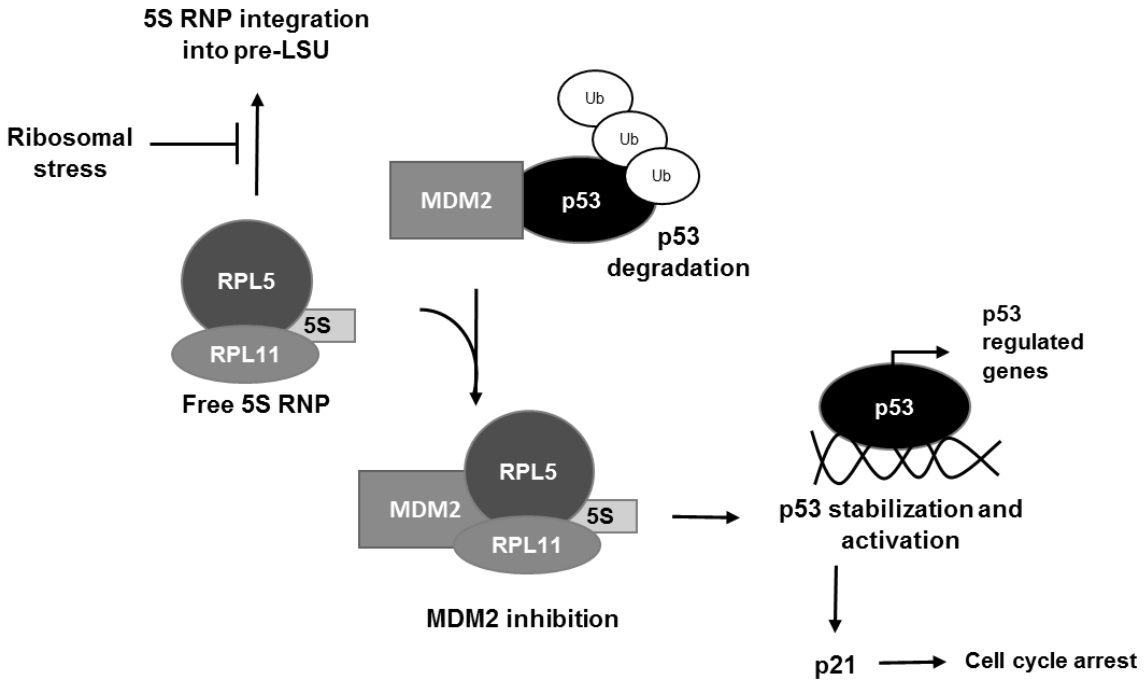

Figure 3.1. Schematic representation of the 5S RNP -p53 signalling pathway.

damage, oxidative stress, oncogene expression and nucleolar stress. In response to these stresses, p53 is stabilised and activated by post translational modifications such as phosphorylation and acetylation. It has been shown that p53 acetylation is linked to apoptosis (Reed and Quelle, 2015).

Being a transcription factor, the activated p53 drives the expression of different p53-regulated genes, resulting in cellular responses such as cell cycle arrest, DNA damage repair, senescence and apoptosis. Being one of the most important downstream factors, the transcription of *CDKN1A* gene, which encodes protein p21, is controlled by the transcription activity of p53 (Figure 3.1). p21, also known as cyclin-dependent kinase (CDK) inhibitor 1, interacts and inhibits CDK complexes which regulate cell cycle at G1 to S phase. Overexpression of p21 results in cell growth arrest and senescence (Xu *et al.*, 2014).

Cellular p53 is maintained at a low level in non-stressed cells. One of the most important negative p53 regulators is MDM2. MDM2 functions as an E3 ubiquitin ligase regulating both the stability and the activity of p53. MDM2 interacts with p53 through its N-terminal binding site, inducing the polyubiquitination of p53 which targets p53 for proteasome-mediated degradation. Furthermore, the binding of MDM2 to p53 inhibits the transcriptional activity of p53. However, being one of the p53 transcriptional target, MDM2 expression is promoted

by p53 activation and it therefore forms a negative feedback loop to regulate p53 (Barak *et al.*, 1993).

Murine double minute X, also known as MDMX or MDM4 protein, also plays an important role negatively regulating p53. MDMX shares 90% homology with MDM2 mainly in its p53-binding domain and the RING domains (Shvarts *et al.*, 1996). However, in contrast to MDM2, MDMX does not have the E3 ligase activity and it lacks the nuclear localisation/export sequence (Biderman *et al.*, 2012). In non-stressed cells, MDMX interacts with MDM2 through its RING domain and they function as one complex in regulating p53. The MDMX association to MDM2 stabilises MDM2 enhancing the MDM2 ubiquitination function of p53. Nevertheless, MDMX is also a polyubiquitination target of MDM2 (Shadfan *et al.*, 2012). In response to ribosomal stress, Gilkes *et al.* found that MDMX degradation was required for ribosomal stress-mediated p53 activation in an MDM2-dependent manner. They also demonstrated that overexpression of MDMX increases cellular resistance to ribosomal stress induced by 5-Fluorouracil (Gilkes *et al.*, 2006).

Recent studies have linked nucleolar stress, caused by defects in ribosome biogenesis, to p53 activation through the inactivation of MDM2. Ribosome biogenesis is affected in almost all stress conditions causing accumulation of free 5S RNP which is not integrated into the LSU. The accumulated non-ribosomal 5S RNP binds to, and inhibits the MDM2 ubiquitination function causing p53 activation (Horn and Vousden, 2008; Chakraborty *et al.*, 2011; Donati *et al.*, 2013; Sloan *et al.*, 2013a; Pelava *et al.*, 2016). Furthermore, the C305F mutation in MDM2 which was originally identified in cancer cells, has been shown to block the interaction between the 5S RNP and MDM2 (Macias *et al.*, 2010). This mutation causes mice to become insensitive to certain anti-cancer chemotherapeutics and being prone to c-Myc driven cancers (Macias *et al.*, 2010). It has also been shown that p53 is activated through the 5S RNP in response to different cellular stresses, including nucleolar stress, oxidative stress, replicative stress, oncogene overexpression and nutrient starvations (Orsolic *et al.*, 2015). The 5S RNP not only activates p53 during nucleolar stress, but was also found to be involved in regulating p53 homeostasis (Sloan *et al.*, 2013a).

Ribosome biogenesis is a tightly regulated and complicated process requiring the coordination of a large number of protein factors. It was therefore proposed that proteins involved in 5S RNP production, localisation and its integration into the ribosome could be

important in the 5S RNP-p53 regulation (Sloan *et al.*, 2013a). For example, it has been demonstrated that the tumour suppressor PICT1, which is involved in the 5S RNP complex integration into the ribosome, determines free 5S RNP abundance and controls cellular p53 levels (Sloan *et al.*, 2013a). In addition to PICT1, there are several other protein factors have been proposed to be involved in p53 regulation via the 5S RNP.

The splicing factor SRSF1 is involved in mRNA splicing, translation initiation, mature mRNA export and nonsense mediated decay (Michlewski *et al.*, 2008; Long and Caceres, 2009). A recent publication showed that SRSF1 binds to RPL5 and that this is essential for the ActD-induced p53 activation (Fregoso *et al.*, 2013). Loren Gibson, a former member of the Watkins lab, further demonstrated the molecular interaction between SRSF1 and the 5S RNP components (unpublished data). Furthermore, it has also been demonstrated that overexpressing SRSF1 promotes p53 stabilisation. Hence it was proposed that SRSF1 assists 5S RNP in p53 activation in response to ribosomal stress.

The positive transcription elongation factor b (P-TEFb) inhibitor, HEXIM1 protein, is involved in translation (Yik *et al.*, 2003; Michels *et al.*, 2004). It was reported that HEXIM1 is not only a p53 binding protein, but also a positive p53 regulator (Lew *et al.*, 2012). They further demonstrated that ActD treatment in MCF7 cells increases the association between HEXIM1 and p53, suggesting regulation of p53 via 5S RNP.

Mybbp1a protein is a ribosome biogenesis factor involved in pre-rRNA transcription (Hochstatter *et al.*, 2012). It has been demonstrated that when ribosomal stress occurs, Mybbp1a translocates from the nucleolus to nucleoplasm where it induces p53 acetylation via the transcription coactivator P300. This results in the tetramerisation of p53 (Kuroda *et al.*, 2011; Ono *et al.*, 2013). Furthermore, ActD treatment in MCF7 cells promotes p53 acetylation resulting in p53 activation (Kuroda *et al.*, 2011), suggesting Mybbp1a could be involved in the 5S RNP-p53 signalling.

NML protein (Nucleomethylin) is a component of the energy-dependent nucleolar silencing complex (eNoSC) which inhibits rDNA transcription when cells are in low glucose conditions (Murayama *et al.*, 2008). NML is also a methyltransferase which is essential for the N^1-methyladenosine (m^1A) modification in the human and mouse 28S rRNAs (Waku *et al.*, 2016). NML is also important in the glucose starvation-induced p53 activation (Kumazawa *et al.*, 2011).

Protein B23 (Nucleophosmin) is a nucleolar protein involved in ribosome biogenesis. It is translocated from the nucleoli to the nucleoplasm in response to ActD treatment (Huang *et al.*, 2005; Yao *et al.*, 2010; Brodská *et al.*, 2016) or UV radiation (Colombo *et al.*, 2002; Kurki *et al.*, 2004). Furthermore, studies showed that it is able to interact with MDM2 and p53 (Kurki *et al.*, 2004; Lambert and Buckle, 2006). It has also been shown to interact with both RPL5 and the 5S rRNA (Yu *et al.*, 2006), therefore it is possible that B23 is involved in 5S RNP-p53 signalling.

Although these protein candidates, HEXIM1, Mybbp1a, NML and B23, are linked to p53 regulation, their involvement in 5S RNP-p53 signalling remains unclear. Hence in this chapter, I aim to investigate how these reported p53 regulators, and SRSF1, are involved in the 5S RNP-p53 regulatory pathway. I first use a siRNA mediated protein depletion approach to validate whether they are essential for the nucleolar stress-induced p53 activation in U2OS cells. I further characterise the importance of SRSF1 function in regulating p53 activity, using the kinase inhibitor compound SRPIN340. Eventually I report evidence showing SRSF1 function is related to p53 activation via the 5S RNP.

3.2 Results

3.2.1 HEXIM1, Mybbp1a, B23 and NML protein levels were depleted by siRNA treatment in U2OS cells

I first validated the effect of siRNAs on depleting cellular protein levels of HEXIM1, Mybbp1a, B23 and NML human osteosarcoma (U2OS) cells. Actinomycin D (ActD) was applied to cells to block ribosome biogenesis and to induce 5S RNP accumulation. ActD is an anti-cancer compound that has been widely used in research. High doses (30 nM or above) of ActD in human tissue culture inhibit RNA Pol II and Pol I function and induce DNA double strand breaks promoting transcription blockage. Low doses (below 30 nM) of ActD specifically inhibit RNA Pol I activity and do not induce DNA damage (Sobell, 1985). RNA Pol I inhibition caused by a low dose of ActD abolishes pre-rRNA transcription, disrupting small and large ribosomal subunit production which results in non-ribosomal (free) 5S RNP accumulation (Donati *et al.*, 2013; Sloan *et al.*, 2013a). Low dose ActD treatment has been commonly used in tissue culture to study the 5S RNP-p53 signalling pathway.

siRNAs targeting the mRNA of each protein of interest (siHEXIM1, siMybbp1a, siNML and siB23) were transfected to U2OS cells for 48 hours, in the presence or absence of 5 nM of ActD during the last 18 hours of culturing. To validate the efficiency of the siRNA on protein knockdown, whole cell lysates were separated by SDS-PAGE and the targeted proteins were probed by western blotting using protein-specific antibodies. An siRNA targeting firefly luciferase protein (siLuc) was used as a control.

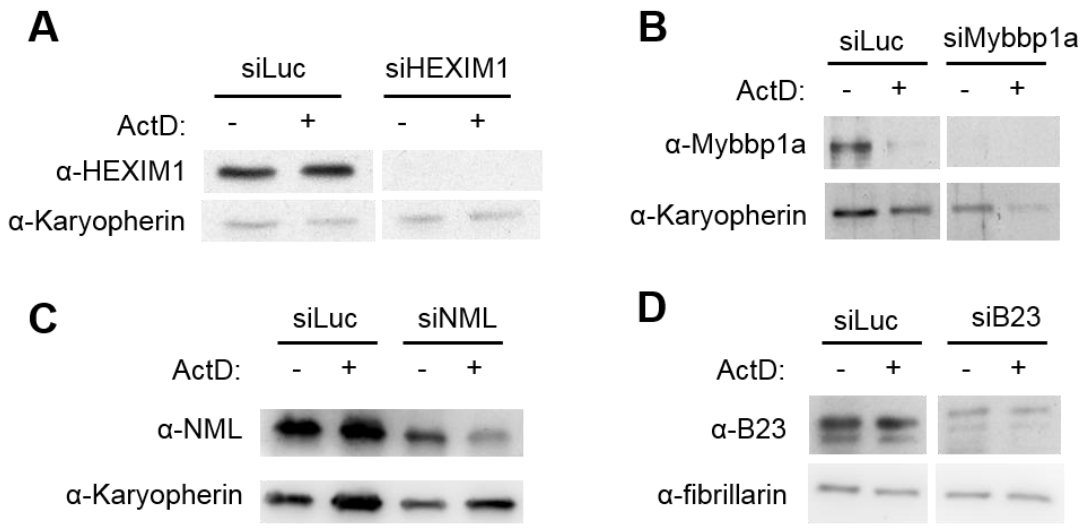

Figure 3.2. Protein levels were efficiently depleted using siRNA.

(A-D) Western blots showing protein levels in U2OS cells that had been treated with siRNAs targeting protein candidates (siHEXIM1, siMybbp1a, siNML and siB23), in the presence or absence of ActD, as indicated above each lane. siRNAs were transfected for 48 hours with addition of 5 nM ActD treatment to the cells during the final 18 hours. An siRNA targeting firefly luciferase mRNA (siLuc) was used as control. Whole cell lysates were separated on SDS-PAGE and proteins were detected by western blotting using indicated antibodies. Signals were detected using HRP/chemiluminescent method exposed on X-ray films. Karyopherin or fibrillarin protein levels were used as loading controls.

Result showed that HEXIM1 and Mybbp1a protein levels became undetectable as a result of the siRNA treatment (Figure 3.2A and B). A fourfold reduction in B23 levels was also observed (Figure 3.2D). Compared to the control, the siRNA targeting NML caused a threefold reduction in NML protein levels (Figure 3.2C). The presence of ActD did not affect the protein levels of HEXIM1, NML and B23 (Figure 3.2A, C and D). However, ActD resulted in a fourfold reduction in Mybbp1a levels (Figure 3.2B).

3.2.2 B23 protein depletion caused rRNA processing defects in U2OS cells

Since defects in ribosome biogenesis promote p53 activation, it is important to confirm whether these protein candidates are involved in ribosome biogenesis before investigating their roles in the 5S RNP-p53 signalling. I therefore used pulse chase labelling to investigate whether knocking down any of these proteins affects rRNA processing.

HEXIM1 and Mybbp1a knockdown and pulse labelling experiments were previously performed by Loren Gibson, a former member in the Watkins lab. The results showed that

while HEXIM1 depletion did not affect rRNA production, Mybbp1a protein knockdown resulted in increased 47/45S and 32S RNA levels and relative reductions in 28S and 18S RNA levels, indicating Mybbp1a is involved in rRNA production (data not shown). Consistent with these findings, Hochstatter *et al.* reported that Mybbp1a is involved in pre-rRNA transcription and also RNA processing (Hochstatter *et al.*, 2012).

To investigate the role of NML and B23 in rRNA processing, U2OS cells were first transfected with control siRNA (siLuc) or siRNAs targeting mRNAs of protein candidates (siNML and siB23) for 48 hours. Next, cellular phosphate was depleted by culturing cells in phosphate-free media for one hour. Cells were then cultured for one hour in media containing inorganic ^{32}P radioactive phosphate. Finally, cells were incubated in normal growth media for a further three hours to allow the production of radiolabelled newly synthesised rRNAs. The extracted cellular RNAs were separated on an agarose gel before being transferred onto a nylon membrane. The signal of the radiolabelled RNAs was visualised using a phosphor imager (Figure 3.3B). Total 28S and 18S rRNA levels were analysed by ethidium bromide (EtBr) staining and used as loading control. The radioactive RNAs were quantified and normalised to the levels of the initial 47/45S precursor RNA (Figure 3.3C-D).

Compared to the control, NML depletion resulted in a slight increase in newly synthesised 32S pre-rRNA, 28S and 18S rRNA levels (Figure 3.3C). However, there was no change in the relative levels of the mature rRNAs and pre-rRNAs. Since NML binds to histone H3 and functions as an rDNA transcription suppressor (Murayama *et al.*, 2008), the increased rRNA levels could be due to increased pre-rRNA when NML was knocked down.

Compared to the control, B23 depletion resulted in a 30% reduction in newly synthesised 32S pre-rRNA levels. However, there was no change in newly synthesised 28S rRNA levels after knocking down B23. Furthermore, there was also a slight reduction in the newly synthesised 18S rRNA levels (Figure 3.3D). My results suggest that knocking down B23 affects 28S rRNA processing because it causes a reduction in 32S pre-rRNA levels but has no effect on 28S rRNA levels, indicating B23 may be involved in 28S rRNA processing.

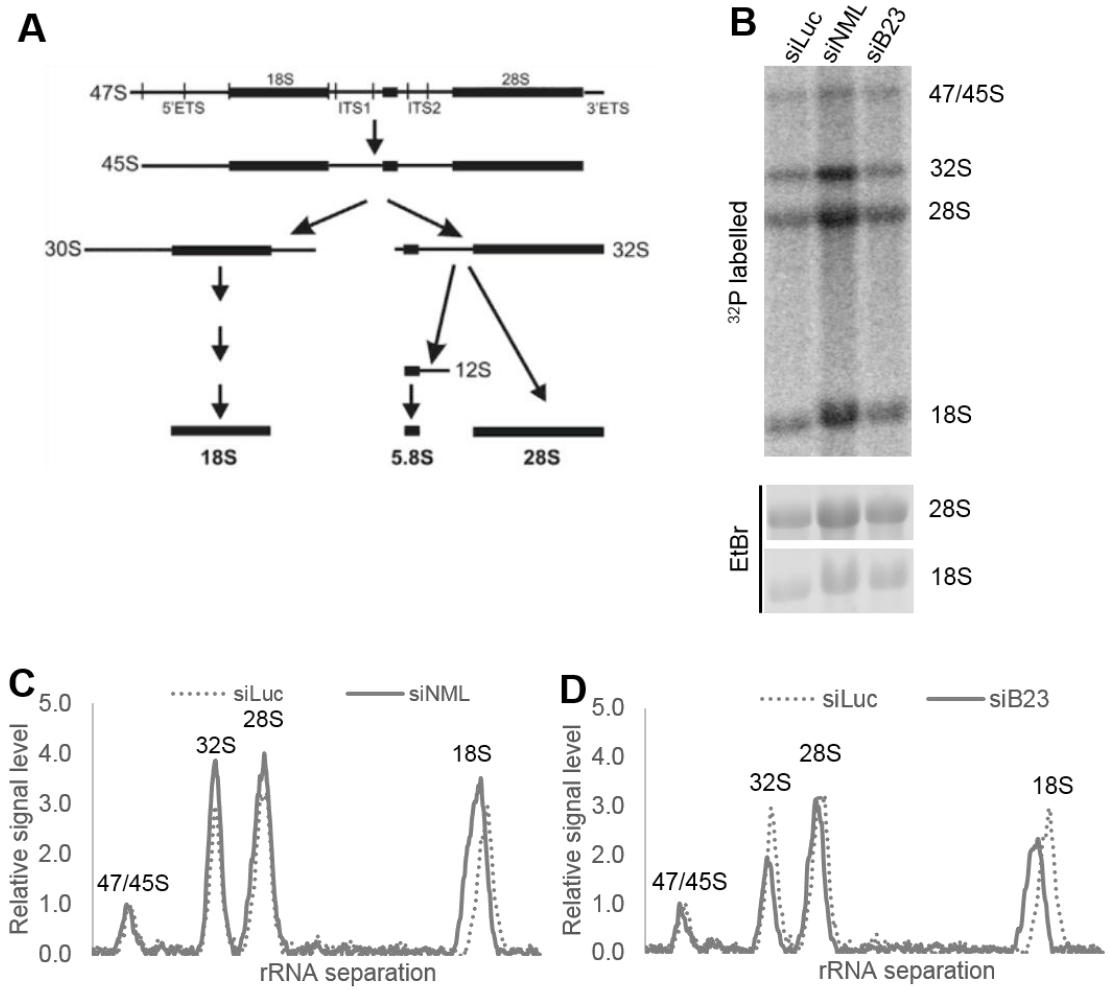

Figure 3.3. B23 depletion resulted in a reduction in 32S pre-rRNA levels in U2OS cells whereas NML protein depletion did not change rRNA processing.

(A) Schematic representation of the human rRNA processing pathway adopted from Sloan *et al.*, 2013a. (B) siRNAs targeting the mRNA of NML (siNML) or B23 (siB23) were transfected into U2OS cells. siRNA targeting the mRNA of firefly luciferase (siLuc) was used as control. 48 hours later, cells were first incubated with phosphate-free media for one hour followed by one additional hour incubation with ^{32}P containing media. Cells were subsequently grown under normal growth conditions for 3 hours before RNA was extracted and separated on a glyoxal-agarose gel and transferred to Hybond-N nylon membrane. The ^{32}P radioactive bands were visualised using a phosphoimager (upper panel). The total 18S rRNA levels on the membrane were detected with ethidium bromide (EtBr) staining (visualised by UV) and used as loading control (lower panel). siRNAs used are indicated above each lane, pre-rRNA and rRNA species are indicated on the right. (C-D) 32P-labelled pre rRNA levels were quantified using ImageQuant software and normalised against the radioactive 47/45S RNA levels. The signal intensity of the detected radioactivity from each lane in (B) are quantitated, plotted in a chart and presented as fold change relative to the radioactive 47/45S RNA levels.

3.2.3 HEXIM1 depletion causes increased p53 activity responding to nucleolar stress in U2OS cells.

HEXIM1 protein is best known as positive transcription elongation factor b (P-TEFb) inhibitor which associates with RNA Pol II (Yik *et al.*, 2003; Michels *et al.*, 2004). A study showed that HEXIM1 physically interacts with p53 and functions as a positive regulator. HEXIM1 knockdown was shown to inhibit p53 stabilisation and activation in response to DNA damage in MCF7 cells (Lew *et al.*, 2012). They also showed that ActD treatment, which blocks ribosome biogenesis, increased association between HEXIM1 and p53 in MCF7 cells (Lew *et al.*, 2012). Furthermore, it was also shown that HEXIM1 interacts with MDM2 and is also a substrate for ubiquitination by MDM2 (Lau *et al.*, 2009). Since HEXIM1 depletion does not affect rRNA processing suggesting it is not involved in ribosome biogenesis (Section 3.2.2), I next investigated if HEXIM1 is essential for 5S RNP-p53 signalling.

To investigate this, I depleted HEXIM1 in U2OS cells and analysed the cellular response to ActD-induced p53 activation. U2OS cells were transfected with control siRNA (siLuc) or siRNA targeting HEXIM1 (siHEXIM1) for 48 hours before they were treated with ActD during the final 18 hours. To evaluate the p53 protein levels and p53 activity, whole cell lysates were separated using SDS-PAGE before p53 and p21 protein levels were analysed by western blotting. Since ActD treatment had been shown to stabilise and activate p53 through the 5S RNP (Sloan 2013), based on the observations of Lew *et al.* (2012), I hypothesised that knocking down HEXIM1 should abolish the ActD-induced p53 activation.

My result showed that p53 and p21 levels were basically undetectable in control cells or cells treated with siRNA targeting HEXIM1 (siHEXIM1, Figure 3.4A). In the presence of ActD, p53 and p21 levels were significantly increased in control cells (Figure 3.4A-C). Knocking down HEXIM1 resulted in a significant reduction in the ActD-induced p53 levels (Figure 3.4B). Moreover, compared to the control, HEXIM1 depletion promoted a threefold increase in the ActD-induced p21 levels (Figure 3.4C).

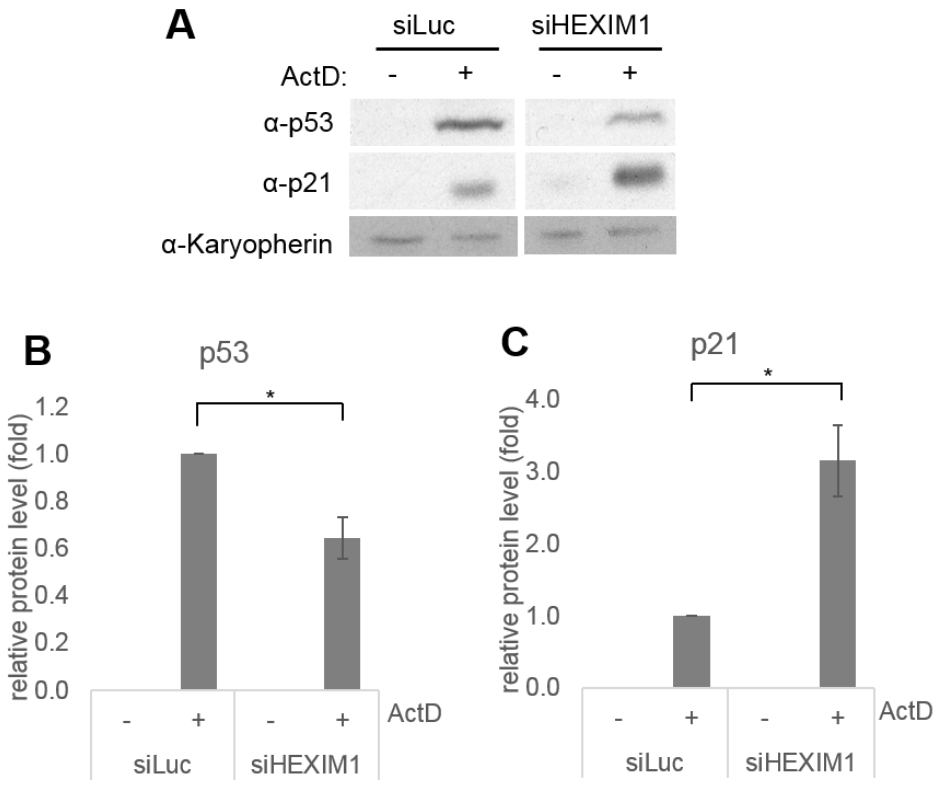

Figure 3.4. HEXIM1 depletion results in p53 reduction and p21 induction.

SiRNAs were transfected to U2OS cells for 48 hours in the presence or absence of 5 nM ActD treatment during the final 18 hours. (A) Whole cell lysates were separated on SDS-PAGE and proteins were detected by western blotting using the indicated antibodies. Signals were visualised by X-ray films. Karyopherin protein levels were used as loading control. (B-C) Protein levels were quantified using ImageQuant software (GE Healthcare) and normalised against the loading control (α-Karyopherin). Graphs are presented as fold change relative to the indicated protein levels (siLuc Act+). Values are presented as mean±standard error of three independent experiments (*p<0.05; student's t-test).

Since p21 is a downstream transcription target of p53, higher p21 protein levels indicate p53 is more active. Therefore, the increased p21 levels but reduced p53 levels suggest an overall increase in p53 activity as a result of knocking down HEXIM1 protein. Hence, my result suggest that HEXIM1 functions as a p53 activity repressor, which contradicts the previously reported role as a positive p53 regulator (Lew *et al.*, 2012). This difference could be due to cell line variations between MCF7 and U2OS cells (see Section 3.3.1).

3.2.4 Mybbp1a protein depletion does not affect p53 stability but results in more active p53

Mybbp1a is a nucleolar protein that is involved in ribosome biogenesis and is associated with RNA Pol I (Hochstatter *et al.*, 2012). A recent study showed that it is necessary for p53 activation through p53 acetylation during nucleolar stress (Kuroda *et al.*, 2011; Ono *et al.*, 2013). Since Mybbp1a is involved in ribosome biogenesis and also p53 signalling, I was interested to determine whether the protein is important for p53 regulation via the 5S RNP.

To investigate this question, siRNA was used to knockdown Mybbp1a in U2OS cells for 48 hours. To block ribosome biogenesis, ActD was added to the cells for the final 18 hours. To evaluate the p53 protein levels and p53 activity, whole cell lysates were separated by SDS-PAGE and p53 and p21 protein levels were analysed by western blotting. Fibrillarin protein levels were used as loading control.

The levels of p53 in the untreated control cells were basically undetectable (Figure 3.5A), while ActD treatment resulted in a significant induction in p53 levels (Figure 3.5B). The same difference in p53 levels was observed in cells with depleted Mybbp1a (Figure 3.5A and B). This suggests that Mybbp1a depletion does not affects ActD-induced p53 stabilisation.

The p21 protein was also undetectable in control cells, and ActD treatment caused a significant increase in p21 levels (Figure 3.5A and C). In the absence of ActD, knocking down Mybbp1a resulted in a noticeable increase in p21 levels compared to the control (Figure 3.5C). In the presence of ActD, Mybbp1a depletion caused a three-fold induction in p21 levels compared to the ActD treated control (Figure 3.5C). Mybbp1a knockdown resulted in increased p21 levels in the presence or absence of ActD treatment (Figure 3.5C), indicating Mybbp1a depletion increases overall p53 activity.

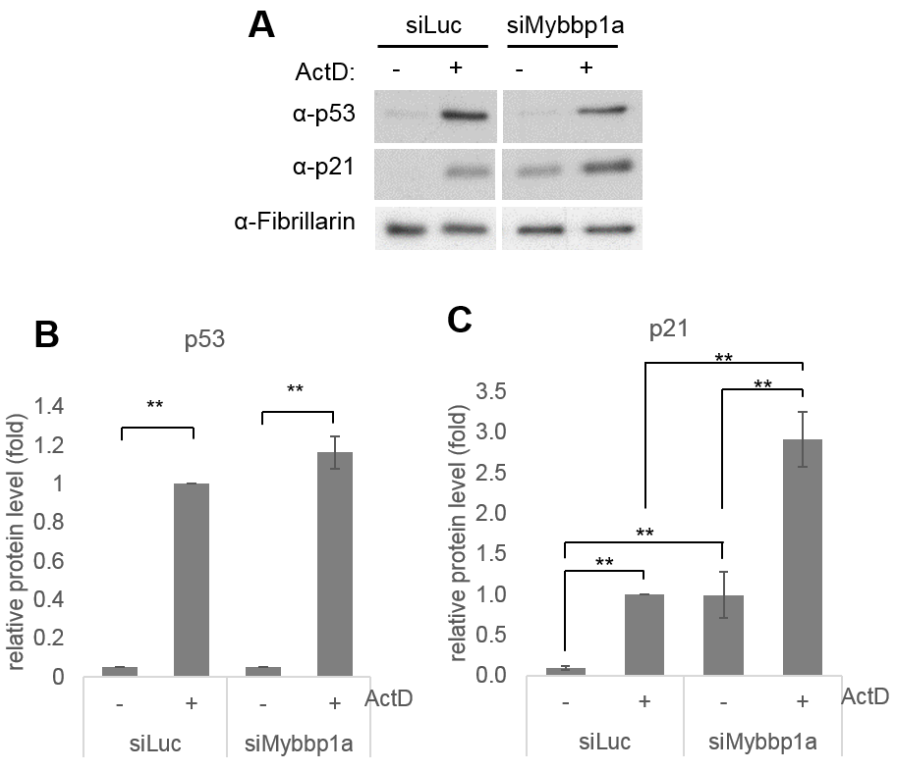

Figure 3.5. Mybbp1a depletion alters p53 activity but not stability.

SiRNAs were transfected to U2OS cells for 48 hours in the presence or absence of 5 nM ActD treatment during the final 18 hours. (A) Whole cell lysates were separated on SDS-PAGE and proteins were detected by western blotting using the indicated antibodies. Signals were visualised by X-ray films. Fibrillarin protein levels were used as loading control. (B-C) Protein levels were quantified using ImageQuant software (GE Healthcare) and normalised against the loading control levels (α- Fibrillarin). Graphs are presented as fold change relative to the indicated protein levels (siLuc Act+). Values are presented as mean±standard error of four independent experiments (**p<0.01; Mann-Whitney U test).

My results showed that Mybbp1a depletion did not stabilise p53, but p21 levels were increased in the presence or absence of ActD (Figure 3.5A-C), hence I conclude that Mybbp1a depletion results in more active p53. My results, however, contradict the published data (Kuroda *et al.*, 2011; Ono *et al.*, 2014) which previously demonstrated that Mybbp1a depletion counteracts the ActD-induced p21 level induction in MCF7 cells (Kuroda *et al.*, 2011). This could be due to cell line variation between U2OS to MCF7 cells or to the different ActD treatment length (8 hours compared to 18 hours) (Kuroda *et al.*, 2011; Ono *et al.*, 2014).

3.2.5 NML depletion results in higher p53 activity

Nucleomethylin (NML; also known as RRP8) was identified as a component of the energy-dependent nucleolar silencing complex (eNoSC). As a methyltransferase, it methylates RNAs and it is also found to be involved in histone remodelling and suppressing rDNA transcription under glucose starvation (Murayama *et al.*, 2008). A recent study showed that NML is involved in p53 activation during glucose starvation-induced nucleolar stress (Kumazawa *et al.*, 2011; Yang *et al.*, 2013). I was therefore interested to find out whether NML is linked to p53 activation through the 5S RNP-p53 pathway in response to nucleolar stress.

SiRNA targeting NML mRNA was used to knockdown NML protein levels in U2OS cells for 48 hours. To block ribosome biogenesis ActD was added for the final 18 hours. To evaluate the p53 protein levels and p53 activity, whole cell lysates were separated by SDS-PAGE and p53 as well as p21 protein levels were analysed by western blotting using protein-specific antibodies. Fibrillarin protein levels were used as loading control.

The p53 protein levels were barely detectable and ActD treatment caused a significant induction of p53 levels (Figure 3.6A). p21 protein was also undetectable in control cells and ActD treatment resulted in a significant induction of p21 levels (Figure 3.6A). In the absence of ActD, knocking down NML caused no change in p53 levels, while promoting a fourfold increase in p21 levels compared to the ActD-free control (Figure 3.6A-C).

In the presence of ActD, NML depletion resulted in inconsistent p53 levels, ranging from 30% lower to 40% higher than the ActD+ control (observed from two repeated experiments), hence more repeat experiments are needed to elucidate the effect of NML depletion on p53 stability. However, in the presence of ActD, NML depletion resulted in a reproducible increase in p21 levels compared to the control (Figure 3.6C), suggesting NML depletion promotes p53 activity.

A recent study reported that NML depletion in HCT116 cells resulted in p53 stabilisation and increased p21 levels (Waku *et al.*, 2016). My results indeed showed increased p21 levels after NML depletion in U2OS cells which is consistent with the previous study (Kumazawa *et al.*, 2011) (Figure 3.6C). However, my work requires further repeat experiments to clarify how NML depletion affects p53 stability (Figure 3.6B).

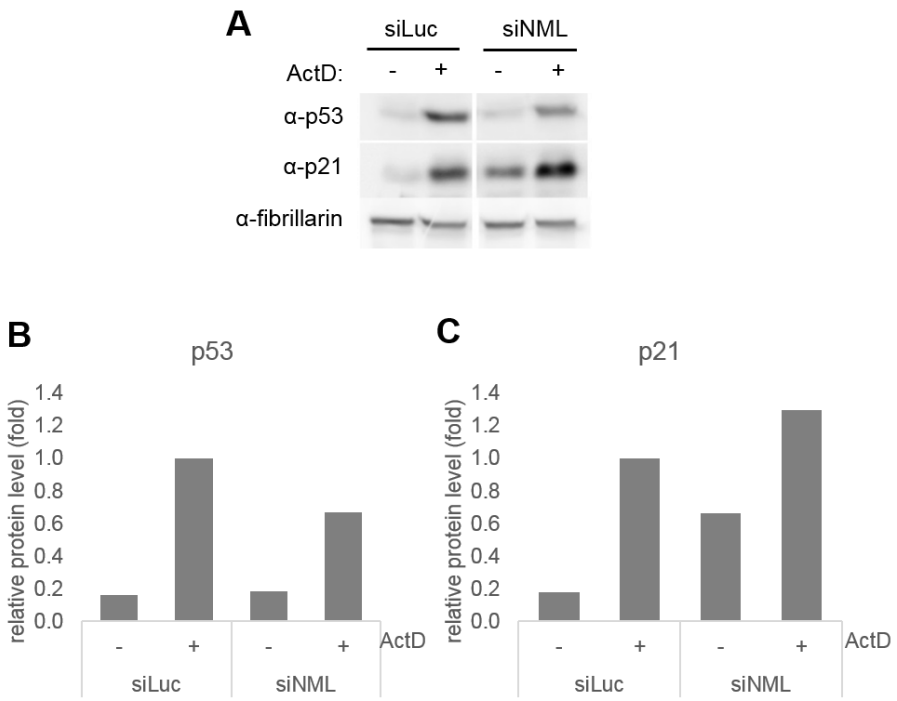

Figure 3.6. NML depletion increases p53 activity.

SiRNAs were transfected into U2OS cells for 48 hours in the presence or absence of 5 nM ActD treatment during the final 18 hours. (A) Whole cell lysates were separated on SDS-PAGE and proteins were detected by western blotting using indicated antibodies. Signals were visualised by X-ray films. Fibrillarin protein levels were used as loading control. (B) Quantitation of the p53 levels shown in (A). Protein levels were quantified using ImageQuant software (GE Healthcare) and normalised against the loading control (α-Fibrillarin). Graphs are presented as fold change relative to the indicated protein levels (siLuc Act+). Due to inconsistent results, data from a single experiment are presented. Refer to result section for further details. (C) p21 levels were quantified as in (B), values are presented as mean from two independent experiments.

Based on previous a study showing that NML is involved in p53 activation in response to glucose starvation-induced nucleolar stress (Kumazawa *et al.*, 2011; Yang *et al.*, 2013), I hypothesised that NML is involved in p53 activation via 5S RNP. However, my results showed that NML depletion causes p21 level induction in the present of ActD. Since p21 protein levels indicate the transcription activity of p53, my results suggest that NML depletion causes higher p53 activity in response to ActD treatment. If NML was essential for p53 activation through 5S RNP, NML depletion could counteract the ActD-induced p53 activation. My result therefore implies that NML is not involved in the 5S RNP-p53 signalling pathway.

3.2.6 B23 protein depletion caused inconsistent p53 and p21 levels.

Nucleolar phosphoprotein B23 is a multifunctional protein involved in multiple steps during ribosome biogenesis (Lindström, 2011). Mutated B23 mislocates to the cytoplasm in acute myeloid leukaemia (AML) cells (Federici and Falini, 2013). B23 has been shown to interact with p53 and is involved in p53 stabilisation and activation in response to DNA damage stress (Colombo *et al.*, 2002).

In order to investigate whether B23 is involved in the 5S RNP-p53 signalling pathway, knockdown experiments using siRNA targeting B23 protein and ActD treatment were performed as described above. However multiple independent experiments showed inconsistent results for p53 and p21 protein levels. Due to time constraints I abandoned these experiments.

3.2.7 Knockdown of HEXIM1, Mybbp1a, NML and B23 results in loss of MDMX.

HEXIM1 and Mybbp1a depletion caused higher p53 activity i.e. increased p21 levels in the presence of ActD (Figure 3.4C; Figure 3.5C) and NML depletion also resulted in increased p21 levels in the presence of ActD (Figure 3.6C). These results are all unexpected and reject my hypothesis. To explain the unexpected results, I investigated whether MDMX is involved in the increased p53 activity. MDMX is a p53 regulator that forms a dimer with MDM2. Although MDM2, is responsible for p53 ubiquitination and degradation, Gilkes *et al.,* reported that the p53 response to nucleolar stress requires the degradation of MDMX (Gilkes *et al.*, 2006). Therefore, I decided to investigate the effect of depleting HEXIM1, Mybbp1a, NML and B23 on cellular MDMX levels.

siRNAs were transfected into U2OS cells followed by ActD treatment as described above. Whole cell lysates were separated by SDS-PAGE and MDMX protein levels were analysed by western blotting.

Knockdown of HEXIM1, Mybbp1a, NML or B23 resulted in a notable reduction in MDMX levels, ranged from 60% to 80%, compared to the siLuc transfected controls (Figure 3.7). ActD treatment caused a notable reduction in MDMX levels in the control as well as in cells

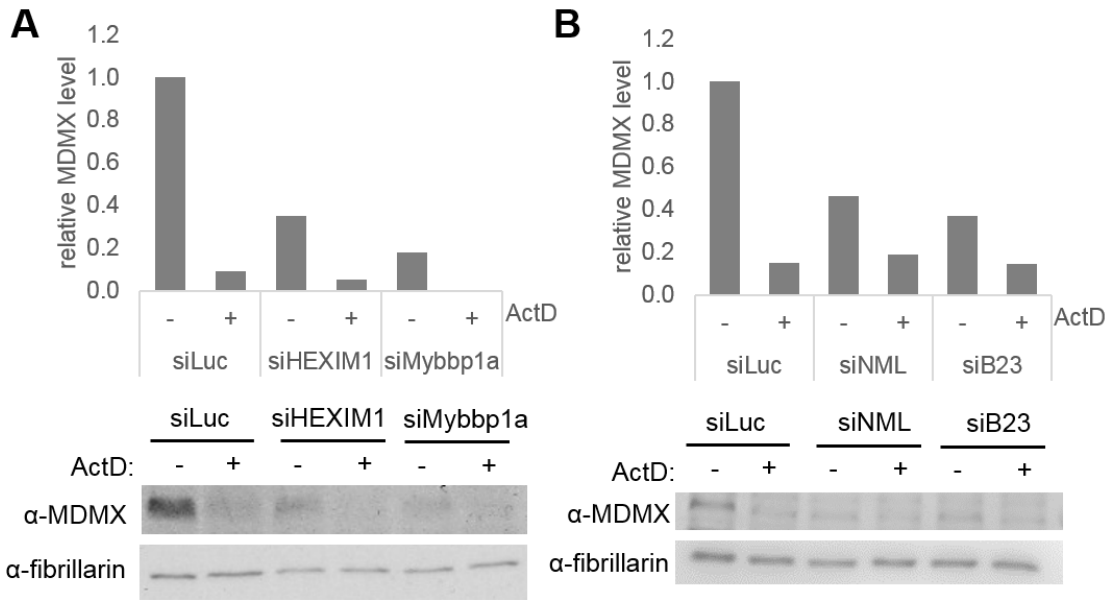

Figure 3.7. Depleting HEXIM1, Mybbp1a, NML and B23 causes a reduction in MDMX protein levels.

siHEXIM1, siMybbp1a, siNML and siB23 were used to deplete their targeted proteins. These siRNAs were transfected to cells for 48 hours in presence or absence of 5 nM ActD treatment during the final 18 hours. Whole cell lysates were separated on SDS-PAGE. Proteins were detected using indicated protein-specific antibodies. Fibrillarin protein levels were used as loading control. MDMX protein levels were quantified using ImageQuant software (GE Healthcare) and normalised against the loading control (α-Fibrillarin). Quantitation are presented from the mean of the two independent experiments. Graphs are presented as fold change relative to the indicated protein levels. (A) Protein bands were visualised using X-ray films and digitalised with an hp professional high-resolution scanner. (B) Protein bands were visualised and digitalised using the ImageQuant bioimager (CCD camera, GE Healthcare). Independent experiments were repeated twice and the average of the consistent results are presented.

with depleted proteins (Figure 3.7A and B). Quantitation analysis showed that, comparted to the ActD untreated control, ActD treated cells resulted in further MDMX reduction in all protein knockdown samples. This suggests that ActD treatment causes a greater reduction in MDMX levels than the knock down of these proteins. To investigate whether MDMX reduction is linked to the increased p53 activity, I next investigate how MDMX depletion affects p53 stability and activity.

3.2.8 MDMX depletion causes both p53 stabilisation and activation

SiRNA targeting MDMX mRNA was used to knockdown MDMX in U2OS cells for 48 hours. Half of the cells were treated with ActD during the final 18 hours. Whole cell lysates were separated by SDS-PAGE. MDMX, p53 and p21 protein levels were analysed by western blotting. MDMX siRNA treatment to U2OS cells resulted in a significant reduction in MDMX levels compared to cells treated with the control siRNA (Figure 3.8A). Additionally, in the presence of ActD, MDMX levels also became undetectable (Figure 3.8A). p53 protein was barely detectable in the untreated control cells (Figure 3.8A).

In control cells, addition of ActD caused a significant induction in p53 levels (siLuc ActD+, Figure 3.8B). In the ActD untreated cells, MDMX depletion also promotes significant increase in p53 levels (MDMX siRNA ActD-, Figure 3.8B), but the p53 level is only half the level caused by ActD treatment (siLuc ActD+, Figure 3.8B).

In cells with MDMX depletion ActD treatment resulted in a threefold increase of p53 levels compared to untreated controls (MDMX siRNA ActD+, Figure 3.8B). The observed level is about 1.5 times higher than the one seen in the ActD treated control cells (siLuc ActD+, Figure 3.8B). My results showed that p53 stabilisation is promoted by either MDMX depletion or ActD treatment, however, ActD results in higher p53 levels than MDMX depletion. My results therefore suggest that MDMX depletion is not the only factor causing ActD-induced p53 stabilisation in U2OS cells.

p21 protein was basically undetectable in control cells (Figure 3.8A). In these cells, the addition of ActD resulted in a noticable increase in p21 levels (siLuc ActD+, Figure 3.8C). After depleting MDMX there was also a significant increase in p21 levels (MDMX siRNA ActD-, Figure 3.8A), which is comparable to the increase seen in the ActD treated control cells (siLuc ActD+, Figure 3.8A). Moreover ActD treatment of the MDMX depleted cells resulted in a twofold increase in p21 levels, compared to the ActD treated control cells (MDMX siRNA ActD+, Figure 3.8C). However, this change in p21 levels was not significant compared to ActD untreated cells with MDMX depleted (MDMX siRNA ActD-, Figure 3.8A). MDMX depletion resulted in increased p21 levels regardless of the presence of ActD, suggesting MDMX depletion indeed activates p53.

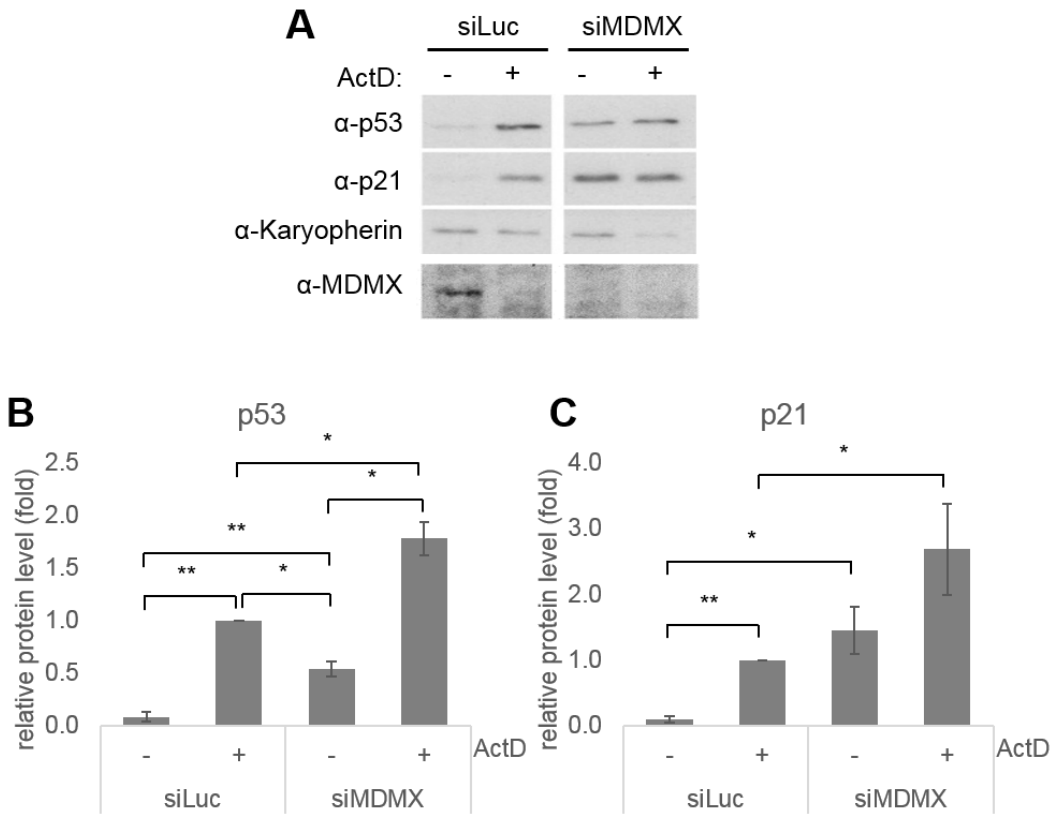

Figure 3.8. MDMX depletion enhances p53 stabilisation and increases p53 activity.

(A) MDMX siRNAs were transfected into U2OS cells for 48 hours in presence or absence of 5 nM ActD treatment during the final 18 hours as indicated above each lane. Whole cell lysates were separated on SDS-PAGE. Proteins were detected using the indicated protein-specific antibodies, as indicated. Proteins were visualised using X-ray films. The karyopherin protein levels were used as loading control. (B-C) p53 and p21 protein levels were quantified using ImageQuant software (GE Healthcare) and normalised against the loading control (α-Karyopherin). Graphs are presented as fold change and normalised to the siLuc ActD+ sample. Values are presented as mean ± standard error of three independent experiments (*p<0.05, **p<0.01; Student's T test).

It had been reported that MDMX degradation was caused by ActD treatment in U2OS cells (Gilkes *et al.*, 2006). Gilks *et al.* also showed that MDMX degradation is essential for p53 activation in response to ActD. My result showed that MDMX depletion increases ActD-induced p53 activation, which is consistent with the published findings. Additionally, from my result also indicate that a loss of MDMX also enhances p53 stability. In conclusion, my result showed that MDMX depletion causes both p53 stabilisation and activation.

I hypothesised that HEXIM1, Mybbp1a and NML protein were involved in the 5S RNP-p53 signalling pathway. However, my results do not support my initial hypothesis, some of them even contradict published findings (REF). Consequently, it is more likely that the investigated

proteins are involved in p53 regulatory pathways that are independent of 5S RNP (see discussion Section 3.3.1). I therefore decided to focus on investigatingprotein factor SRSF1.

3.2.9 Recombinant SRSF1 binds to both RPL11 and RPL5 in vitro

It was previously shown that SRSF1 interacts with RPL5 and stabilises p53 via RPL5 in response to nucleolar stress (Fregoso *et al.*, 2013). Unpublished data from the Watkins lab further demonstrated that SRSF1 depletion counteracts ActD- induced p53 activation (Loren Gibson, unpublished data). Furthermore, SRSF1 depletion resulted in free 5S RNP accumulation and affected the localisation of 5S RNP components. Lauren Gibson proposed a model that SRSF1 protein functions as a chaperone during the 5S RNP assembly and is essential in 5S RNP-p53 signalling in response to nucleolar stress.

To further characterise SRSF1, I first investigated the interaction between SRSF1, RPL5 and RPL11 using recombinant proteins. Compared to the wild type, the recombinant His-tagged SRSF1 construct lacks the RS domain, since E*. coli* lacks human kinase that phosphorylates the RS domain leading to insoluble protein products (Figure 3.10A) (Gallego *et al.*, 1997). The protein Tip48, an ATPase and a pre-snoRNP complexes component, does not bind RNA as previously shown in the lab. Tip48 was therefore used as a negative control in these experiments.

Recombinant proteins, either GST-tagged (RPL5, RPL11 and Tip48) or His-tagged (SRSF1), were over-expressed in *E. coli* and purified, before being analysed by coomassie stained SDS-PAGE. The intensity of the stained protein bands was quantified and used as a loading control for the pull-down experiments (Figure 3.10B). To investigate the interaction between SRSF1 and the 5S RNP *in vitro*, equal amounts of GST-tagged RPL5, RPL11 or Tip48 were incubated individually with an equal amount of His-SRSF1 at 4 °C for 5 hours before purification with glutathione sepharose resin. Resins were subsequently washed and the bound material was denatured and separated by SDS-PAGE and analysed by western blotting. Bound SRSF1 was revealed using anti-polyHis antibody. 50% of His-SRSF1 input levels were used as a positive control.

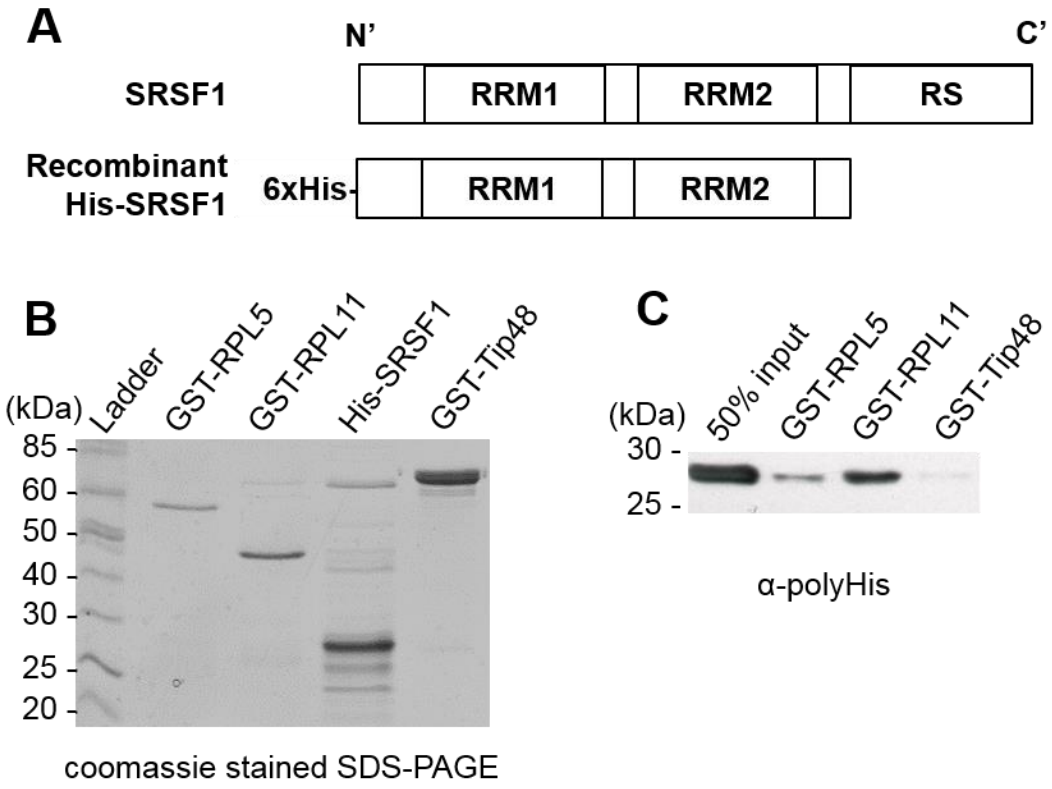

Figure 3.9. SRSF1 interacts with RPL11 and RPL5 in vitro.

(A) Schematic representation of the domain composition of wild type and recombinant His-SRSF1. (B) Affinity-tagged proteins expressed in *E. coli* BL21 strains were purified using GST or Ni resin. Yield and quality of the eluted product were analysed by SDS-PAGE and stained with coomassie. The molecular weight for these recombinant proteins are: GST-RPL5 (60 kDa), GST-RPL11 (46 kDa), His-SRSF1 (28 kDA) and GST-Tip48 (77 kDa). (C) GST-tagged RPL5, RPL11 and Tip48 were incubated with His-SRSF1 *in vitro* before pull- down by glutathione sepharose resin. The eluate was separated by SDS-PAGE before western blotting using an anti-PolyHis antibody to confirm the interaction with His-SRSF1. Tip48 protein was used as a negative control.

In the pull- down experiment results, His-SRSF1 was detected in the bound material from both GST-tagged RPL5 and RPL11 (Figure 3.10C). The levels of His-SRSF1 bound to RPL11 was noticeably higher than to RPL5. There were barely detectable His-SRSF1 levels interacting with the control protein Tip48, suggesting the interaction is non-specific (Figure 3.10C). These results suggest that SRSF1 interacts with both ribosomal proteins and may have a higher affinity to RPL11 than RPL5 *in vitro*. This evidence provides support to the 'chaperone' model previously proposed by Loren Gibson suggesting that SRSF1 is involved in the 5S RNP assembly (see Section 3.3.2).

3.2.10 Splicing inhibitor SRPIN340 reduces ActD-induced p53 activation in a dose dependent manner

I next investigated how SRSF1 function is involved in 5S RNP-p53 signalling. It has been reported that SRSF1 is highly phosphorylated which controls the function and localisation of SRSF1 (Gonçalves *et al.*, 2015). The serine/arginine-rich protein-specific kinase (SRPK) is one of the kinases responsible for the phosphorylation of SRSF1, and SRPK phosphorylates SRSF1 specifically on multiple serine residues located in the RS domain (also known as the arginine/serine rich region) of the protein (Ma *et al.*, 2008; Giannakouros *et al.*, 2011).

SRPIN340 is a specific chemical inhibitor of SRPK. Inhibition of SRPK blocks phosphorylation of SRSF1 and subsequently affects SRSF1 function (Gammons *et al.*, 2013). Therefore, I decided to use SRPIN340 to further investigate how SRSF1 function links to the 5S RNP-p53 signalling via phosphorylation of the protein.

In order to investigate how different doses of SRPIN340 affect the ActD-induced p53 activity, U2OS cells stably expressing a p53-driven firefly luciferase reporter were treated with 5 nM ActD in combination with different concentrations of SRPIN340 (0 to 20 µM). Subsequently, p53 activity was analysed by measuring luciferase levels in the whole cell lysate using a luminometer. Total protein concentration in each sample was determined by Bradford assay and used as an input control.

First, cells treated with 5 nM ActD only (0 µM SRPIN340) were referred as 100% (control). As SRPIN340 dose increased, the luciferase levels decreased linearly in the ActD treated cells (Figure 3.11). A SRPIN340 dose of 5 µM resulted in 20% reduction of cellular luciferase levels compared to cells treated with only ActD. 10 µM SRPIN340 caused 50% luciferase reduction compared to the control. 20 µM SRPIN340 resulted in luciferase levels lower than 10% compared to the control.

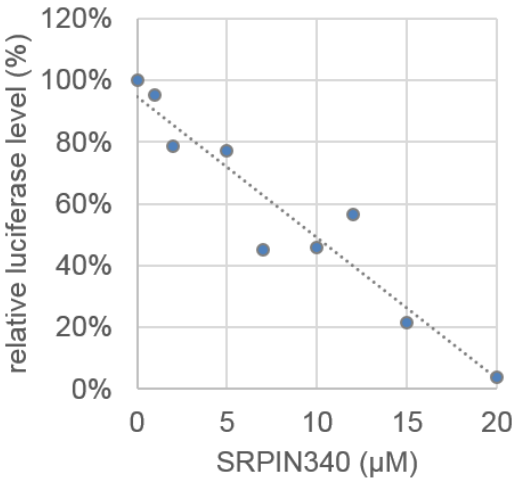

Figure 3.11. ActD-induced p53 activity is inhibited by SRPIN340 in a dose dependent manner.

U2OS cells expressing a p53-driven firefly luciferase reporter was treated with 5 nM ActD and a range of SRPIN340 concentrations (0 to 20 μM) for 12 hours. Luciferase levels in the whole cell lysate were measured by the single-tube luminometer with supplement of luciferase assay substrate (Promega). The signal was normalised to total protein concentration of the sample measured using Bradford assay. Cells treated with 5 nM ActD was referred as 100% control and experimental data (mean from two repeated experiments) was plotted relative to these control levels. A linear trend line was generated from the experimental data using Microsoft Excel.

My result showed that the ActD-induced p53 activation was inhibited by the SRPIN340 treatment in a dose dependent manner in U2OS cells. Since SRPIN340 prevents SRSF1 phosphorylation by SRPK, it implies that SRSF1 phosphorylation is important in p53 activation in response to nucleolar stress. Moreover, SRPIN340 had a significant effect on cell survival. I therefore used 10 μM SRPIN340 in later experiments to reduce the impact of SRPIN340 on cell survival.

3.2.11 SRPIN340 inhibits ActD-induced p53 activation and stabilisation

Since blocking SRSF1 phosphorylation with SRPIN340 inhibits p53 activation in response to ActD treatment in a dose dependent manner, I next decided to investigate whether this effect observed was specific to 5S RNP-mediated activation of p53. I also investigated whether SRPIN340 affects p53 stability.

Nutlin-3a (Nut3a) which inhibits the interaction between MDM2 and p53 was used as a control to ActD treatment, allowing me to determine if SRPIN340 affects p53 specifically via 5S RNP. Nut3a causes p53 stabilisation by counteracting negative p53 regulation by inhibition of MDM2. Hence, in contrast to ActD, MDM2 activates p53 in a 5S RNP-independent manner.

U2OS cells stably expressing p53-driven luciferase were used. They were treated with 2.5 µM Nut3a or 5 nM ActD for 12 hours, in the presence or absence of 10 µM SRPIN340. Luciferase levels were measured using a luminometer by supplying whole cell lysates with luciferase substrate. The signal was normalised to total protein concentration measured by the Bradford assay.

Treating cells with SRPIN340, in the absence of ActD or Nut3a, had no effect on luciferase levels and therefore, no change in p53 activity (Figure 3.12A). Addition of ActD resulted in an eight-fold increase in luciferase levels. This increase was not seen in cells treated with SRPIN340 (Figure 3.12A).

Similarly, addition of Nut3a to these cells resulted in a fivefold increase in luciferase levels. However, compared to cells treated with SRPIN340 alone, the Nut3a treatment-induced luciferase change was not statistically significant (Figure 3.12A). The high variation in luciferase levels in the cells treated with a combination of Nut3a and SRPIN340 meant these result are unconclusive and repeated experiments are needed. In conclusion, my results show that SRPIN340 inhibits ActD-induced p53 activation, however more experiments are needed to elucidate whether this effect is ActD specific.

I also investigated p53 and p21 levels in these cells, to test if SRPIN340 affects p53 stability. To evaluate p53 protein levels and p53 activity, whole cell lysates prepared from U2OS cells

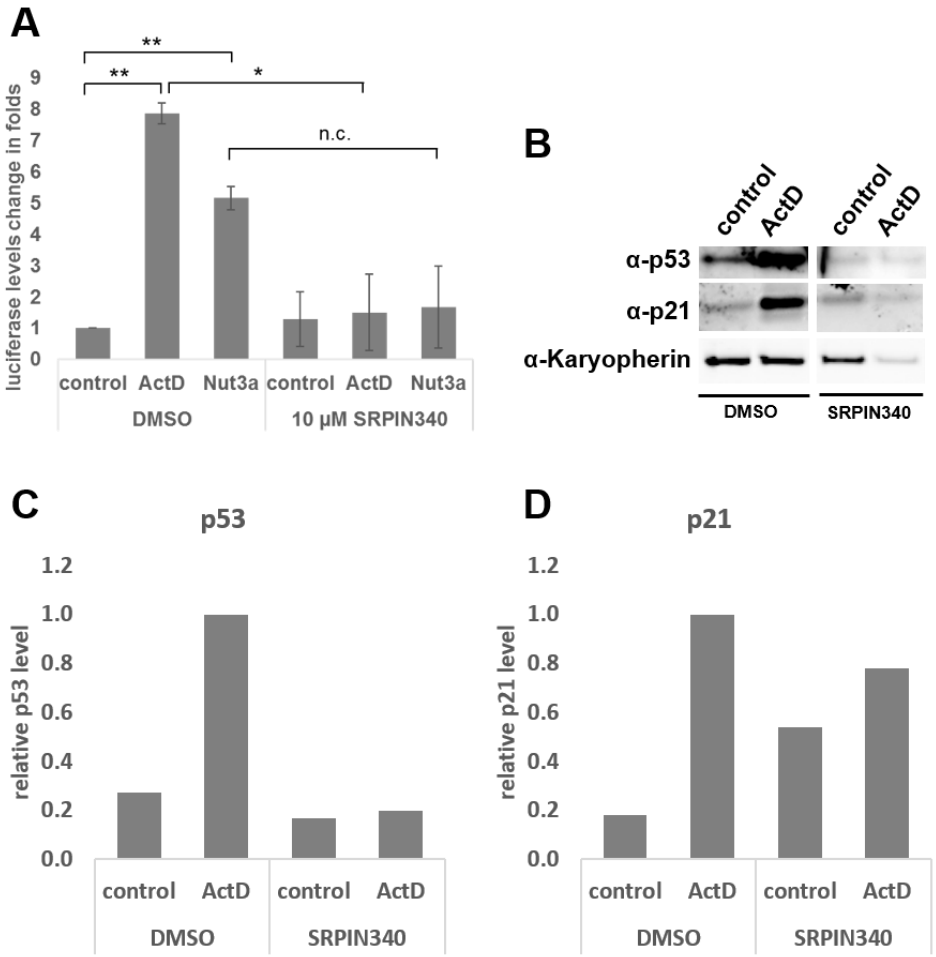

Figure 3.12. SRPIN340 inhibits ActD-induced p53 activation

U2OS cells expressing p53-driven luciferase reporter were incubated in media containing 2.5 µM Nut3a or 5 nM ActD, in the presence or absence of 10 µM SRPIN340 for 12 hours. (A) Luciferase levels in the whole cell lysate were measured using the single-tube luminometer with supplement of luciferase substrate (Promega). The signal was normalised to the total protein concentration of the sample measured using Bradford assay. The graph is presented as fold change relative to the control treatment level. Values are presented as mean ± standard error of three independent experiments. *p<0.05, **p<0.01; Student's T test. Non-significant difference is indicated as n.c. (B) p53 and p21 protein levels were analysed using western blotting. Whole cell lysates were separated on SDS-PAGE. Proteins were detected using indicated protein-specific antibodies. Protein were visualised using ECL methods and the ImageQuant bioimager (CCD camera, GE Healthcare). Karyopherin protein levels were used as loading control. (C-D) p53 and p21 protein levels were quantified using ImageQuant software (GE Healthcare) and normalised against the loading control (α-Karyopherin). Graphs are presented as fold change relative to the indicated protein levels. Results are consistent in repeated experiments. Presented quantitation is from one representative experiment.

stably expressing p53-driven luciferase reporter were separated by SDS-PAGE and p53 and p21 protein levels were analysed by western blotting. Karyopherin protein levels were

analysed likewise and used as loading control. However, due to toxicity of the combination treatment of Nut3a and SRPIN340, cell numbers were greatly reduced. As a consequence, there was insufficient protein material to allow conclusive results to be observed from these samples. Therefore, the Nut3a treated samples were excluded from this section.

The SRPIN340 only treatment of the cells did not stabilise p53 compared to the control, but it caused a two to three-fold increase in p21 levels (Figure 3.12 C, D). The combination of ActD and SRPIN340 treatment resulted in a five-fold reduction in p53 levels compared to the ActD only treatment (Figure 3.12C), whereas it only caused slight reduction in p21 levels (Figure 3.12D).

Taken together, I conclude that SRPIN340 inhibits both ActD-induced p53 activation and stabilisation. However, as the combination treatment of these drugs affects cell survival, it causes protein levels to be insufficient for protein analysis in the samples. Hence in the further experiment, method development should focus on use less dose of drug and improve the protein detection in order to generate more reliable data.

3.3 Discussion and future work

3.3.1 HEXIM1, Mybbp1a and NML are not required for the 5S RNP-mediated p53 signalling in U2OS cells

In this chapter, I aimed to characterise protein factors HEXIM1, Mybbp1a and NML, and their involvement in the 5S RNP mediated-p53 regulatory signalling. Previously they all have been reported in the literature to be important in activating p53 in response to DNA damage (HEXIM1; (Lew *et al.*, 2013)), ActD treatment (Mybbp1a; (Kuroda *et al.*, 2011)) or glucose starvation (NML; (Kumazawa *et al.*, 2011)). Based on their published data, I hypothesised that they are important in 5S RNP-induced p53 activation.

Using siRNA mediated-protein depletion, I tested if their depletion in U2OS cells affects the p53 activation in response to ActD treatment. However unexpectedly, I observed that knocking down any of these proteins in U2OS cells results in upregulated p21 protein levels in response to ActD treatment.

Low doses of ActD treatment promote p53 activation specifically through accumulation of the non-ribosomal 5S RNP (Donati *et al.*, 2013; Sloan *et al.*, 2013a). p53 stabilisation is mainly achieved by inhibiting the MDM2 ubiquitination function on p53 which targets p53 for proteasome-mediated degradation. Non-ribosomal 5S RNP interacts with the acidic domain of MDM2, which inhibits its E3 ubiquitin ligase activity (Golomb *et al.*, 2014). This results in p53 stabilisation and activation as a transcription promoter, binding to p53 responsive elements in the DNA. p21 protein is encoded by the *CDKN1A* gene, which is controlled by the transcriptional activation of p53 (Section 3.1). As one of the most important downstream factors, the cellular protein levels of p21 is commonly used as a read-out of p53 activity. Therefore, the increased p21 levels in my results suggest that depletion of HEXIM1, Mybbp1a or NML in U2OS cells resulted in higher p53 activity in response to ActD treatment.

Since depletion of essential protein factors in the 5S RNP-mediated p53 pathway would counteract the ActD effect on p53 activation, I concluded that these proteins are not needed in the 5S RNP-mediated p53 signalling pathway, which does not support my hypothesis.

	HEXIM1 (regulates transcription)		**Mybbp1a** (repress rRNA transcription)		**NML** (regulates pre-rRNA transcription)	
published data						
	30 nM ActD on MCF7 cells: ⬆ HEXIM1/p53 association		KD in MCF7 cells: ⬇ p53 activation upon ActD		KD in WI-38 cells: ⬇p53 ⬇p21 upon glucose starvation	
	KD in MCF7 cells: ⬇p53 activity on DNA damage					
reference	(Lew *et al.*, 2012)		(Kuroda *et al.*, 2011)		(Kumazawa *et al.*, 2011)	
experimental result						
U2OS	p53	p21	p53	p21	p53	p21
KD ActD-	N/A	N/A	n.c.	⬆10x	n.c.	⬆3x
KD ActD+	⬇1/3	⬆3x	n.c.	⬆3x	⬇1/4	⬆20%
MDMX	⬇		⬇		⬇	

Table 3.1. Summary of the published data and my experimental result for HEXIM1, Mybbp1a and NML.

(N/A: not detectable level; n.c.: no change; ⬆ increased in level; ⬇ reduced in level)

Lew *et al.* (2012) demonstrated that treatment with 30 nM ActD promotes p53-HEXIM1 interaction in MCF7 cells. They also showed that knockdown HEXIM1 counteracts the doxorubicin or flavopiridol-induced p53 stabilisation and activation (Lew *et al.*, 2012). My result showed that HEXIM1 depletion results in 40% less of stable p53 in response to ActD treatment but three times more p21 (Figure 3.4), suggesting HEXIM1 is needed to stabilise p53 during nucleolar stress, which is consistent with previous Lew's findings. However, I could not show HEXIM1 depletion counteracts the ActD-induced p53 activation as predicted, which suggests HEXIM1 could be selectively involved in DNA damage response signalling.

Doxorubicin is a DNA damage inducing compound and flavopiridol inhibits P-TEFb function promoting mRNA transcription blockage. On the other hand, ActD induces nucleolar stress. My results in conjunction with the published studies suggest that HEXIM1 is involved in p53 activation specifically induced by DNA damage, but not by nucleolar stress. It also implies that p53 activity may be differentially regulated in a stress-dependent manner.

p53 activity is determined by its modification status, such as acetylation and phosphorylation, and it has been demonstrated that in response to DNA damage signals, phosphorylation of serine 15 and serine 20 regulate p53 by affecting the MDM2/p53 interaction (Lakin and Jackson, 1999). Unfortunately, the modifications of p53 directly linked to a response to nucleolar stress remain unknown. Therefore, it is possible that HEXIM1 mediated p53 regulation depends on modifications triggered in response to a DNA damage signal but not to a nucleolar stress signal.

Kuroda *et al.* (2011) demonstrated that Mybbp1a depletion counteracts the ActD-promoted p21 induction in MCF7 cells (Kuroda *et al.*, 2011), suggesting the protein is needed for p53 activation in response to ActD in these cells. However, I could not reproduce the result previously demonstrated by Kuroda using U2OS cells. In contrary, my result showed that Mybbp1a depletion caused an opposite effect on p21 levels. Since Kuroda *et al.* (2011) have only tested HEXIM1 in MCF7 cells whereas U2OS cells were used in my work, the difference in results could also be due to the different cell types.

Although both U2OS and MCF7 cells express wild type p53, both cell lines have highly altered chromosomes with hypertriploid to hypotetraploidy range chromosome counts (ATCC database). These uncontrollable differences may cause them to respond differently to particular drugs. Hence ActD treatment in MCF7 cells should be used to clarify the discrepancy in future investigations.

My result also showed that in the absence of ActD, depleting Mybbp1a or NML in U2OS cells caused p21 level induction, tenfold or threefold respectively, without causing p53 stabilisation. This suggests that these proteins could mediate p53 activity but may not be involved in regulating p53 stability. In order to understand the unexpected results better, I further investigated the involvement of MDMX, one of the p53 negative regulator, in the ActD-induced p53 activation.

My subsequent results demonstrated that depleting Mybbp1a or NML promotes MDMX reduction (Section 3.2.7), and I also demonstrated that MDMX depletion results in p53 stabilisation and activation in U2OS cells (Section 3.2.8). MDMX functions as a negative p53 regulator which inhibits p53 activity by directly blocking its transactivation domain. It also dimerises to MDM2 which enhances the MDM2-

mediated ubiquitination and degradation of p53 (Section 1.3.2, Chapter 1). Therefore, it is possible that Mybbp1a or NML depletion promotes p53 activation via reducing MDMX levels.

However, the association between Mybbp1a or NML depletion and MDMX level reduction remains unclear. Both these co-factor proteins are nucleolar proteins linked to rDNA transcription repression. Mybbp1a associates with RNA Pol I and it represses rDNA transcription (Section 1.5.3, Chapter 1) and NML helps recruiting other protein factors resulting in histone methylation and deacetylation which inhibits rDNA transcription (Section 1.5.4, Chapter 1). Hence, it is possible that knocking down either of the protein results in misregulated rDNA transcription, which causes a stress signal resulting in p53 activation and p21 level induction.

3.3.2 SRSF1 could facilitate RPL11 recruitment during 5S RNP assembly

Given that the results could not establish a link between HEXIM1, MYBBP1A and NML and p53 signalling through 5S RNP, I subsequently focused on characterising the involvement of splicing factor SRSF1 in both 5S RNP assembly and 5S RNP-p53 signalling.

SRSF1 has been previously shown to interact with the 5S rRNA in BJ-TT cells (Fregoso *et al.*, 2013). Loren Gibson, a past member of the Watkins Lab, further confirmed that SRSF1 interacts with RPL5 and the 5S rRNA in U2OS cells (data unpublished). It was also demonstrated that SRSF1 depletion reduced RPL11 levels in the cytoplasm and decreased RPL5 levels in the nucleolus in U2OS cells, suggesting SRSF1 is required for 5S RNP assembly (Gibson, data unpublished). Dr Gibson therefore proposed that SRSF1 is involved in 5S RNP assembly functioning as a chaperone, facilitating the recruitment of RPL11 to the 5S rRNA/RPL5 complex during the 5S RNP assembly process.

In this work I demonstrated that recombinant SRSF1 interacts with both RPL11 and RPL5 *in vitro* (Figure 3.10C). My result confirmed the association between SRSF1 and RPL11, supporting the proposed role of SRSF1 in 5S RNP assembly. Since it showed that noticeably more RPL11 is pulled down by SRSF1 than RPL5, my result suggests that SRSF1 could have a higher affinity to RPL11 than that to RPL5. It is not yet clear why

SRSF1 has higher affinity for RPL11 than RPL5. One explanation could be that SRSF1 functions as a chaperone which binds to and stabilises RPL11 during the 5S RNP assembly.

Since RPL11 is produced in excess and the unbound molecules are degraded by the proteasome (Lam *et al.*, 2007), high SRSF1 binding affinity to RPL11 could help stabilising RPL11. It was also shown that RPL11 recruitment to the 5S RNP is a rate limiting step during the LSU production (Sloan *et al.*, 2013a), therefore SRSF1 may also facilitate the recruitment of RPL11 to the 5S rRNA/RPL5 complex, ensuring the efficiency of the 5S RNP assembly. In addition, since the recombinant His-tagged SRSF1 protein lacks the RS domain, my result further suggests this domain is not required for the interaction between SRSF1 and these 5S RNP components.

3.3.3 SPRIN430 affects p53 stability and p53 activity in response to ActD and Nutlin 3 treatment.

SRSF1 is a highly phosphorylated protein and its phosphorylation determines its functions (Gonçalves *et al.*, 2015). SRPK is one of the kinases responsible for the phosphorylation of SRSF1 and SRPIN340 is a kinase inhibitor specifically targeting SRPK. It has been proposed that SRSF1 is also important in p53 activation via the 5S RNP (Fregoso *et al.*, 2013), I therefore investigated, using the kinase inhibitor SRPIN340, whether SRSF1 function is involved in the 5S RNP-p53 signalling pathway.

SRPIN340 is a selective inhibitor only specific to SRPK1 and SRPK2, but not to Clk1 and Clk4 (database: Selleckchem; caymanchem). Its specificity for inhibiting SRPK2 is found to be 10-fold less than that for SRPK1 (Fukuhara *et al.*, 2006). SRPK1 targets selectively on SR protein family which includes SRSF1 (Ghosh and Adams, 2011). Compared to SRSF1, the SRPK1 function on other members in SR family is less understood. Hence SRPIN340 affects selectively to SRSF1 via specifically inhibiting SRPK1.

I first showed that in U2OS cells, blocking SRSF1 phosphorylation by SRPIN340 treatment inhibits p53 activation in response to ActD treatment in a dose dependent manner, suggesting SRSF1 phosphorylation could be important in the 5S RNP-p53 signalling pathway (Figure 3.11).

My result also showed that SRPIN340 treatment significantly reduces ActD-induced p53 activation, whereas it does not affect p53 activity in response to Nutlin3a treatment (Figure 3.12). Because Nutlin3a treatment stabilises and activates p53 via dissociating the MDM2/p53 interaction, my results imply that the SRPIN340 effect on counteracting p53 activation could be specific to nucleolar stress. I also demonstrated that ActD-induced p53 stabilisation was inhibited by SRPIN340 treatment in these cells (Figure 3.12). However, more future repeats of these experiments are required to fully address the SRPIN340 effect on p53 stability.

It has been shown that the SRPK1-mediated phosphorylation of the RS domain of SRSF1 is essential for its nuclear localisation (Lai *et al.*, 2001; Cazalla *et al.*, 2002). Therefore, it is likely that SRPIN340 keeps SRSF1 in a non-phosphorylated state, which cannot be translocated into the nucleus and accumulates in the cytoplasm. Accordingly, in my experiments, SRPIN340 may have caused depletion of nuclear SRSF1 in U2OS cells. In support of these results, it has been demonstrated that SRPIN340 blocks the insulin-like growth factor 1 (IGF-1) induced-nuclear localisation of SRSF1 in epithelial cells (Nowak *et al.*, 2010).

SRSF1 has been proposed to be important for the 5S RNP-p53 signalling. If SRPIN340 results in nuclear depletion of SRSF1, my results imply that a physical interaction between SRSF1 and the 5S RNP may be required for activating p53 in the nucleus. The requirement for a physical interaction between SRSF1 and the 5S RNP may also provide an explanation for the loss of stable p53 when treating cells with SRPIN340 and ActD (see Chapter 5). Therefore, in the future investigations, immunofluorescence could be used to confirm the SRPIN340 effect on the cellular localisation of SRSF1.

3.3.4 Future work

In this chapter I provided evidence showing HEXIM1, Mybbp1a and NML are not essential for the 5S RNP-mediated p53 signalling pathway. I also aimed to characterise the involvement of nucleolar protein B23 in this pathway, however due to inconsistent data no conclusive results could be shown. Because evidence suggested that B23 is important for p53 stabilisation and activation in response to cellular stress (Section 1.5.5), future investigation in its involvement in the 5S RNP-p53 pathway would be of interest. This could include repeating the experiment of B23 depletion and test how it

affects Actinomycin D-induced p53 and p21 levels in U2OS cells. Furthermore, the same workflow could be applied on U2OS cells expressing the p53-driven luciferase reporter to measure the p53 activity directly.

Moreover, this work also provided evidence supporting the 'chaperone' role of SRSF1 in the 5S RNP assembly, which was previously proposed by Loren Gibson in the Watkins Lab (Section 3.3.2). My results also suggested that phosphorylation of SRSF1 is important in regulating p53 activity in response to ribosomal stress. However, the dose of SRPIN430 and/or treatment time on U2OS cells should be reduced in future experiments, in order to minimise cell loss during the experiment (Section 3.2.11).

Future investigations should also focus on characterising the effect of splicing inhibitor SPRIN430 on SRSF1. For example, immunofluorescence could be used to confirm whether SPRIN430 treatment affects cellular localisation of SRSF1.

Finally, further studies should be carried out using SPRIN430 in optimised experiments, to elucidate whether SRSF1 phosphorylation affects specifically the 5S RNP-p53 signalling pathway (see Chapter 5).

Chapter 4. Understanding the Role of RPL11 Phosphorylation in Regulating Large Ribosomal Subunit Biogenesis and p53 Signalling

4.1 Introduction

4.1.1 The 5S RNP is important in ribosome biogenesis and p53 signalling

In eukaryotic cells, the mature ribosome consists of the small ribosomal subunit (SSU; 40S) and the large ribosomal subunit (LSU; 60S). The eukaryotic SSU contains the 18S rRNA and about 30 ribosomal proteins (RPs) whereas the eukaryotic LSU contains 28S, 5.8S, 5S rRNAs and 45 RPs (Gamalinda and Woolford, 2015).

In humans, ribosome biogenesis starts in the nucleolus, a membrane-less subcellular compartment within the nucleus, where rDNA is transcribed into the 47S pre-rRNA by RNA Pol I (Mullineux and Lafontaine, 2012). The 47S pre-rRNA undergoes multiple cleavage and modification processes. After incorporating to RPs it eventually becomes 28S and 5.8S rRNA in the LSU, and the 18S rRNA in the SSU (Figure 4.1) (Tafforeau *et al.*, 2013a).

The 5S rRNA, which is 120 nucleotides in length, is transcribed by RNA Pol III in the nucleoplasm. Once being produced, it is stabilised by binding to the ribosomal protein RPL5, before the RPL5/5S rRNA complex recruits RPL11. The 5S rRNA, RPL5 and RPL11 together form the 5S RNP which is subsequently integrated into the pre-LSU. The pre-LSU subsequently undergoes a series of maturation processes becoming the mature LSU (Figure 4.1) (Bursać *et al.*, 2012).

Ribosome biogenesis is linked to the stability and activation of the tumour suppressor p53 through the 5S RNP and MDM2 (Chakraborty *et al.*, 2011; Sloan *et al.*, 2013a). In response to different cellular stresses, p53 is activated and promotes the expression of its downstream regulated genes. Depending on severity and stress type, it leads to different cellular responses such as cell cycle arrest, DNA damage repair, apoptosis and senescence (Vousden and Prives, 2009). For example, one of the p53-regulated genes

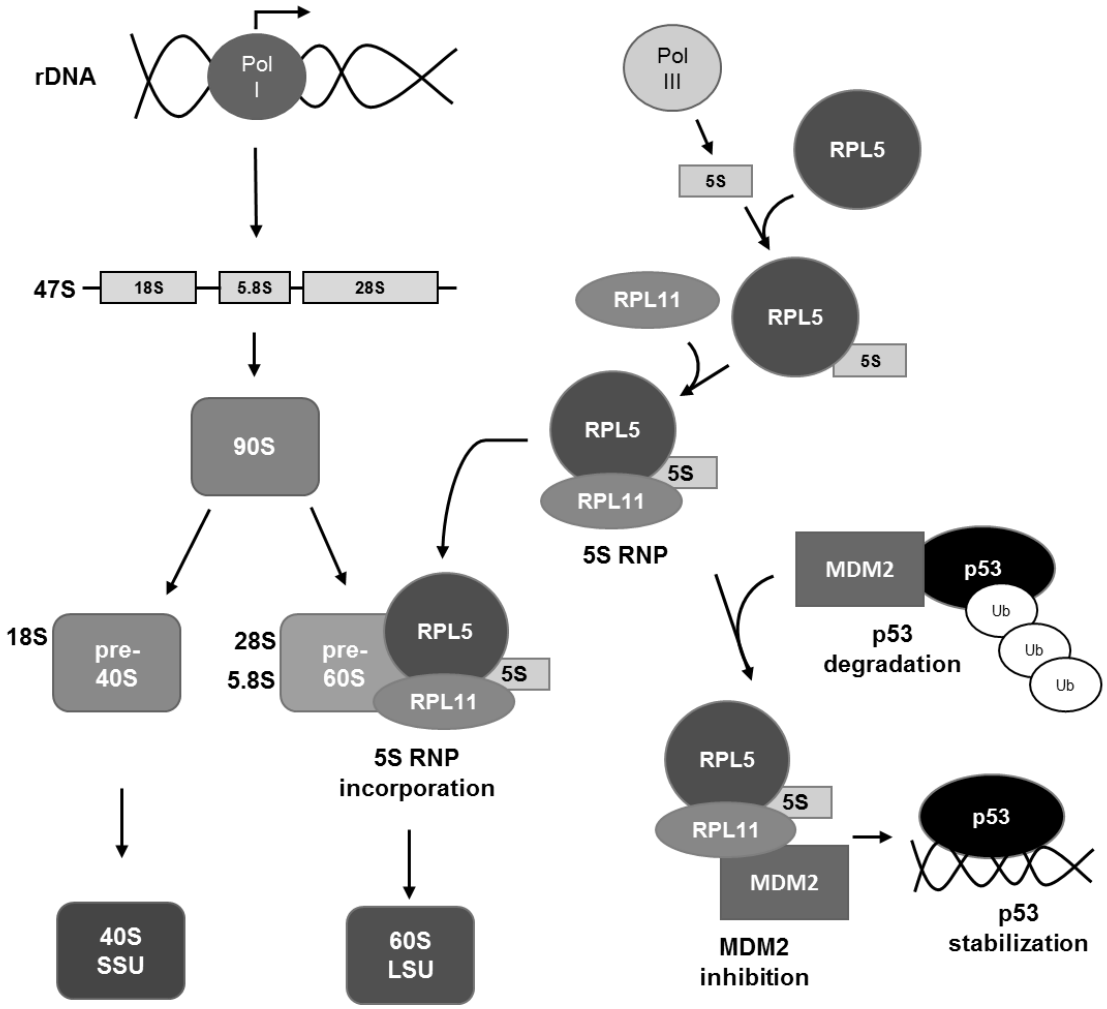

Figure 4.1. Schematic representation of how the 5S RNP is involved in ribosome biogenesis and MDM2-p53 regulatory signalling.

The pathway of the small and large ribosomal subunit biogenesis is shown on the left. 5S RNP biogenesis and integration pathway is shown in the middle. p53 stabilisation via the 5S RNP-MDM2 pathway was shown on the right.

is *CDKN1A* which translates to p21 protein, and p21 expression promotes cell cycle arrest (Vousden and Prives, 2009).

Normally p53 protein is tightly regulated and maintained at a low level in human cells by the proteins MDM2 and MDMX (Shadfan *et al.*, 2012). MDM2 functions as an E3 ubiquitin ligase polyubiquitinating p53 for proteasome mediated degradation (Figure 4.1). MDM2 and MDMX are highly conserved in their p53 binding domain located at the N-terminal and the RING domain located at the C-terminal (Zhang *et al.*, 2011). They bind to each other through the RING domain and function as a complex when regulating p53 (Chavez-Reyes *et al.*, 2003).

In the event of a ribosome biogenesis defect, free 5S RNP accumulates in nucleoplasm where it binds to and inhibits MDM2, causing p53 to be stabilised and activated (Figure 4.1) (Chakraborty *et al.*, 2011; Sloan *et al.*, 2013a; Pelava *et al.*, 2016).

4.1.2 RPL11 is important in ribosome biogenesis and 5S RNP-MDM2 binding

Ribosomal protein RPL11 plays a vital role in 5S RNP assembly and 5S RNP integration into the ribosome. The interaction between RPL11 and the 5S rRNA is important during the 5S RNP assembly. It was reported that the R75Q mutation in RPL11 inhibits RPL11-5S rRNA binding, blocking RPL11 recruitment to the 5S RNP (Horn and Vousden, 2008). In addition, RPL11 binding to the RPL5/5S rRNA complex was shown not only to be crucial for 5S RNP integration into the ribosome, but also for being a rate limiting step during LSU maturation (Sloan *et al.*, 2013a).

On the other hand, there is emerging evidence to suggest that RPL11 also plays an important role in p53 signalling, likely through the interaction between RPL11 and MDM2. Firstly, all three 5S RNP components, RPL5, RPL11 and the 5S rRNA interact with MDM2, and they are all required for the 5S RNP-p53 signalling (Sun *et al.*, 2010; Bursać *et al.*, 2012; Fumagalli *et al.*, 2012; Donati *et al.*, 2013; Sloan *et al.*, 2013a).

Secondly, mutated MDM2 abolishes 5S RNP-p53 signalling by inhibiting RPL11-MDM2 interaction. Interactions between RPL11 and MDM2 were first biochemically demonstrated in 2003 (Zhang *et al.*, 2003). Since then, the zinc finger domain in MDM2 has been shown to be crucial for the RPL11-MDM2 interaction. The C305F mutation in MDM2, located in the zinc finger domain (Figure 4.2A), was subsequently demonstrated to inhibit the interaction between MDM2 and RPL11 (Macias *et al.*, 2010). Furthermore, the disruption of RPL11-MDM2 interaction caused by this mutation also blocks p53 activation in response to nucleolar stress in a mouse model (Macias *et al.*, 2010), indicating the importance of RPL11-MDM2 binding in p53 regulation.

In addition, amino acid residues in RPL11 are important for the RPL11/MDM2 interaction. It has been reported that certain basic residues in RPL11, including K52,

R54 and R136, are important for RPL11 binding to MDM2 and also inhibit MDM2 activity (Zhang *et al.*, 2011).

4.1.3 Binding sites for MDM2 and the 28S rRNA colocalise in RPL11

A most recent piece of evidence supporting the important role that RPL11 plays in both ribosome biogenesis and p53 signalling, was reported by Zheng *et al.* in 2015. The structure of the human RPL11 associated with a short peptide fragment of human MDM2 (E293 to K334) has been resolved at 2.4 Å (Zheng *et al.*, 2015). Firstly, this work revealed that binding to RPL11 causes a conformational change in MDM2. In addition, the structural comparison of the MDM2 peptide bound on RPL11 with the 28S rRNA structures from *T. thermophiles* (PDB ID: 4W2E), *S. cerevisiae* (PDB ID: 4V88) and *D. melanogaster*, revealed a high degree of structural similarity between the bound MDM2 peptide and the 28S rRNA structure (Zheng *et al.*, 2015). This finding implies that MDM2 competes for the RPL11 binding site with the 28S rRNA which further suggests that RPL11 could be important controlling the 5S RNP-p53 signalling via MDM2 when ribosome biogenesis defect occurs.

To further validate this, I first analysed the structural similarity between Zheng's MDM2 peptide-RPL11 structure and the RPL11 that associates the 28S rRNA in the human ribosome. The Human 80S ribosome structure was resolved at 3.6 Å and published by Khatter *et al*. in 2015 (PDB ID: EMD-2938). To conduct the structural comparison, RPL11 and its associated 28S rRNA structure was isolated from the human ribosome (Figure 4.2C). The MDM2 E293-K334 peptide (PDB: 4xxb; Zheng *et al.,* 2015) was also isolated and overlaid on the 28S rRNA binding site of human RPL11 structure (Figure 4.2B).

The structural comparison showed a high degree of similarity between the MDM2 peptide and the 28S rRNA when binding to RPL11 in the ribosome (Figure 4.2B and C). In addition, 28S rRNA and MDM2 share the same RPL11 binding interface, except a small region of the Zinc finger domain in MDM2 (sticks in blue, Figure 4.2B). This suggests that RPL11 could be an important factor determining the 5S RNP integration into the LSU, or stabilising and activating p53 through binding to MDM2.

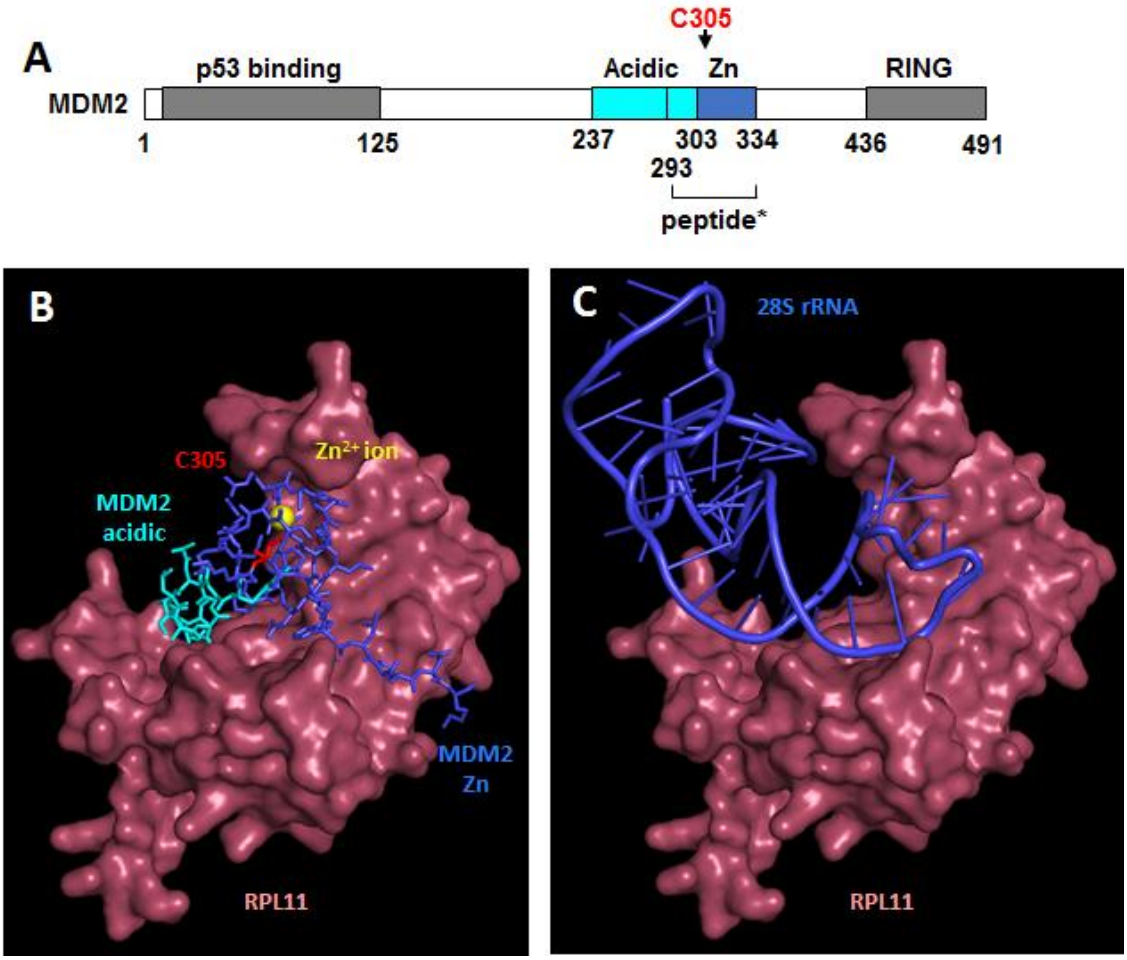

Figure 4.2. MDM2 interacts with RPL11 through its zinc finger domain; MDM2 and the 28S rRNA share the same binding interface on RPL11 in the ribosome.

(A) Schematic representation of protein domains in MDM2. The number of amino acid residues are indicated below the representation. p53 binding domain and RING finger domains (RING) are shown in grey. Acidic domain and the zinc finger domain (Zn) are coloured in cyan and blue respectively. Cysteine 305 (C305) is indicated in red. The peptide E293-K334 resolved in the MDM2-RPL11 crystal structure by Zheng *et al.* is indicated with an asterix. (B) Structure of RPL11 (pink, molecular surface, PDB ID: EMD-2938, Khatter *et al*., 2015) bound to MDM2 short peptide E293 to K334 (represented in sticks), with the acidic domain in cyan and the zinc finger domain (Zn) in blue (PDB ID: 4XXB, Zheng *et al.*, 2015). Cystine 305 (C305) is coloured in red whereas the Zn ion is coloured in yellow. (C) Structure of RPL11 (pink, molecular surface, PDB ID: EMD-2938, Khatter *et al*., 2015) bound to 28S rRNA (C4237 to C334, in blue, shown in sticks, PDB ID: EMD-2938, Khatter *et al*., 2015). The molecular structures were generated using PyMOL.

RPL11 mutations have also been linked to ribosomopathies. Ribosomopathies are a group of genetic disorders associated with impaired ribosome production and function, such as Diamond-blackfan anaemia (DBA), 5q syndrome, and Treacher-Collins Syndrome (Narla and Ebert, 2010) (see Chapter One for details). DBA, one of the best

studied ribosomopathies, is a form of inherited congenital bone marrow failure syndrome. It is characterised by macrocytic or normocytic anaemia with absence or insufficient erythroid precursors in the bone marrow (Gazda *et al.*, 2008; Narla and Ebert, 2010; Jaako *et al.*, 2015). In one recent study, DBA was associated to abnormalities in nine different ribosomal protein genes in approximately 53% of total 96 patients. Among them, RPL11 and RPL5 gene mutations were identified in 4.8% and 6.6% of total patients respectively (Gazda *et al.*, 2008). Furthermore, studies using a DBA mouse model suggested that the development of anaemia symptom was dependent on the 5S RNP-MDM2 interaction (Jaako *et al.*, 2015). These evidence once again implies the importance of 5S RNP-MDM2 interaction in connection with p53 regulatory signalling.

4.1.4 Aims and objectives

Although a number of studies have demonstrated the importance of RPL11 in relation to both ribosome biogenesis and the p53 regulation via MDM2, the involvement of post translational modification (PTM) in RPL11 function has not been described. There are numerous reports identifying RPL11 PTMs, including phosphorylation, acetylation, ubiquitination, methylation, neddylation and sumoylation according to the database PhosphoSitePlus® (see Section 4.2.1). Given the different functions of RPL11, such as binding to and stabilising the RPL5/5S rRNA complex during the 5S RNP assembly, associating with 28S rRNA to form the pre-60 large ribosomal subunit and interacting with MDM2 to stabilise p53, PTM could play a vital role in these functions.

In this chapter, I therefore aimed to establish the importance of RPL11 phosphorylation in ribosome biogenesis and 5S RNP/p53 signalling. I first validate the reported phosphorylated residues in RPL11. Thereafter, human cell lines expressing RPL11 phospho-mutants are created, and they are used to investigate whether the mutant protein affects ribosome biogenesis and the p53 signalling pathway.

4.2 Results

4.2.1 Reported phosphorylated sites in RPL11 are highly conserved

To start with my investigation, I first focused on the reported RPL11 phosphorylation sites in the database PhosphoSitePlus®. Up to the date of writing this thesis, a total of eight phosphorylated residues have been in reported in the literature using proteomic discovery-mode mass spectrometry (Table 4.1).

According to the database, the residue which scored the highest number of reports is threonine 47 (T47, 29 reports, Table 4.1). One of the recent studies reported the phosphorylation on both serine 29 (S29) and serine 51 (S51) using mass-spectrometry-based proteomic and phosphoproteomic analysis (Mertins *et al.*, 2016). In addition, serine 140 (S140) phosphorylation was reported twice (Rigbolt *et al.*, 2011; Luerman *et al.*, 2014).

Site	Number of reports
S29	6
T44	2
T47	29
S51	3
T73	1
Y119	1
S140	2
Y170	2

Table 4.1. Number of reports of each phosphorylated residue in Human RPL11 identified using proteomic discovery mass spectrometry. (PhosphoSitePlus® database, March 2018)

I further aligned RPL11 protein sequences from different eukaryotes, including human, mouse, zebrafish, fruit fly, yeast, Tetrahymena and mouse-ear cress, to analyse whether these reported phosphorylation sites are conserved in different species. The alignment showed that among these eukaryotic RPL11 sequences, S29, S51 and T73 are highly conserved (Figure 4.3). S140 however, is also conserved but interestingly

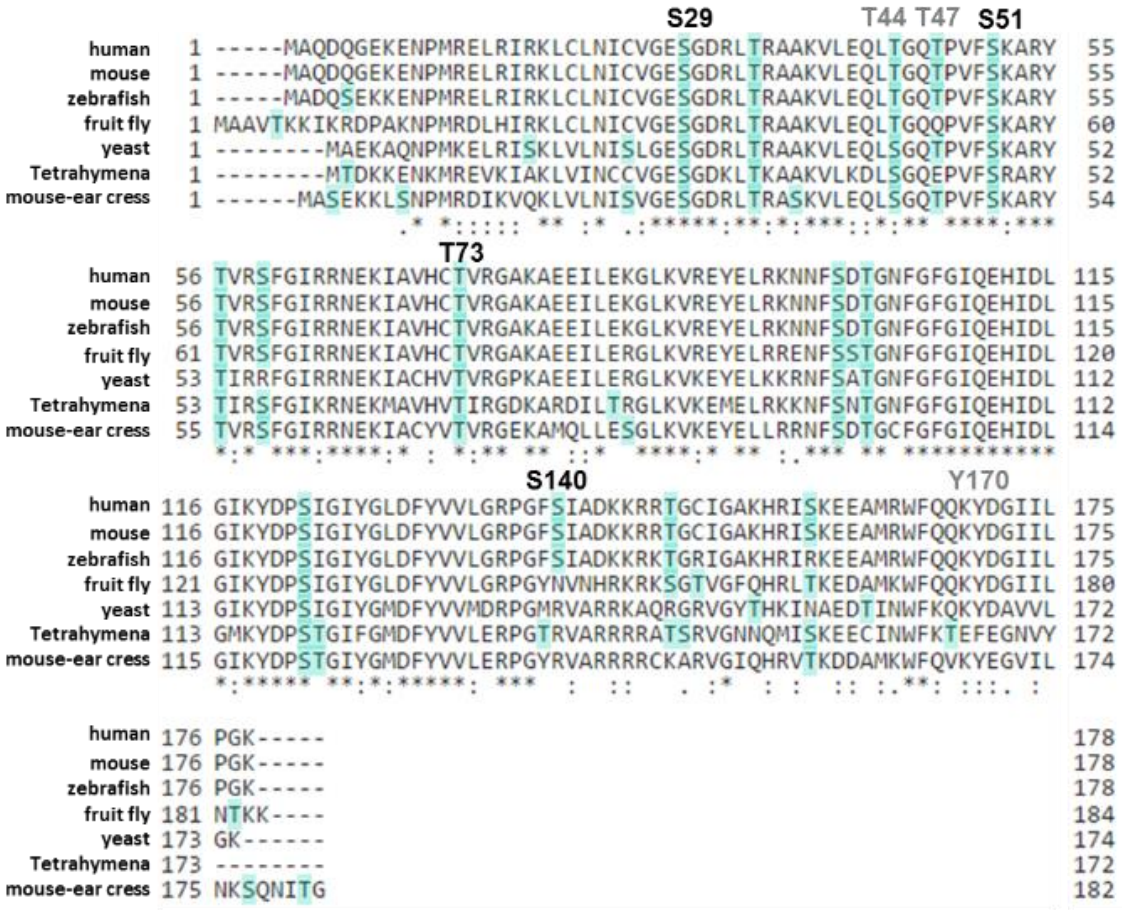

Figure 4.3.Reported phosphorylated sites on RPL11 are highly conserved.

Multiple sequence alignment of RPL11 protein sequences from different species. Serine and threonine residues are highlighted in cyan. Phospho-sites of interest (S29, S51, T73 and S140) are indicated above the sequence alignment in bold.

only among vertebra RPL11 sequences (Human, mouse and zebrafish). In yeast, Tetrahymena and plant RPL11, there is an arginine instead of a serine, whereas in fruit fly there is an asparagine (Figure 4.3).

4.2.2 Serine 51 interacts with 28S rRNA while threonine 73 and serine 140 locate close to the 5S rRNA in the ribosome structure

In mature ribosomes, RPL11 associates with the 5S rRNA of the 5S RNP and it also contacts the 28S rRNA from the LSU (see Chapter 1). In addition, Zheng *et al.* provided evidence suggesting MDM2 competes with the 28S rRNA for the same RPL11 binding site (Zheng *et al.*, 2015). I therefore decided to investigate whether these phosphorylated residues locate to the rRNA binding sites in RPL11.

I mapped the reported phosphorylated residues on RPL11 using the 60S ribosome structure resolved by Khatter *et al*. (Figure 4.4). Zheng *et al.* compared the RPL11 structure with a hand indicating the 'thumb', 'palm' and 'fingertips' (Figure 4.4) (Zheng *et al.*, 2015). Firstly, S29 is located in the RPL11 'thumb' region whereas S51 is located in the 'palm' region (Figure 4.4A). The 'palm' region was also reported to be the shared interface for RPL11/28S rRNA or RPL11/MDM2 binding (Zheng *et al.*, 2015). In the magnified view, the side chain of S51 is in close proximity to the backbone from cytosines 4258 and 4259 of the 28S rRNA (Figure 4.4B). Furthermore, T73 and S140 are located in the RPL11 'fingertip' region, where the 5S rRNA binds to RPL11 (Figure 4.4A). Interestingly, a magnified view locates T73 adjacent to the R75 and a previous study showed that a R75Q mutation abolishes RPL11/RPL5 interaction (Horn and Vousden, 2008). The sidechain of both R75 and T73 share an interaction interface with the base of the 5S rRNA (Figure 4.4C). Finally, the magnified view also revealed that the sidechain of S140 is in close proximity to the phosphate backbone of the 5S rRNA (Figure 4.4D).

As these highly conserved phospho-sites S29, S51, T73 and S140 are located on key regions in RPL11, it is likely that they are involved in ribosome biogenesis and/or MDM2-p53 signalling. I therefore decided to investigate how phosphorylation of these residues affects both processes.

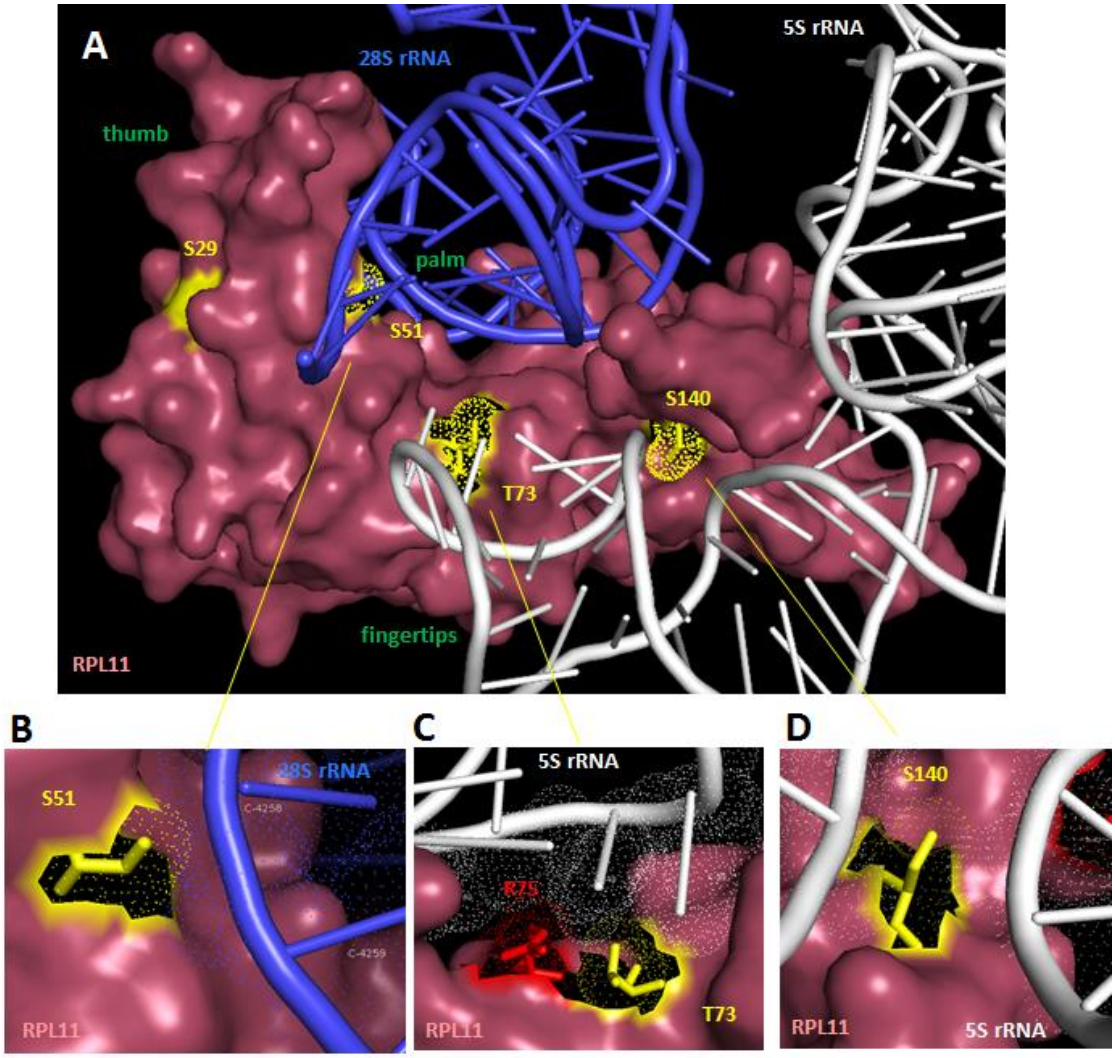

Figure 4.4. Threonine 73 and serine 140 are located close to the 5S rRNA whereas serine 51 is located close to the 28S rRNA in the ribosome.

(A) Reported phosphorylated sites are mapped in the region of RPL11/5S rRNA/28S rRNA within the ribosome (PDB ID: EMD-2938; Khatter *et al.*, 2015). RPL11 (in pink) is shown as a molecular surface view, with the phospho-sites of interest highlighted in yellow. 28S rRNA (blue) and 5S rRNA (white) are shown as sticks. The 'thumb', 'palm' and 'fingertips' descriptions are adopted from Zheng *et al.* (2015). (B-D) Magnified view from (A) showing a tight molecular interface (represented in dots) between RPL11 (pink) and 28S (blue) or 5S rRNAs (white). The side chain of the phosphorylated sites (S51, T73 and S140 in yellow) are represented as sticks. R75 is coloured in red. The molecular structures were generated using PyMOL.

4.2.3 Creation of inducible U2OS stable cell lines expressing FLAG-tagged RPL11 wild type and mutants

I first created U2OS cell lines stably expressing FLAG-tagged RPL11 wild type (WT) or RPL11 mutants in which the phosphorylated amino acid residues were mutated. I mutated the RPL11 encoding sequence such that serine (at 29, 51 and 140) was changed to an aspartic acid (D) as a negatively charged residue in order to mimic the phosphorylated serine side chain. At the same sites, I also replaced the serine to an alanine (A) so that the side chain cannot be phosphorylated (denoted phospho-null mutant). With the same idea and approach, I mutated the threonine (at 73) into a glutamic acid (E) which contains a negatively charged side chain, or to a valine (V) as a phospho-null mutant.

The pcDNA5 plasmid containing the RPL11 cDNA was used as the vector for these mutagenesis experiments as it enables the creation of stable mammalian cell lines expressing the RPL11 mutants in an inducible manner. A pcDNA5 plasmid containing an insert of FLAG-tagged wild type RPL11 has been previously constructed in the lab, and it is used as a template for site-directed mutagenesis to create vectors containing RPL11 mutants using mutagenesis PCR. The detail of this method is described in Section 2.1.4.

Eight pcDNA5 plasmids containing FLAG-tagged RPL11 mutants S29A, S29D, S51A, S51D, T73V, T73E, S140A and S140D were successfully created. I also aimed to create phospho-mutants at T47 in RPL11 because the phosphorylation of this threonine residue has been reported 29 times in database PhosphoSitePlus®. Unfortunately, despite significant time and effort spent, the site-directed mutagenesis PCR did not produce plasmids containing the desired sequence.

U2OS Flp-In™ cells were subsequently used and these plasmids were transfected to create inducible U2OS stable cell lines. An induction test was performed with the transfected cells by treating them with tetracycline for 48 hours. Cells were then harvested and whole cell lysates were separated using SDS-PAGE. FLAG-tagged protein levels were analysed by western blotting using the anti-FLAG antibody. U2OS cells transfected with the empty pcDNA5 plasmid were used as a control.

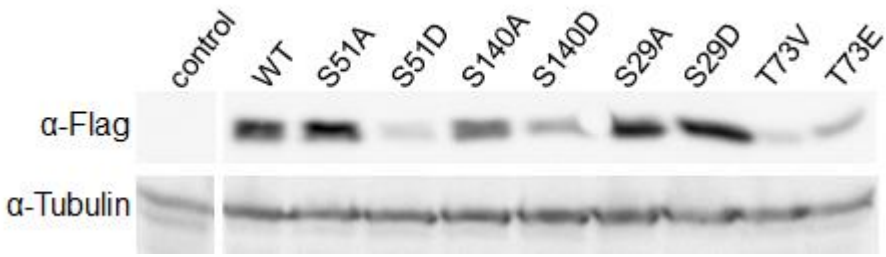

Figure 4.5.FLAG-tagged RPL11 mutants were detected in U2OS stable cell lines at different levels.

FLAG-tagged RPL11 wild type or mutant protein levels in U2OS stable cell lines were determined by western blotting. U2OS stable cell lines transfected with plasmid encoding FLAG-tagged RPL11 wild type or mutants (indicated above the panel) were treated with 1 µg/ml tetracycline for 48 hours. Whole cell lysates were separated using SDS-PAGE and analysed by western blotting using specific antibodies. U2OS cells transfected with empty plasmid were used as control. Tubulin levels were determined and used as loading reference. Antibodies used are indicated to the left of each panel.

FLAG-tagged proteins with the expected size were observed in all cell lines created, whereas FLAG levels were undetectable in the cells that contain the empty pcDNA5 control plasmid (Figure 4.5). In cells expressing S51A, S29A and S29D RPL11 mutants, FLAG-tagged protein levels were similar to those in WT cells. In contrast, the rest of mutant cells showed significantly less FLAG-tagged RPL11 protein, especially for T73V (Figure 4.5). This suggests that these mutant proteins may be less stable than their WT counterparts.

4.2.4 FLAG-tagged RPL11 mutants associate with the 5S and 5.8S rRNAs

As shown previously, S51, T73 and S140, are located close to either the 5S or 5.8S rRNA in the LSU (Figure 4.4). Therefore, it is important to evaluate whether the point mutations introduced to RPL11 affect the protein's ability to associate with the 5S and 5.8S rRNAs.

To analyse this question, U2OS cells were treated with tetracycline for 48 hours in order to express WT or mutant FLAG-tagged RPL11 before whole cell lysates were prepared. Anti-FLAG affinity agarose beads were then incubated with the whole cell lysates. The unbound material was removed and the beads were washed. RNA was subsequently extracted from the bound material and analysed by Northern blotting using ^{32}P labelled probes specific to the 5S and 5.8S rRNAs. FLAG-tagged protein levels bound to the beads were also detected by western blotting using anti-FLAG antibody. U2OS cells transfected with empty pcDNA5 plasmid were analysed as control.

For reasons that we could not explain 5S and 5.8S rRNAs were not reliably detected from both T73 mutants, therefore their results were left out from this analysis (Figure 4.6),. A possibility could be the low protein levels of T73E and T73V, which was observed previously (Figure 4.5), which resulted in too little protein for immunoprecipitation.

When analysing 10% of the total input, FLAG-tagged proteins were detected in all samples except in the control (Figure 4.6). Among all samples, the protein levels of FLAG-tagged RPL11 S51D and S140D bound to the beads were noticeably lower than the other mutants (Figure 4.6), in agreement with cellular protein levels from previous results (Figure 4.5). This suggests that the FLAG-tagged S51D and S140D mutant proteins may be less stable than other mutant proteins.

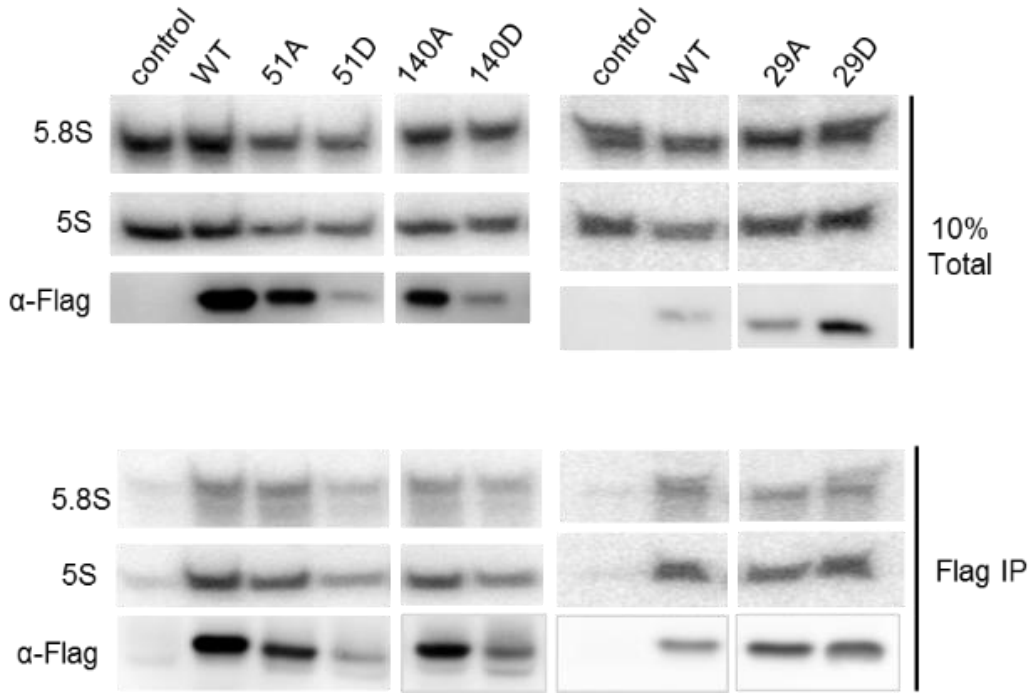

Figure 4.6. The 5S and 5.8S rRNAs associate with some of the RPL11 mutants.

U2OS cells stably expressing RPL11 WT or mutants (indicated above the panels) were induced for 48 hours. Anti-FLAG affinity agarose beads were used to capture the FLAG-tagged protein and the associated rRNAs. After removal of the unbound material, the rRNAs associated to the FLAG-tagged protein were detected by Northern blotting using specific probes (indicated on the left). 10% of the total IP input material was also analysed and used as a positive control. U2OS cells transfected with empty plasmid were used as negative control. FLAG-tagged protein levels were determined by western blotting with anti-FLAG antibody. Independent experiments were repeated twice and a representative result is presented.

The FLAG IP showed background levels of the 5.8S and 5S rRNAs that were co-purified in the control sample (Figure 4.6). It also showed that in all mutant samples, the both 5S and 5.8S rRNAs were detected in the FLAG-IP material.

These results confirm the association between the mutant proteins and the 5S and 5.8S rRNAs. In conclusion, except for the T73 site, RPL11 mutant proteins are able to bind to the 5S and 5.8S rRNAs, and the cells are able to utilise the mutated FLAG-tagged RPL11 proteins to produce ribosomes.

4.2.5 The 5S RNP incorporation of FLAG-RPL11 wild type is less efficient than seen with the endogenous RPL11

I next investigated whether the reported phosphorylated amino acid residues in RPL11 are involved in the ribosome biogenesis. The idea is to analyse whether the expression of RPL11 phospho-mutant proteins affect the ribosome production in U2OS cells as compared to the WT protein.

Glycerol gradient centrifugation was used to separate the free 5S RNP from the mature and the pre-ribosomes, before determining the distribution of FLAG-tagged protein found in both free and ribosomal complexes. I also analysed ribosomal protein RPL7 which provides a marker for the ribosomal complexes. Ribosomal protein RPL7 is a large subunit protein component which exists in pre-60S and the mature 60S ribosomal subunits (Russo *et al.*, 1997). RPL7 protein distribution was measured in each gradient experiment for two reasons: Firstly, RPL7 distribution indicates those fractions containing ribosomes and pre-ribosomes. Secondly, it also serves as an internal control between different gradient runs.

Stable U2OS cell lines were treated with tetracycline for 48 hours in order to express FLAG-tagged RPL11 WT or mutant proteins. The cells were then harvested and whole cell lysates were prepared. The extracts were subsequently separated in a 10-40% glycerol gradient using ultracentrifugation. The gradient was subsequently fractionated into 20 samples (labelled as 1-20, from top to bottom, Figure 4.7A). Proteins in each fraction were separated by SDS-PAGE followed by western blotting using anti-FLAG and anti-RPL7 antibodies. The probed proteins were visualised using either ECL or fluorescence detection. A quantitative distribution analysis was perform based on the western signals, by first quantitating the protein signal (FLAG-tagged RPL11 or RPL7) detected in each fraction. The protein signal intensity in each fraction was then converted into a percentage of total, before data from all fractions were presented in a histogram which represents the protein distribution from free and ribosomal complexes (protein level percentage over gradient fraction, Figure 4.7B). Finally, the FLAG-tagged RPL11 distribution in free and ribosomal complexes are calculated by combining the percentage number in free (fraction #1-6) or ribosomal fractions (fraction #8-20) (Figure 4.7C).

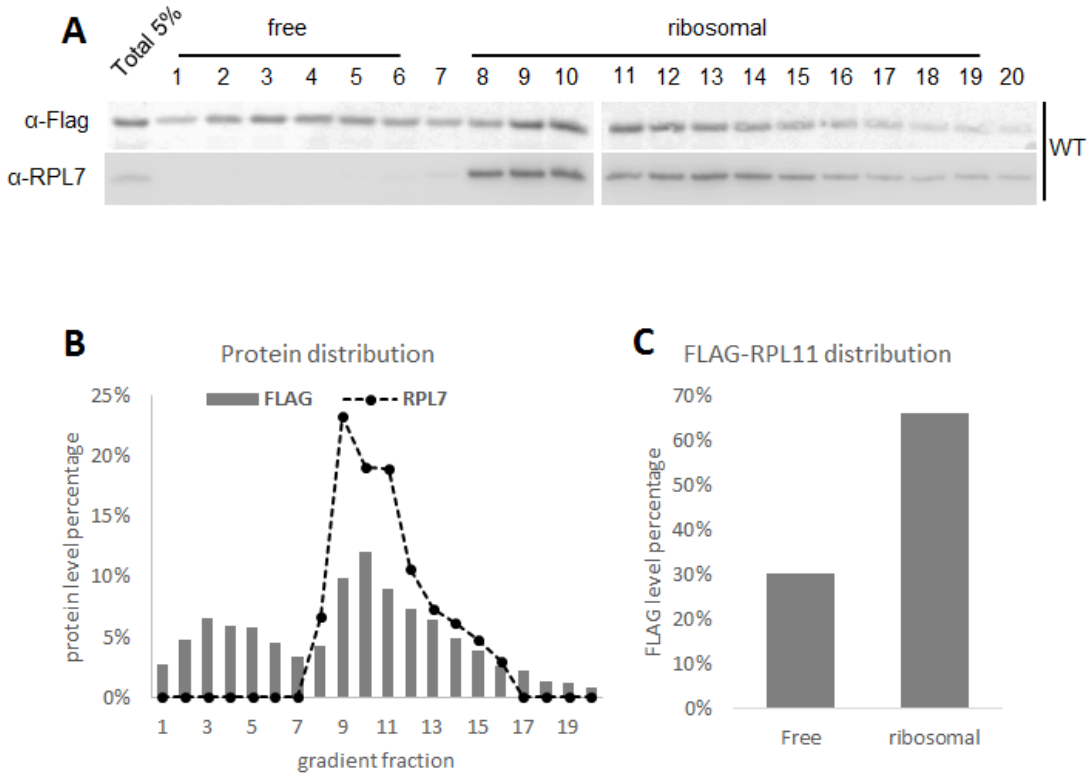

Figure 4.7. One third of FLAG-RPL11 wild type protein was found in the 'free' fractions in U2OS cells overexpressing the protein.

(A)Extracts from stable U2OS cells expressing FLAG-RPL11 wild type were analysed using glycerol gradient centrifugation followed by western blotting. The glycerol gradient was fractionated into 20 samples (indicated above the panel). Fractions #1-6 and #8-19 are indicated as 'free' and 'ribosomal', respectively. Antibodies used are indicated to the left of each panel. (B)Protein levels detected in each gradient fraction was quantitated. Protein distributions are presented as the percentage of total protein levels (sum of all fractions #1-20). (C) FLAG-RPL11 levels in 'free' or 'ribosomal' fractions are presented. FLAG levels from fractions #1-6 in (B) were combined and indicated as 'free', fractions #8-19 is indicated as 'ribosomal'. Multiple independent experiments were performed and a representative result is presented.

Before focusing on the mutant proteins, I first analysed gradient samples from cells expressing the FLAT-tagged RPL11 WT protein. In these cells, RPL7 protein was detected in the fractions #8 – 20 which coincides with the distribution of ribosomal complexes in the gradient (Figure 4.7A). The signal quantitation showed a primary peak in fractions #9 – 11, followed by a relative smaller 'shoulder peak' in fractions #13 – 16 where the RPL7 distribution declined gradually (Figure 4.7B).

The quantitative distribution analysis also showed that about 30% of the total FLAG-RPL11 was present in free fractions whereas about 70% was detected in the ribosomal

counterparts (Figure 4.7C). Among the non-ribosome associated fractions, #3 contained the highest percentage of FLAG-RPL11 protein and the percentage declined gradually from #4 to #7 (Figure 4.7B). In ribosomal fractions, FLAG-RPL11 distributed in a similar way compared to RPL7. They were both distributed from fractions #8 to #20, with a peak at fractions #9-11 (Figure 4.7A-B).

Previous study had shown that the free, or non-ribosomal, 5S RNP is distributed in the 'free' fractions (Sloan *et al.*, 2013a). Sloan *et al.* also reported that in HEK293 cells there was almost no endogenous RPL11 found in the free complexes but primarily detected in the ribosomal complexes (Sloan *et al.*, 2013a). The FLAG-tagged RPL11 is primarily found in ribosomal complexes in my result, which is largely consistent with what has been previously reported by Sloan *et al*. However, the increased levels of FLAG-RPL11 in the 'free' fractions may suggests that the FLAG-tagged protein is not being incorporated into the ribosome as well as the endogenous form. This could be due to the charged nature of the FLAG tag which may affect the recruitment of 5S RNP into the LSU.

4.2.6 Threonine 73 mutations in RPL11 cause accumulation in free 5S RNP in U2OS cells

Having characterised the wild type RPL11, I next analysed RPL11 mutations T73V and T73E. In U2OS cells expressing the FLAG-RPL11 T73V mutant, more than 60% of the total FLAG-tagged protein was detected in the free fractions (#1-6), significantly more than that observed from WT protein (Figure 4.8B). In free fractions, the FLAG-RPL11 T73V mutant showed a similar distribution to the WT protein (#1-6, Figure 4.8B). In the ribosomal fractions (#8-18), FLAG levels from fractions #10 to #15 were slightly lower than from the WT protein (Figure 4.8B). Taken these results together, they suggest that 5S RNP containing the RPL11 T73V mutation incorporates less efficiently into the large subunit precursor than the RPL11 WT.

In U2OS cells expressing the FLAG-RPL11 T73E mutant protein, the majority of T73E protein was detected in free fractions whereas the protein was present only at low levels in the ribosomal fractions (Figure 4.8A). Over 80% of total T73E protein was present in free fractions, approximately three fold as much as seen with the WT protein (Figure 4.8D).

The majority of the FLAG-RPL11 T73E mutant was detected in fraction #2. In the 'free' fractions the T73E mutant was accumulated in fractions #1-3, resulting a relatively narrower peak, whereas the WT protein distributed broadly in fractions #1-6 (Figure 4.8C). This 'shifted' peak seen in the 'free' fractions suggests that the RPL11 containing T73E mutation is not being assembled efficiently into the 5S RNP.

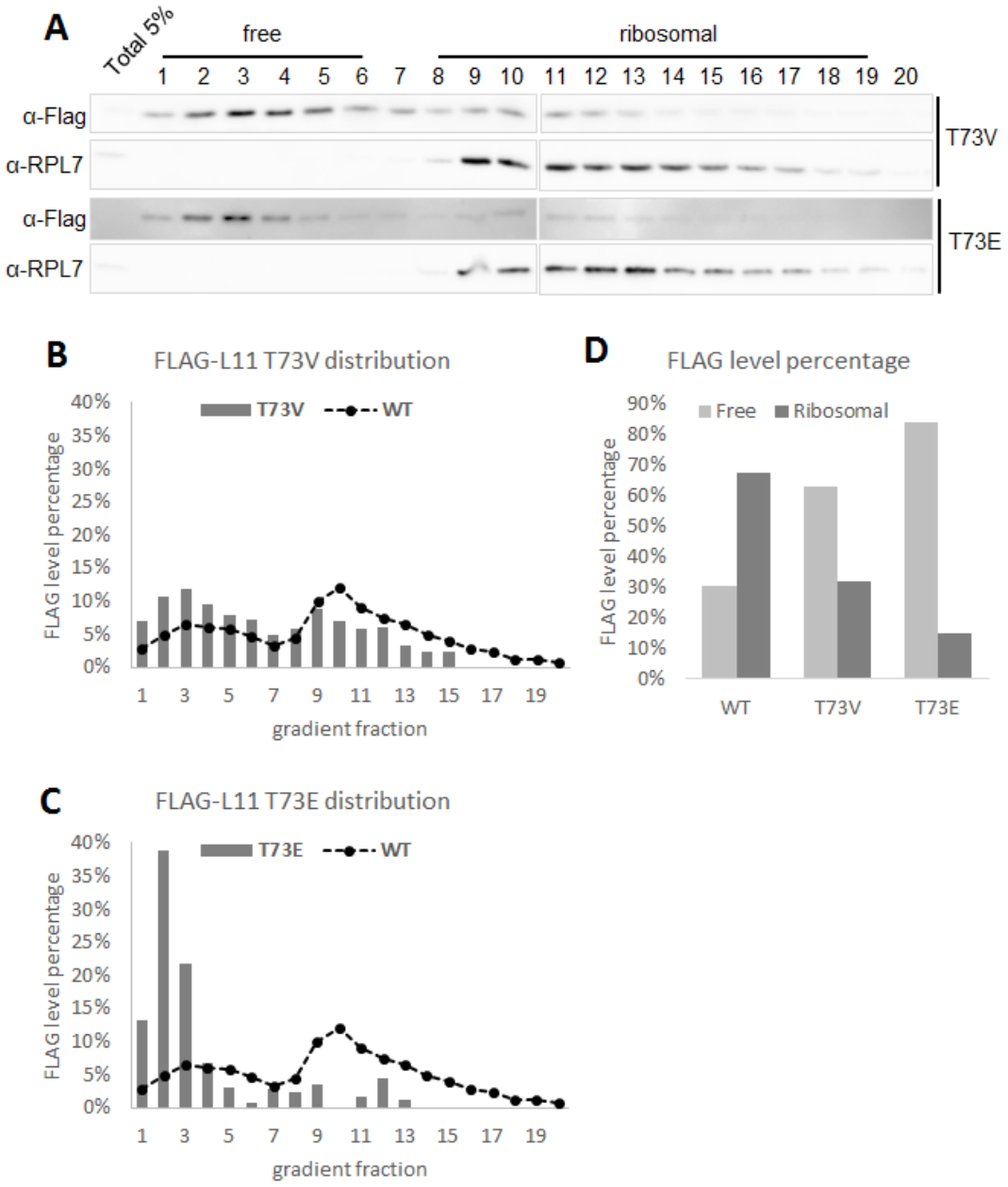

Figure 4.8. T73 RPL11 mutants are defective at integration into ribosomes.

(A) Extracts from U2OS cells overexpressing FLAG-RPL11 T73V (T73V) or T73E (T73E) were analysed using glycerol gradient centrifugation followed by western blotting. The gradient fractions are indicated above the panel. Antibodies used are indicated to the left of each panel. (B-C) FLAG-tagged RPL11 levels detected in each gradient fraction from U2OS cells expressing RPL11 T73V or T73E mutants were quantitated. FLAG-tagged RPL11 distributions are presented as percentage of total protein levels (sum of fractions #1-20). Extracts from U2OS cells expressing RPL11 wild type were analysed likewise and FLAG-RPL11 wild type levels were used as control (indicated as WT). (D) FLAG-tagged RPL11 levels distributed in 'free' or 'ribosomal' fractions were presented as percentage of the total FLAG levels. FLAG levels from fractions #1-6 (in B or C) were compiled and presented as 'free',

4.2.7 Serine 51 mutations in RPL11 affect 5S RNP integration into the ribosome

In stable U2OS cells expressing the S51A RPL11 mutant, there was almost 50% of the total FLAG-RPL11 S51A mutant protein accumulated in the 'free' fractions which was a noticeable increase compared to the WT protein (Figure 4.9D). When looking at FLAG-RPL11 distributions in the 'free' fractions (#1-6), the mutated protein predominantly located in fractions #2 to #4 and was almost enriched two fold as compared to the WT protein (Figure 4.9B). Furthermore, in the ribosomal fractions, the FLAG-RPL11 S51A protein peak was shifted slightly compared to the WT protein peak (Figure 4.9B). However, when comparing the RPL7 distribution between S51A and WT cell extracts, the RPL7 peak was shifted similar to the altered distribution seen with FLAG-RPL11. Therefore, the FLAG peak difference between S51A and WT extracts was likely due to fractionation inconsistency. The accumulated FLAG-RPL11 S51A mutant in 'free' fractions suggest that this mutation leads to a less efficient 5S RNP incorporation into the ribosome.

In U2OS cells expressing the S51D RPL11 mutant, the majority of the protein was detected in the 'free' fractions and made up almost 70% of the total mutant protein, more than the double of the WT protein (Figure 4.9D). In contrast, the FLAG-RPL11 mutant protein was almost undetectable in the ribosomal fractions (fractions #8-18, Figure 4.9A).

When analysing the gradient fractions, most of the S51D mutant protein appeared accumulated in fractions #2-4, whereas the distribution of the mutant in the following fractions #5-7 was similar to the WT protein (Figure 4.9C). Comparable to the cells expressing the S51A RPL11 mutant, the S51D mutant accumulated in fractions #2-4 whereas fractions #3-7 contained similar levels compared to the WT protein (Figure 4.9B and C). In the 'ribosomal' fractions, the S51D mutant distributed similarly to the WT protein, in which the peak of FLAG-tagged protein locates to fraction #10 (Figure 4.9 C).

The accumulation of the S51D mutant in 'free' fractions suggest that this mutation blocks 5S RNP incorporation into the ribosome. In summary, these result suggest that serine 51 is important for 5S RNP incorporation into the ribosome.

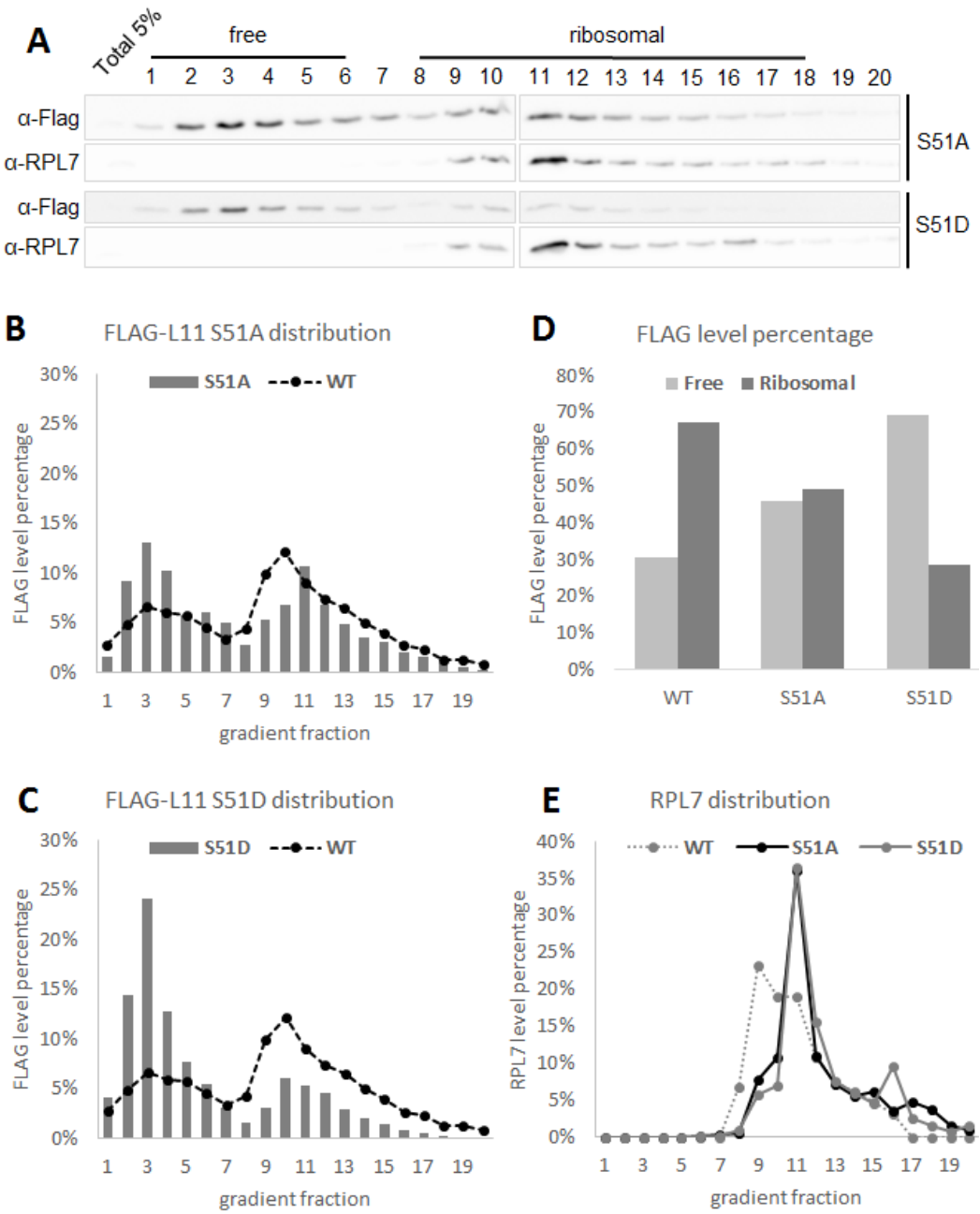

Figure 4.9. Serine 51 mutations promote increased RPL11 in 'free' fractions.

(A) Extracts from U2OS cells overexpressing FLAG-RPL11 S51A (S51A) or S51D (S51D) were analysed using glycerol gradient centrifugation followed by western blotting. The gradient fractions are indicated above the panel. Antibodies used are indicated to the left of each panel. (B-C) FLAG-tagged RPL11 levels detected in each gradient fraction from U2OS cells expressing RPL11 S51A or S51D mutants were quantitated. The FLAG distribution is presented as the percentage of total protein levels (sum of fractions #1-20). Extracts from U2OS cells expressing RPL11 wild type were analysed in parallel and FLAG-RPL11 wild type distributions were used as control (indicated as WT). (D) The level of FLAG-tagged RPL11 distributed in 'free' or 'ribosomal' fractions are presented in percentage of the total FLAG levels. FLAG levels from fractions #1-6 (in B or C) were compiled and presented as 'free', fractions #8-19 are presented as 'ribosomal'. (E) The distribution of RPL7 was determined by quantitating RPL7 levels decribed in (A). Data are presented in percentage of the total RPL7 levels. U2OS cells expressing RPL11 wild type were analysed in parallel and the corresponding RPL7

4.2.8 Serine 140 mutations in RPL11 affect ribosome maturation

When analysing the protein distribution in extracts harvested from U2OS cells expressing S140A or S140D FLAG-RPL11 mutants, both of the FLAG-tagged proteins were detected predominantly in the 'ribosomal' fractions (Figure 4.10A). In general, the overall FLAG-RPL11 distributions between 'free' and 'ribosomal' fractions were very similar among the S140A, S140D mutants and the WT protein (Figure 4.10D).

In the 'ribosomal' fractions, S140D RPL11 mutant distributed in two peaks in fractions #9 and #11, which was distinctively different from the single peak seen for the WT RPL11 in fraction #10 (Figure 4.10C). Referencing to the RPL7 distribution, as RPL7 in WT cell extracts is primarily detected in fractions #9 and #10 and RPL7 marks fractions containing mature ribosomes, those two peaks seen with the S140D RPL11 mutant suggest that this mutation results in pre-60S accumulation and less mature ribosomes. However, the defect seen with the S140D mutants was less pronounced with the S140A mutants. This implies that serine 140 phosphorylation could be important for ribosome biogenesis (see Section 4.3 Discussion).

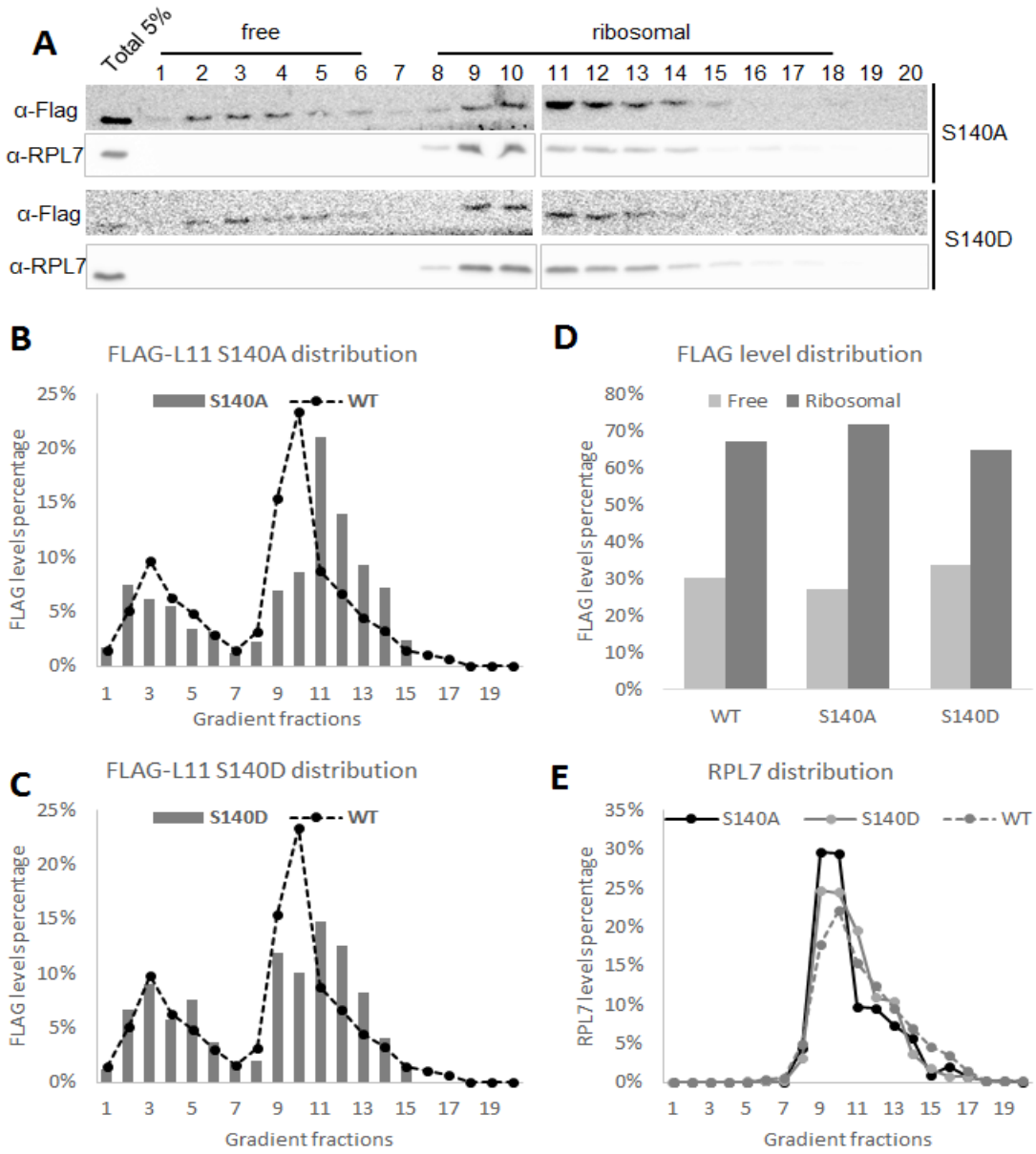

Figure 4.10. Serine 140 mutations promote accumulation of pre-60S ribosomes.

(A) Extracts from U2OS cells overexpressing FLAG-RPL11 S140A (S140A) or S140D (S140D) were analysed using glycerol gradient centrifugation followed by western blotting. The gradient fractions are indicated above the panel. Antibodies used are indicated to the left of each panel. (B-C) FLAG-tagged RPL11 levels detected in each gradient fraction from U2OS cells expressing RPL11 S140A or S140D mutants were quantitated. The FLAG distribution is presented in the percentage of total protein levels (sum of fractions #1-20). Extracts from U2OS cells expressing RPL11 wild type were analysed in parallel and FLAG-RPL11 wild type distributions were used as control (indicated as WT). (D) The level of FLAG-tagged RPL11 distributed in 'free' or 'ribosomal' fractions are presented in percentage of the total FLAG levels. FLAG levels from fractions #1-6 (in B or C) were compiled and presented as 'free', fractions #8-19 are presented as 'ribosomal'. (E) The distribution of RPL7 was determined by quantitating RPL7 levels detected in (A). The RPL7 distribution is presented in percentage of the total RPL7 levels. U2OS cells expressing RPL11 wild type were analysed in parallel and its RPL7 distribution was used as control

4.2.9 RPL11 S29D mutant causes accumulation of pre-LSU complexes in U2OS cells

I next analysed the FLAG-RPL11 mutant distribution in 'free' and 'ribosomal' fractions in U2OS cells expressing S29A mutants. Approximately 40% of the total FLAG-tagged RPL11 mutants were present in the free fractions (Figure 4.11A and D). This was slightly higher than the WT protein (Figure 4.11D). Compared to the WT, despite the slight quantitative increase, the distribution of FLAG-RPL11 S29A in the 'free' fractions was comparable to the WT protein (Figure 4.11B).

When looking at the ribosomal fractions (#8-18, Figure 4.11B), fraction #11 contained the highest amount of S29A mutant protein. Compared to the WT, this 'ribosomal' peak was slightly shifted (Figure 4.11B). Furthermore, RPL7 distribution in the ribosomal fractions of cells expressing RPL11 S29A was also shifted compared to the WT (Figure 4.11E). Hence the shifted FLAG peak of the mutants are likely due to gradient or fractionation inconsistencies.

In cells expressing the S29D mutant, there were barely detectable levels of FLAG-RPL11 mutants in the 'free' fractions, whereas the majority was detected in the 'ribosomal' fractions #10 to #15 (Figure 4.11A). Overall, there was only less than 10% of total FLAG-RPL11 mutant protein present in 'free' fractions, which was noticeably less than in WT or RPL11 S29A expressing cells (Figure 4.11D). In the 'ribosomal' fractions, the FLAG-RPL11 S29D mutant peak was broader and shifted compared to the WT protein (Figure 4.11C). When comparing the RPL7 distribution in WT and RPL11 S29D extracts, a similar broader and shifted peak could also be observed (Figure 4.11E). The shifted RPL7 peak from RPL11 S29D cell extracts indicated that the observed shifted FLAG-RPL11 S29D peak was likely due to inconsistent gradient fractionation. However, both the FLAG-RPL11 mutant and RPL7 proteins from the RPL11 S29D extract distributed differently to the control.

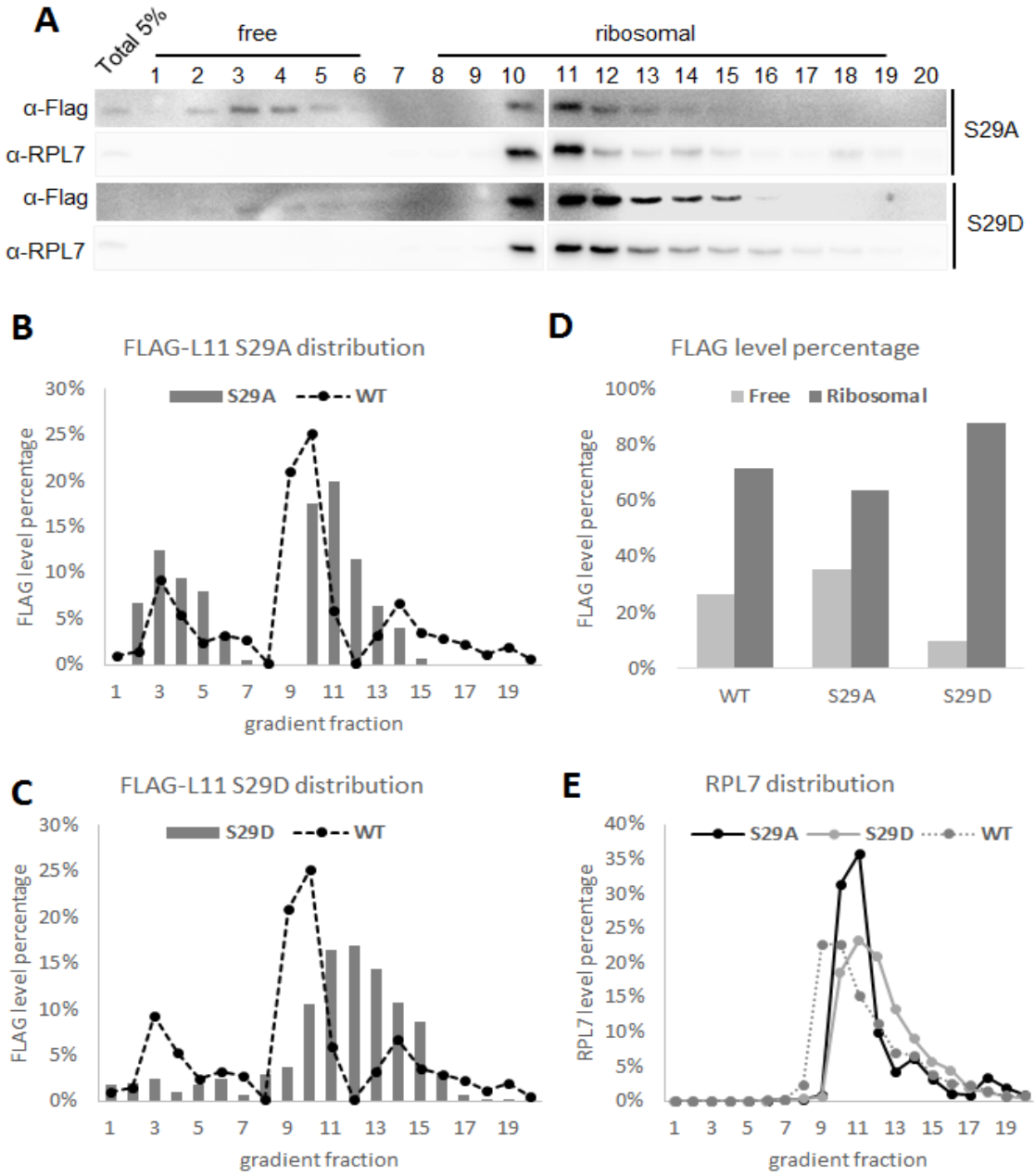

Figure 4.11. S29A and S29D RPL11 mutations cause different FLAG-RPL11 distributions.

(A) Extracts from U2OS cells overexpressing FLAG-RPL11 S29A (S29A) or S29D (S29D) were analysed using glycerol gradient centrifugation followed by western blotting. The gradient fractions are indicated above the panel. Antibodies used are indicated to the left of each panel. (B-C) FLAG-tagged RPL11 levels detected in each gradient fraction from U2OS cells expressing RPL11 S29A or S29D mutants were quantitated. FLAG-tagged RPL11 distributions are presented as the percentage of total protein levels (sum of fractions #1-20). Extracts from U2OS cells expressing RPL11 wild type were analysed in parallel and FLAG-RPL11 wild type distributions were used as control (indicated as WT). (C) The level of FLAG-tagged RPL11 distributed in 'free' or 'ribosomal' fractions are presented as percentage of the total FLAG levels. FLAG levels from fractions #1-6 (in B or C) are compiled and presented as 'free', and fractions #8-19 are presented as 'ribosomal'. (E) The distribution of RPL7 was determined by quantitating RPL7 levels detected in (A). Data is presented in percentage of the total RPL7 levels. U2OS cells expressing RPL11 wild type were analysed in parallel and the RPL7 distribution was used as control (indicated as WT). Independent experiments were repeated three times and a representative result is presented.

The disappearance of the FLAG-RPL11 S29D peak in the 'free' fractions, suggests that 5S RNP containing this mutant RPL11 is incorporated into the large ribosomal subunit more efficiently than the WT protein. Furthermore, the broader 'ribosomal' FLAG and RPL7 peak from the S29D extracts suggests that, relative to the mature 60S, the mutation resulted in accumulated pre-60S ribosomes. Taken together, serine 29 phosphorylation and/or dephosphorylation could be important to both 5S RNP integration and large ribosomal subunit biogenesis.

4.2.10 Analysing subcellular localisations of RPL11 phospho-mutants.

Glycerol gradient analysis suggested that some RPL11 mutations affect ribosome biogenesis. Hence, I next performed immunofluorescence in the U2OS cells expressing RPL11 mutants in order to investigate whether RPL11 mutations affect the protein's subcellular localisation.

Tetracycline was applied to the stably transfected U2OS cell lines grown on glass coverslips for 48 hours in order to express FLAG-tagged RPL11 WT or mutant proteins. After fixing the cells, anti-FLAG antibody was used detect the FLAG-RPL11 proteins and DAPI staining was used to identify the nucleus. The signal was subsequently visualised with fluorescence microscopy. U2OS cells transfected with empty pcDNA5 plasmid were used as control.

In the pcDNA5 control cells, no FLAG signal was detected. In the cells expressing WT RPL11, the FLAG signal was predominantly located in nucleoli and the cytoplasm (WT, Figure 4.12). This is consistent with previous observations performing immunofluorescence that RPL11 was predominantly located in the nucleoli and cytoplasm of HEK293 cells (Sloan *et al.*, 2013a).

In the U2OS cells expressing the S140A RPL11 mutant, the cytoplasmic levels of RPL11 S140A were significantly reduced compared to control cells (S140A, Figure 4.12). This observation agrees with the gradient results which showed a decrease of mutant protein in mature ribosome (Figure 4.10B). In the cells expressing the S140D mutant, the FLAG-tagged protein localisation was largely similar to the WT protein, with a slightly stronger nucleolar FLAG signal (S140D, Figure 4.12).

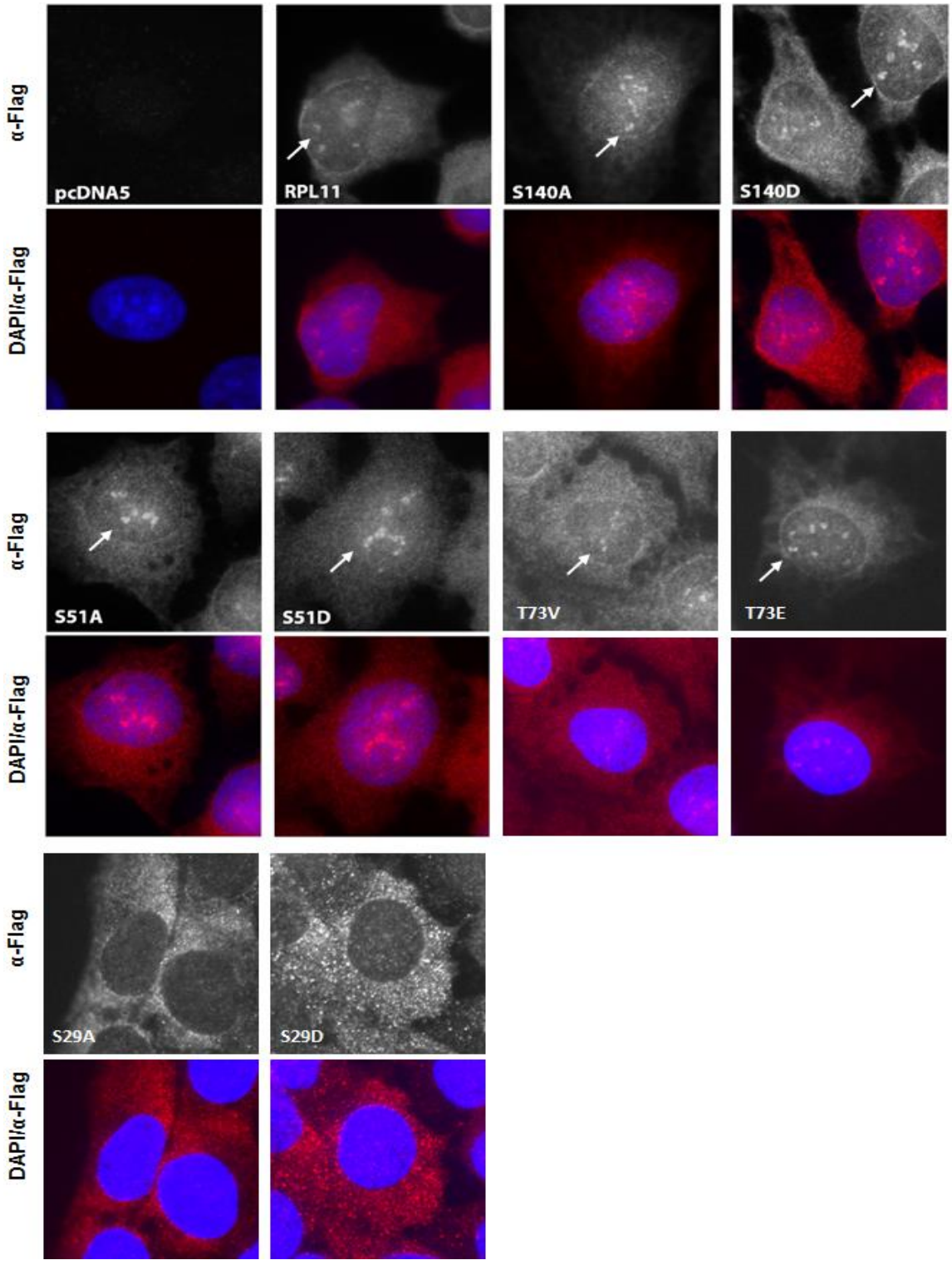

Figure 4.12. Mutations in phospho-sites change the localisation of RPL11.

The localisation of newly synthesised FLAG-RPL11 wild type (RPL11) or mutants (S51A, S51D, S140A, S140D, S29A, S29D, T73V and T73E) in U2OS cell lines was determined by immunofluorescence. Stably transfected U2OS cells were grown on coverslips and the expression of tagged protein was induced by tetracycline. After fixing the cells, immunofluorescence was performed using α-FLAG antibody (red/grayscale). DAPI staining was used to visualise nuclear material (blue). Nucleoli are indicated with arrows (white). Independent experiments were repeated twice and a representative result is presented.

In cells expressing either the S51A or S51D RPL11 mutant, there was increased nuclear and nucleolar FLAG-specific signal compared to the WT protein (S51A and S51D, Figure 4.12). Compared to the S51A mutant, RPL11 S51D was localised more predominantly in the nucleoplasm (Figure 4.12). The gradient result suggests that both these mutations block 5S RNP incorporation into the ribosome (Figure 4.9B). The increased nuclear FLAG-RPL11 levels observed in these cells agrees with the gradient results, since free 5S RNP accumulates in nucleoplasm.

In the cells expressing T73V or T73E mutant proteins, overall FLAG related fluorescence was noticeably weaker than for the WT protein (T73V and T73E, Figure 4.12), which coincides with to the low cellular levels of FLAG tagged RPL11 previously observed (Figure 4.8). According to the gradient results, the T73E mutant does not associate well with the 5S RNP. Moreover, the FLAG signal in cells expressing T73V was distributed similar to the WT protein (T73V, Figure 4.12). Compared to the T73V protein, the FLAG-RPL11 T73E signal was noticeably increased in the nucleoli, but reduced in the cytoplasm (T73E, Figure 4.12).

Compared to the WT protein, cells expressing the S29A and S29D RPL11 showed a complete absence of nucleolar FLAG signal (S29A, S29D; Figure 4.12). Furthermore, there were almost undetectable nuclear FLAG signals in the cells expressing the RPL11 S29A mutant (Figure 4.12). The gradient results for these cells suggest that S29A causes free 5S RNP accumulation, whereas S29D results in the accumulation of pre-60S RNA. Neither of the immunofluorescence result relates well to the corresponding gradient results.

In summary, although the reduced nucleolar FLAG signal in RPL11 S29A and S29D expressing cells were unexpected and difficult to explain, the immunofluorescence results from the rest of the mutant proteins provide additional evidence supporting the glycerol gradient results. The accumulated nucleolar signal of RPL11 S140A and S140D supports the gradient results which indicated that serine 140 is important for ribosome biogenesis. Furthermore, the increased nuclear RPL11 S51D signal concurs with the accumulated protein in ‘free’ gradient fractions, both findings support the hypothesis that this mutation inhibits the integration of RPL11 into 5S RNP. Finally, the

reduced cytoplasmic T73E signal also agree with the gradient results which indicate almost complete absence of FLAG tagged RPL11 protein in ribosomal fractions.

4.2.11 A relation between RPL11 mutant expression and p53 activation?

When ribosomal defects occur, free 5S RNP accumulates and promotes p53 stabilisation and activation via the inhibition of MDM2 (Sloan *et al.*, 2013a). As a component of the 5S RNP, RPL11 had been associated with MDM2 interaction and p53 signalling in a number of studies. Previously, Loren Gibson from the Watkins lab showed that mutations of phosphorylated tyrosine residues in ribosomal protein RPL5 resulted in p53 stabilisation and activation via MDM2 (unpublished data). Given the importance of RPL11/MDM2 interactions to p53 regulatory signalling, it is conceivable that the phosphorylation of RPL11 could also play a vital role in regulating p53. I, therefore, investigated how overexpressing the RPL11 mutants described above affect p53 and p21 cellular levels.

U2OS cells were treated with tetracycline for 48 hours in order to overexpress FLAG-tagged RPL11 WT or mutant proteins. Whole cell lysates were separated by SDS-PAGE followed by detecting p53 and p21 protein levels using western blotting with specific antibodies. Protein signals were visualised using ECL detection. U2OS cells transfected with empty plasmid were included as controls and karyopherin protein levels were measured as loading control. To quantitate the signal p53 and p21 bands were normalised to the loading control and data were plotted in relation to the empty plasmid control. Since both T73 mutants did not reliably express, they are omitted from this analysis.

When expressing RPL11 WT, S51D or S140A mutants in U2OS cells, there was a slight induction in p53 levels compared to the control (Figure 4.13B). Although the result is not statistically significant, expression of other RPL11 mutants also indicated p53 stabilisation to various degrees. For example, there was a threefold increase in p53 levels when expressing S29A or S29D mutants (Figure 4.13B). Expression of these mutant proteins also caused increased cellular p21 levels; again, the differences were not significant (Figure 4.13C).

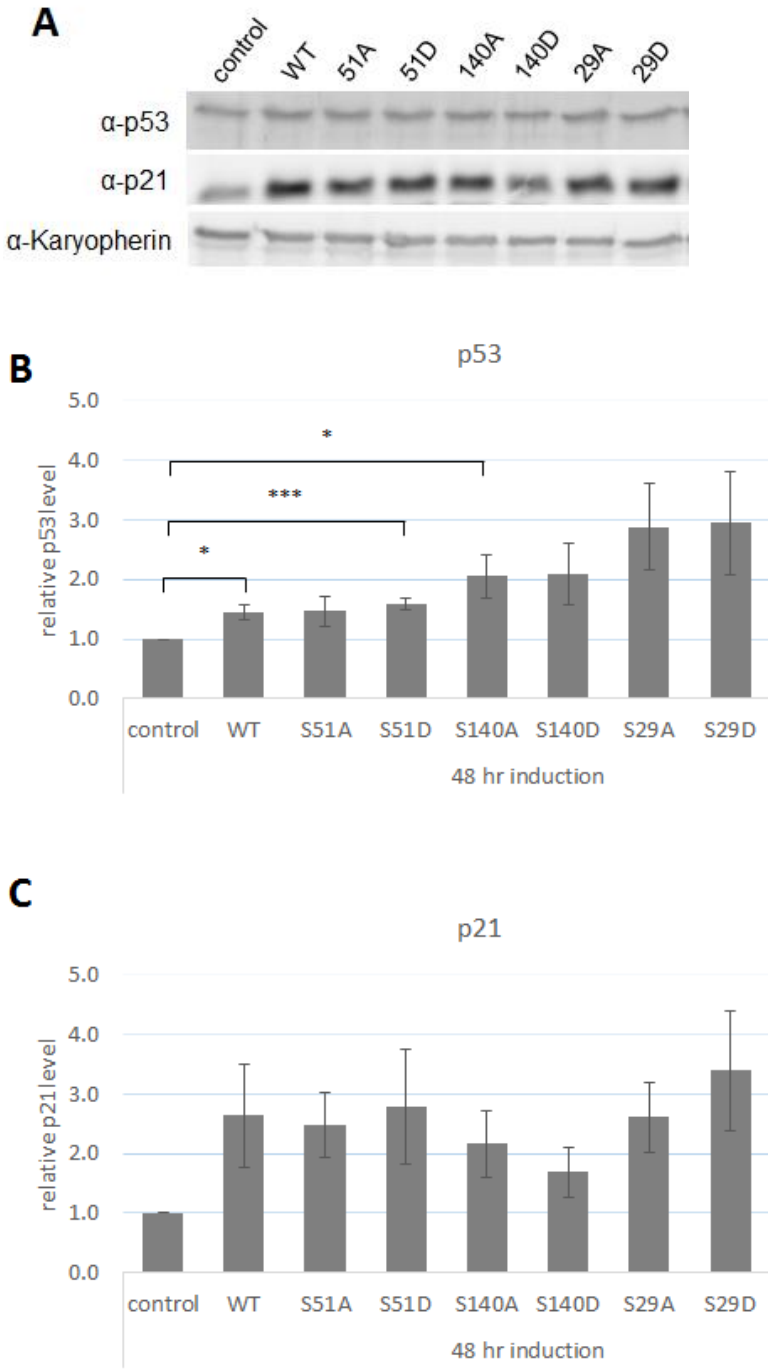

Figure 4.13. Effect of RPL11 mutants on p53 and p21 protein levels.

(A) Western blot determining cellular p53 and p21 levels. U2OS cells stably expressing RPL11 WT or mutants (indicated above the panel) were induced for 48 hours. Whole cell lysates were separated on SDS-PAGE and specific antibodies were used to determine protein levels (indicated on the left). U2OS cells transfected with empty plasmid were used as control. Karyopherin protein levels is used as a loading control. (B-C) Quantitation of p53 or p21 bands from (A). The mean value and standard error from three repeats is presented in related to the control (*$p<0.05$, ***$p<0.005$).

4.3 Discussion and Future Work

The 5S RNP is a crucial component for both ribosome biogenesis and the p53 regulatory pathway in response to the nucleolar stress (Sloan *et al.*, 2013a). In human cells, the 5S RNP is assembled in the nucleoplasm, where RPL5 stabilises the 5S rRNA, before RPL11 binds to the RPL5/5S rRNA dimeric complex to form the 5S RNP. The 5S RNP is then integrated into the pre-LSU in the nucleolus. After that, the pre-LSU undergoes further processing and the matured LSU is exported into the cytoplasm.

When ribosome biogenesis is disrupted, the accumulated 'free' 5S RNP binds and inhibits MDM2 functions resulting in p53 stabilisation and activation. It has been reported that the zinc finger and acidic domain of MDM2 interacts with RPL11 (Zheng *et al.*, 2015), and when MDM2 binds to the 5S RNP, the MDM2 binding interface on RPL11 is the same region that contacts the 28S rRNA in the ribosome (Khatter *et al.*, 2015). Since RPL11 has been reported to be phosphorylated at multiple serine and threonine residues, I aimed to establish the importance of RPL11 phosphorylation in relation to both ribosome biogenesis and p53 signalling in this chapter.

I first identified the reported phosphorylated sites and analysed their location on RPL11 in relation to the 5S and 28S rRNAs (Figure 4.4). Since serine 29 of RPL11 is distant to either the 28S or the 5S rRNA, phosphorylation of this site is likely to be involved at later stages of ribosome biogenesis. Hence, I hypothesised that phospho-mutants of this residue could affect the 5S RNP integration into the LSU or subsequent steps in LSU processing (Table 4.2).

Because the sugar-phosphate backbone of RNAs is negatively charged, based on the observation that serine 51 and serine 140 of RPL11 are located close to the sugar-phosphate backbone of the 28S and 5S rRNA in the ribosome structure (Figure 4.4), I predicted that the phospho-mimic mutation of these residues could inhibit RPL11 association with the rRNAs (Table 4.2).

Number of reports	Location	Site	Mutant	Predicted effects
6	contacts RPs in LSU	**S29**	A D	affects the 5S RNP integration into LSU
3	contacts 28S backbone	**S51**	A D	Phospho-mimic inhibits 28S binding
1	contacts nitrogenous bases in 5S	**T73**	V E	affects 5S binding and 5S RNP assembly
2	contacts 5S backbone	**S140**	A D	Phospho-mimic inhibits 5S binding

Table 4.2. Summary of the predicted effects of the introduced point mutations on RPL11 protein function based on the location analysis of the reported phosphorylated amino acid residues.

Finally, the structure analysis showed that threonine 73 of RPL11 is located close to the nitrogenous bases in the 5S rRNA of the ribosome. Therefore, I hypothesised that mutating this residue could affect the binding between RPL11 and the 5S rRNA, which could further affect the assembly of the whole 5S RNP (Table 4.2).

4.3.1 Threonine 73 in RPL11 could be important for RPL11 binding to 5S RNP components

I have first observed that in U2OS cells, the over-expressed T73E mutants of RPL11 distributed significantly less in the ribosomal complexes compared to the WT counterpart (Table 4.3). These results suggest that the RPL11 T73E mutant may be integrated into ribosome less efficiently than the WT counterpart.

Using immunofluorescence, I also showed that the T73E RPL11 mutant becomes accumulated in the nucleus and reduced in the cytoplasm (Figure 4.12). Because the 5S RNP is integrated into the pre-LSU in the nucleus, and mature ribosomes are mainly located in the cytoplasm, this result supports the speculation that T73E inhibits the integration of 5S RNP into the ribosome.

Site	Mutant (protein level)	Distribution (gradient result)		Localisation (IF result)
		Free	Ribosomal	
S29	A	⬆	n.c.	undetectable nucleolus, ⬇ nucleus
	D	⬇	⬆	undetectable nucleolus, ⬇ nucleus
S51	A	⬆	⬇	⬆ nucleolus, ⬆ nucleus
	D (⬇)	⬆	⬇	⬆ nucleolus, ⬆ nucleus
T73	V (⬇)	⬆	⬇	n.c.
	E (⬇)	⬆	⬇	⬆ nucleolus, ⬇ cytoplasm
S140	A	n.c.	⬆ pre-ribosome	⬇ cytoplasm
	D (⬇)	n.c.	⬆ pre-ribosome	n.c.

Table 4.3. Summary of the gradient and IF results for over-expressed RPL11 mutant proteins in U2OS cells. (n.c. no change; ⬆ increased levels; ⬇ reduced levels)

Based on the structural analysis showing that threonine 73 in RPL11 locates close to the nitrogenous bases of the 5S rRNA in the ribosome, I predicted that mutations of this residue interfere with the binding between RPL11 and the 5S rRNA and could further affect the 5S RNP assembly (Table 4.2). My results support this hypothesis, as the negatively charged glutamic acid substituting the serine 73 residue likely abolishes the RPL11 binding to the 5S rRNA. It inhibits the RPL11 mutant from being assembled into the 5S RNP in the first place, before the 5S RNP can be integrated into the ribosome.

Furthermore, my results suggest that threonine 73 could be an important residue for RPL11 binding to the 5S rRNA. Previously Horn *et al.* reported that a mutation of the arginine 75 residue (R75Q), adjacent to threonine 73, inhibits the interaction between RPL11 and RPL5, by blocking RPL11 binding to the 5S rRNA (Horn and Vousden, 2008). Since my result showed that T73E mutant inhibits 5S RNP integration into the ribosome, it is possible that threonine 73, like arginine 75, provides essential interactions between RPL11 and the 5S rRNA.

I have also demonstrated that both FLAG-tagged T73 RPL11 mutants are less stable than WT and other mutants when over-expressed (Figure 4.5). This could also be the

result of mutants not being assembled into the 5S RNP, since it has been shown that RPL11 protein is produced in excess and the unbound molecules are degraded by the proteasome (Lam *et al.*, 2007). A recent study also has demonstrated that RPL11 incorporation into the 5S RNP is a rate limiting step during LSU production (Sloan *et al.*, 2013a). Taken together, it is likely that those T73 mutants which cannot bind to the 5S rRNA are turned over rapidly in the cell, resulting in less stable protein as observed in my results.

Since T73E is designed to mimic a phosphorylated threonine, it is possible that the phosphorylation of threonine 73 in RPL11 influences RPL11 turn-over in U2OS cells. Because RPL11 is produced in excess and those proteins unbound to the RPL5/5S rRNA complex are turned over, the low cellular levels of the T73E RPL11 mutant suggest that threonine 73 phosphorylation could target RPL11 for degradation. This could also explain why only one proteomic study reporting the phosphorylation of this residue has been published (Table 4.2), as a transient phosphorylation event on 'free' RPL11 with a low abundance (less than 10% free in U2OS cells) would be technically difficult to observe.

In fact, this work has not directly established a phosphorylation event at threonine 73 in the free RPL11 proteins, to confirm this will require additional investigations in the future, such as utilising kinase inhibitor (see Chapter 5 for detail). As a conclusion, my work suggests that threonine 73 in RPL11 could be important for RPL11 binding to the 5S rRNA.

4.3.2 Serine 51 phosphorylation in RPL11 could inhibit 5S RNP integration into the large subunit

In RPL11 serine 51 is located on the RPL11-MDM2/28S rRNA interface and when RPL11 interacts with the 28S rRNA in the ribosome, the sidechain of serine 51 is in close proximity to the sugar-phosphate backbone of the 28S rRNA (Figure 4.4). Because the sugar-phosphate backbone of RNA is negatively charged, I therefore predicted that the phospho-mimic mutant of this residue (S51D) inhibits RPL11 binding to the 28S rRNA (Table 4.2).

My glycerol gradient results suggest that the 5S RNP containing the S51A or S51D RPL11 mutant is not incorporated into the ribosome. The S51D mutant is barely detectable in ribosomal complexes, indicating that the S51D mutation in RPL11 severely affects the 5S RNP incorporation into the ribosome (Figure 4.9). This assumption is supported by the immunofluorescence data which showed reduced S51D mutant levels in cytoplasm, suggesting there were few mature ribosomes containing the mutant RPL11 (Figure 4.12).

As a phospho-mimic mutation of S51D, the negatively charged aspartic acid sidechain of the mutant likely disrupts the association between RPL11 and the sugar-phosphate backbone of the 28S rRNA. My observations presented in the results section supports this hypothesis. In contrast, as a phospho-null mutant, S51A is supposed to have a lesser effect on RPL11 binding to the 28S rRNA, because the similarity between alanine and a serine residues. In deed when over-expressing S51A in U2OS cells, the mutant did cause less free 5S RNP accumulation than the RPL11 S51D mutant (Figure 4.12).

Therefore, phosphorylation of serine 51 is likely to block the RPL11 binding to 28S rRNA, which could constitute an important step in controlling 5S RNP integration into the LSU. Consequently, to integrate the 5S RNP into the LSU, dephosphorylation could be needed. Nonetheless further investigation is required to confirm this speculation (See Chapter 5 for detail). In conclusion, my work suggests that serine 51 phosphorylation in RPL11 could inhibit 5S RNP integration into the large subunit.

4.3.3 Phosphorylation of serine 140 in RPL11 could be important for LSU maturation

It was shown by structural analysis that the sidechain of serine 140 locates close to the sugar-phosphate backbone of the 5S rRNA in the ribosome. I therefore predicted that mutations of this residue may affect RPL11 binding to the 5S rRNA, which may subsequently affect the 5S RNP assembly (Table 4.2).

Although the structural analysis leads to the prediction that mutations of this residue may affect RPL11 binding to the 5S rRNA, the IP experiments performed demonstrated otherwise. They showed that S140A and S140D mutants associate with the 5S and 5.8S rRNAs at comparable levels to the WT (Figure 4.6). Interestingly, the gradient centrifugation result suggested that these mutants may instead affect ribosome maturation.

Using glycerol gradient analysis, I showed that both of S140A and S140D RPL11 mutants accumulated in fractions containing pre-ribosomes. Compared to the WT protein, there was a notable reduction of RPL11 S140A in the mature ribosomes (Table 4.3, Figure 4.10). This suggests that while both mutants affect LSU maturation, the phospho-null mutation RPL11 S140A causes a more severe defect. The subsequent IF results showed that RPL11 S140A accumulated in the nucleus and that there was a significant reduction of cytoplasmic RPL11 S140A levels, which was not seen for the S140D mutant (Figure 4.12). Since the mature ribosomes are exported from the nucleus into the cytoplasm, the IF data support the gradient results and further suggest that the S140A mutation give rise to more severe LSU maturation defects than the S140D mutant.

Because a serine to alanine mutation creates a residue which is not a substrate for protein kinases, my results imply that phosphorylation of serine 140 residue is needed for the LSU maturation. In the mature ribosome structure (PDB ID: EMD-2938; Khatter *et al.*, 2015), serine 140 contacts the sugar-phosphate backbone of 5S rRNA in the LSU (Figure 4.4). Since a phosphorylated serine 140 residue is likely to disrupt the RPL11 5S rRNA interaction, it is likely that the phosphorylation on this residue is a transient event during LSU maturation.

Furthermore, yeast studies have demonstrated that after integration into the large subunit, 5S RNP undergoes a 180-degree rotation during LSU maturation (Leidig *et al.*, 2014). Up to now this remodelling event has not been fully documented in human, however the rotation is believed to initiate a cascade of large subunit protein maturation events, leading to the final conformation of the mature LSU (Baßler *et al.,* 2014; Nicolas *et al.,* 2016). Therefore, it is possible that a transient phosphorylation on serine 140 is involved to this process, allowing the 5S RNP to adopt the final conformation within the CP region of the mature LSU, followed by dephosphorylation of the residue to stabilise RPL11 binding to the 5S rRNA after LSU maturation. In conclusion, phosphorylation of serine 140 in RPL11 could be important for LSU maturation, and further studies are needed to characterise the involvement of this modification in LSU maturation (See Chapter 5 for detail).

4.3.4 Phosphorylation of serine 29 in RPL11 could be involved in the 5S RNP integration into the ribosome

The structural analysis showed that S29 of RPL11 is not interacting with either 5S or 5.8S rRNA (Figure 4.4). After 5S RNP assemby, S29 is located to the CP region of the large subunit during the late stages of the ribosome biogenesis. Since this process involves different ribosome biogenesis factors, S29 could regulate interactions with these biogenesis factors. I therefore predicted that the phospho-mutants for this residue could affect the 5S RNP integration into the LSU and the LSU maturation process (Table 4.2).

My results first showed that the RPL11 S29D mutant is barely detectable in the ‘free’ fractions (Figure 4.11). Given that the cellular protein levels of both mutants are comparable to WT (Figure 4.5), it is unlikely that the absent S29D RPL11 mutant protein in the ‘free’ fractions are due to protein instability. It rather suggests that the 5S RNP containing S29D RPL11 is integrated into the pre-LSU more efficiently than the WT protein. Interestingly RPL11 S29D mutant protein also accumulated in fractions containing pre-ribosome material, whereas the S29A mutant was distributed similar to the WT protein (Figure 4.11). Taken together, my results suggest that the S29D mutant may result in more efficient 5S RNP integration into the ribosome, however this mutation also slows down the following ribosome maturation process.

My results suggest that the phosphorylation of serine 29 could enhance 5S RNP incorporation into the ribosome. However, this phosphorylation may slow down the ribosome maturation process. Since ribosome biogenesis is a complicated process involving a large number of co-factors at different stages of the processes, a phosphorylated serine 29 residue in RPL11 could provide binding surface for ribosome biogenesis factors which yet to be identified in further investigations (See Chapter 5).

My IF results further showed a notable reduction of RPL11 S29A and S29D mutant proteins localised in the nucleus of cells (Table 4.3, Figure 4.12). More surprisingly, these mutant proteins were not detected in the nucleolus as the WT protein (Table 4.3, Figure 4.12). The reduction in both mutant protein levels in the nucleus could mean more efficient nuclear export of mature ribosomes containing the mutant RPL11, however this hypothesis does not concur with the gradient result. Furthermore, the lack of nucleolar signal of the mutant proteins is difficult to explain as the gradient analysis showed the RPL11 S29D mutant retained in the pre-ribosomes. It is possible that mutation of serine 29 causes a structural change in RPL11, which blocks antibody access to the FLAG epitope after RPL11 integration into the pre-LSU, resulting in the loss of signal. Because ribosome maturation takes place in the nucleoli and the process involves multiple protein co-factors, it is conceivable that the RPL11 mutations block certain interactions between the ribosome and protein co-factors, resulting in reduced FLAG-tag detection in IF. In conclusion, phosphorylation of RPL11 at S29 could favour 5S RNP integration into the ribosome, and become dephosphorylated during later steps of LSU maturation.

4.3.5 Result summary and proposed model

Based on the conclusions drawn from my results, I propose a model summarising the roles that the phosphorylated residues play during ribosome biogenesis.

Accordingly, during the 5S RNP assembly, RPL11 binds to and stabilises the RPL5/5S rRNA intermediate complex in nucleoplasm. It has been shown that RPL11 protein is produced in excess and the RPL11 integration into the 5S RNP is tightly regulated (Lam *et al.*, 2007) (Sloan *et al.*, 2013a). Unbound RPL11 is targeted for degradation which is mediated by the phosphorylation on threonine 73 residue.

After the assembly of the 5S RNP, it is integrated into the large ribosome subunit precursor in the nucleolus. During this process, the phosphorylation of serine 29 in RPL11 promotes 5S RNP integration into the ribosome, by providing an interface for the binding of ribosome biogenesis factors. At the same time, serine 51 in RPL11 is dephosphorylated to allow the RPL11 binding to the sugar-phosphate backbone of the 28S rRNA.

During the large ribosomal subunit maturation, 5S RNP undergoes a 180-degree rotation to adopt the final conformation in the mature ribosome, likely to trigger the continuation of the ribosomal protein maturation process (Nicolas *et al.,* 2016). In this process, serine 140 in RPL11 is transiently phosphorylated to allow 5S RNP to adopt the conformational changes. After that this residue is dephosphorylated to allow the stable binding between RPL11 and the sugar-phosphate backbone of the 5S rRNA again.

Finally, once the maturation of large ribosomal subunit completes, serine 29 dephosphorylation is required to allow the export of the mature large ribosomal subunit to the cytoplasm.

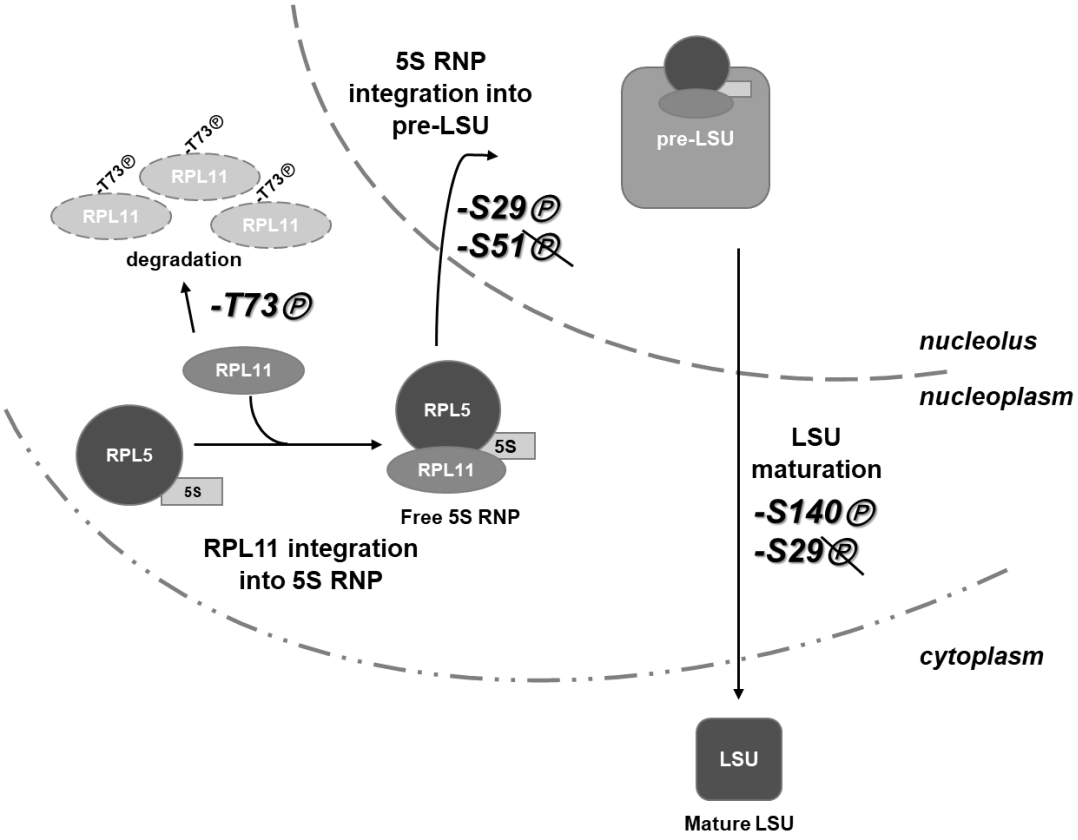

Figure 4.14. Proposed model of the phosphorylation events in RPL11 during different stages of large ribosomal subunit biogenesis.

The phosphorylation event is indicated using Ⓟ. The dephosphorylation event is indicated as ~~Ⓟ~~.

4.3.6 Future work

In this work I provided evidence suggesting that phosphorylation takes place at serine 29 and 51 residues of RPL11. Furthermore, my work also implies that during LSU maturation, it is possible that a transient phosphorylation takes place at the serine 140 residue. However, further investigations are required to confirm these conclusions.

Preliminary data from the Watkins lab showed that, using Phos-tag™ SDS-PAGE, stably phosphorylated RPL11 was not detected in whole cell lysates of U2OS cells (Justine Lee, personal communication). This suggests that the reported phosphorylation of RPL11 found by Mass-spectrometry-based phosphoproteomic analysis could be a

transient phosphorylation event. Therefore, kinase or phosphatase inhibitor compounds could be used prior to the future analysis to either block phosphorylation or stabilise the phosphorylated residues in the cell. Alternatively, methods to more accurately separate and enrich free and ribosomal complexes may also be needed to be developed in order to differentiate the phosphorylation status in free or ribosomal 5S RNP. After that, mass spectrometry could be used to identify the different phosphorylation status in pre-ribosomal complexes.

In human cells, the process of 5S RNP integration into the LSU, including the 5S RNP remodelling event which was described in yeast, is still not fully understood. It is known that ribosome biogenesis factors BXDC1 and RRS1 are required for the assembly of the CP region in the LSU (Sloan *et al.*, 2013a; Nicolas *et al.*, 2016). Therefore, further investigations into how these reported phosphorylation steps are related to the different factors could yield insight into the 5S RNP integration and remodelling process in humans. To characterise this, the association and interaction between RPL11 phospho-mutant protein and BXDC1 or RRS1 could be tested by using immunoprecipitation or immunofluorescence in U2OS cell lines.

In this work I also attempted to characterise how RPL11 mutant expression affects p53 activation in U2OS cells and due to time constraints the results remained inconclusive (Section 4.2.11). It has been proposed that proteins involved in 5S RNP production, localisation and integration into the ribosome could be important in regulating p53, indirectly via 5S RNP (Sloan *et al.*, 2013a). The results I presented in this work on RPL11 serine 51 and 29 (Section 4.2.11), implied a potential involvement of these residues in 5S RNP integration into the ribosome. Hence, further investigations are needed to establish how phosphorylation of RPL11 serine 51 and 29 tie into p53 regulation via the 5S RNP-MDM2 pathway. Since serine 51 is proposed to locate at the RPL11/MDM2 binding interface, co-immunoprecipitation could be used to validate the association between MDM2 and the RPL11 S51 mutants. In addition, recent studies suggested that RPL11 and RPL5 couple nucleolar structural integrity and p53-dependent nucleolar stress (Nicolas *et al.*, 2016). My results showed that the serine 29 mutant of RPL11 affects nucleolar localisation of the protein (Figure 4.12), and its over-expression also causes increased cellular p53 levels (Figure 4.13). Together, these results are

compatible with a connection between serine 29 in RPL11 and p53 signalling, though more repetitions of experiments are required to be conclusive.

Furthermore, it remains unclear that how much RPL11 mutant protein in U2OS cells is required to reach the threshold of significant p53 activation. Therefore, in future investigations, the concentration of tetracycline used to induce the expression of mutant protein in the U2OS cell lines should be reviewed. Alternatively, the potential of using CRISPR-Cas9 genome editing technique to create phospho-mutations in the RPL11 gene in the cells could also be explored in future investigations.

Finally, other documented phosphorylated residues in RPL11, such as threonine 44 and 47, should also be characterised for their involvement in ribosome biogenesis and p53 signalling in future studies.

The findings in this work have led to better understanding of the role of RPL11 phosphorylation in ribosome biogenesis. This work also raised unanswered questions about the involvement of RPL11 phosphorylation in the 5S RNP-regulated p53 signalling, which deserved attention in future investigations (Chapter 5).

Chapter 5. Final Discussion

5.1 Final Discussion

The 5S RNP is made up of three components, ribosomal protein RPL5, RPL11 and the 5S rRNA. Structurally it is relatively simple compared to the rest of the ribosome. However its uniqueness does not only refer to being a core component of the ribosome, as it is required for a functional ribosome, but it is also able to regulate p53 through interacting with MDM2.

In this work, I aimed to investigate the role of 5S RNP in ribosome biogenesis and 5S RNP-mediated p53 signalling. This work first characterised protein factors suspected to be involved in the 5S RNP-mediated p53 signalling pathway. It demonstrated the important role of splicing factor SRSF1 in this pathway, while showing that protein factors HEXIM1, Mybbp1a and NML are not essential in p53 activation through the 5S RNP (Chapter 3). This work also provided experimental evidences suggesting the phosphorylation of serine and threonine residues of ribosomal protein RPL11, a 5S RNP component, could be involved in various stages of ribosome biogenesis such as the 5S RNP assembly and its integration into the ribosome as well as large ribosomal subunit maturation (Chapter 4). Results presented in this work provide valuable insights into the complex relationship between ribosome biogenesis and p53 signalling connected via the 5S RNP. This work hence points to future research directions to further the understanding of the molecular basis of diseases like ribosomopathies and cancer.

5.1.1 The 5S RNP assembly

In humans the newly transcribed 5S rRNA is stabilised by binding to ribosomal proteins in the nucleoplasm, before recruiting RPL11 in the nucleolus forming the stable 5S RNP. The results presented in this work, as discussed in the previous chapter, may suggest that threonine 73 is important for RPL11 binding to the 5S rRNA during 5S RNP assembly (Section 4.3.1). It has been previously reported that in RPL11, mutation of the arginine 75 (R75Q), adjacent to threonine 73, inhibits the interaction between RPL11 and the 5S rRNA (Horn and Vousden, 2008). Indeed, structural analysis showed that both of residues in RPL11, arginine 75 and threonine 73, are located close to the nitrogenous bases of the 5S rRNA (Section 4.2.2). Taken together the data presented in

this work strongly suggest that residues in this region of RPL11 play crucial roles in stabilizing the 5S rRNA during 5S RNP assembly.

Furthermore, it is believed that ribosomal proteins are produced in excess to accommodate the rapid process of biogenesis, and those ribosomal proteins unbound to pre-ribosomes are targeted for proteasome mediated degradation (Lam *et al.*, 2007). Moreover, the recruitment of RPL11 into the 5S RNP is a rate limiting step for 5S RNP integration into the ribosome (Sloan *et al.*, 2013a). However it remains unclear which factors control the RPL11 availability that regulates 5S RNP synthesis. Phosphorylation of threonine 73 in RPL11 has been identified by high-resolution Mass-spectrometry-based phosphoproteomic analysis (Mertins *et al.*, 2016) and suggested to affect RPL11-5S rRNA interaction.

Results in this work showed that expressing RPL11 protein mimicking the phosphorylated threonine 73 in human cells, prevents 5S RNP assembly, likely by blocking the 5S rRNA binding which results in RPL11 degradation (Section 4.3.1). Hence, evidence presented in this work may suggest that phosphorylation of threonine 73could be an important signal controlling the turnover of RPL11 and regulating RPL11 availability in 5S RNP assembly.

Data presented in this work may also provide a better understanding of the role that splicing factor SRSF1 plays in 5S RNP assembly (Section 3.3.2). Previously in the Watkins Lab, SRSF1 was shown to interact with both RPL5 and the 5S rRNA in U2OS cells (Loren Gibson, unpublished data), which was consistent with the result demonstrated by Fregoso et al. (2013) in BJ-TT cells. Gibson's work also showed that SRSF1 is important for regulating the cellular localisation of RPL5 and RPL11. Hence Loren Gibson proposed a chaperone role for SRSF1 facilitating the recruitment of RPL11 to the RPL5/5S rRNA subcomplex (unpublished data).

This work demonstrated that recombinant SRSF1 interacts with RPL5 and RPL11 in vitro (Chapter 3). The binding between SRSF1 and RPL11 provided important evidence which is complementary to Gibson's work. Furthermore, this work suggested that RPL11 exhibits a higher binding affinity to SRSF1 than RPL5. Since RPL11 is likely to be produced in excess and the molecules unbound to the 5S RNP are turned over by degradation, it is possible that SRSF1 binding to RPL11 may block the modification on

RPL11 targeting it for degradation (such as threonine 73 phosphorylation, see above). Hence the high binding affinity between SRSF1 and RPL11 may ensure RPL11 availability during 5S RNP assembly. The data presented in this work not only support the proposed 'chaperone model' from Loren Gibson's work, it also suggests a direct role that SRSF1 may play in recruiting RPL11 to the 5S RNP precursor.

In summary, data presented in this work could explain how the 5S RNP assembly is tightly regulated by protein factors such as SRSF1 and/or phosphorylation of the ribosomal protein RPL11.

5.1.2 The ribosome integration of 5S RNP and the large ribosomal subunit maturation

After its assembly, the 5S RNP is recruited, as one single complex, into the central protuberance region of the large ribosomal subunit. Structural analysis showed that RPL11 is located closely to the 28S rRNA in the mature large ribosomal subunit. Furthermore, the 5S RNP is an essential component for producing a mature ribosome, and its recruitment is thought to take place during the later stages of large subunit maturation. This work also aimed to establish how phosphorylation of RPL11 residues is related to ribosome biogenesis. Although multiple phosphorylated residues in RPL11 had been identified in proteomic studies (Section 4.2.1), it remains unclear whether these modifications are important in the 5S RNP integration into the large ribosomal subunit.

The data presented in this work may suggest that phosphorylation of serine 51 of RPL11 blocks the 5S RNP integration into the large ribosomal subunit, possibly by blocking the binding between RPL11 and the 28S rRNA located within the large ribosomal subunit precursor (Section 4.3.2). Indeed, previously the Watkins Lab showed that the helix 83-85 of the 28S rRNA interacts with RPL11 in vitro (Gibson, unpublished data). Taken together, this work showed the importance of RPL11/28S rRNA interaction in 5S RNP integration into the large ribosomal subunit. Moreover, since the 28S rRNA binding site and the interface that interacts with MDM2 are co-localised in RPL11 (Zheng *et al.*, 2015), the finding in this work could also be exploited in developing new therapeutic strategies for the treatment of ribosomopathies in the future (see below). The data presented in this work further showed that

phosphorylating serine 29 of RPL11 favours the 5S RNP integration into the large ribosomal subunit. Interestingly, results also showed that dephosphorylation of this residue could be required for large subunit maturation (Section 4.3.4), suggesting that this residue is transiently phosphorylated during large subunit maturation.

The findings discussed above were related to the human ribosome structure demonstrating that serine 29 of RPL11 does contact neither the 5S rRNA nor 28S rRNAs (Section 4.2.2). This suggested that phosphorylation of this residue may not provide a binding surface for the protein-rRNA association. It is, in turn, more likely that serine 29 phosphorylation provides a binding surface for other ribosome biogenesis factors during large ribosomal subunit maturation.

Moreover, data presented in this work may also suggest a transient phosphorylation of serine 140 in RPL11 (Section 4.3.3), as phosphorylating the residue is required for large subunit maturation. In support of this hypothesis, the structural analysis predicted that a phosphate group could abolish the binding between RPL11 and the 5S rRNA.

As mentioned in the introduction chapter, the current understanding of the 5S RNP recruitment into the large ribosomal subunit is primarily based on studies in yeast, while in humans this process is not yet fully understood (Section 1.2.6). In yeast, the CP region of the large subunit undergoes a conformational change in which the 5S RNP rotates 180 degrees during the final stages of large ribosomal subunit maturation. In contrast, there is still little known about the 5S RNP remodelling event in humans and whether phosphorylation of RPL11 is involved in this process. The data presented in this work could mean that during the 5S RNP remodelling event, the transiently phosphorylated residues in RPL11 provide binding interfaces which are needed by specific ribosome biogenesis factors during the complicated process of large subunit maturation. However, future investigations are needed to elucidate the relationship between RPL11 phosphorylation and the 5S RNP remodelling in humans. In summary, data presented in this work may reveal a highly sophisticated network of phosphorylation events in RPL11 which regulate large ribosomal maturation.

5.1.3 The 5S RNP-p53 pathway

When ribosome biogenesis is blocked, non-ribosomal 5S RNP accumulates in the nucleoplasm where it binds to MDM2. In human cells, MDM2 is responsible for maintaining p53 at low levels by ubiquitinating it for protease degradation. 5S RNP binding to MDM2 inhibits its function thus resulting in p53 stabilization and increased expression of the p53-regulated genes.

Previously Fregoso et al. (2013) showed that the splicing factor SRSF1 is needed for p53 activation by RPL5 and the study suggested that SRSF1, RPL5 and MDM2 function as a complex to stabilise and activate p53. Accordingly, it was demonstrated that all three 5S RNP components are required for ribosomal stress-induced p53 activation (Donati *et al.*, 2013; Sloan *et al.*, 2013a). Recently, Loren Gibson from the Watkins Lab attempted to elucidate if SRSF1 and 5S RNP function as a single complex. Gibson's work demonstrated that SRSF1 binds to the 5S rRNA and its depletion causes abnormal nuclear re-localization of RPL5 and RPL11 in human cells, suggesting that SRSF1 indeed plays an essential role in the 5S RNP-p53 pathway (unpublished data). However it remains unclear whether SRSF1 directly regulates p53 via the 5S RNP. SRSF1 is known to be a highly phosphorylated protein and protein phosphorylation directly controls protein functions. Hence in this work, the splicing inhibitor compound SPRIN430 specifically targeting SRPK, a kinase phosphorylating the RS domain in SRSF1, was utilised to investigate if SRSF1 function is required for p53 activation.

The data presented in this work demonstrated that the Actinomycin D-induced p53 activation reduces by SPRIN430 at a dose dependent manner (Section 3.2.10), suggesting that SRSF1 phosphorylation is indeed required for p53 activation in response to ribosomal stress. Furthermore, results from this work also showed that SRSF1 reduces p53 levels (Section 3.2.11) suggesting that SRSF1 phosphorylation is required for stabilising p53. Previous studies also showed that in SRSF1, SRPK-mediated RS domain phosphorylation is important for the nuclear localisation of the protein (Lai *et al.*, 2001; Cazalla *et al.*, 2002; Ghosh and Adams, 2011). Therefore data presented in this work may imply that the nuclear localisation of SRSF1, as a result of the RS domain phosphorylation, is crucial in 5S RNP-p53 signalling. This may also suggest that the physical interaction between SRSF1 and the 5S RNP in the nucleus is required for stabilising p53 in response to ribosomal stress. However it requires further

investigation, such as immunofluorescence, to elucidate how nuclear SRSF1 level reduction correlates to RPL5 and RPL11 localisation when cells are treated with SPRIN430.

Besides investigating protein factors such as SRSF1, this work also aimed to investigate how RPL11 phosphorylation is involved in the 5S RNP-p53 signalling (Chapter 4). Phosphorylation is one of many post translational modifications found in proteins, and it is widely regarded that phosphorylation controls protein functions and signalling pathways. As demonstrated previously (Chapter 4), several phosphorylated residues (serine 51, threonine 73 and serine 140) in RPL11 were found located at the RPL11-MDM2 binding surface through structural analysis. Hence it was predicted that RPL11 phosphorylation is involved in the 5S RNP-p53 signalling by affecting its interaction with MDM2.

Result presented in this work demonstrated that a mutation mimicking phosphorylated RPL11 at its serine 140 residue results in a significant approximately twofold increase in p53 levels in U2OS cells (Section 4.2.11). However, despite the observed difference being statistical significant, arguably other factors than RPL11 S140 phosphorylation must contribute to the tenfold p53 induction in response to Actinomycin D treatment. Therefore, future investigations are required to understand how RPL11 phosphorylation ties to p53 levels, for example, titrating the RPL11 mutant expression level in cells to account for dosage effects.

This work also characterised protein factors HEXIM1, Mybbp1a and NML, which were predicted to be involved in the 5S RNP-p53 pathway based on previous publications (Chapter 3). However results presented in this work showed that in this experimental context they are not required for the Actinomycin D-induced p53 activation via the 5S RNP, as discussed in Chapter 3 (Section 3.3.1).

5.2 Future Directions and Preliminary Data

5.2.1 Future Directions

In summary, findings presented in this work highlight the complex nature of different components, such as protein factors (SRSF1) and RPL11 phosphorylation, in regulating ribosome biogenesis and the 5S RNP-p53 pathway. Moreover, this work also points to

future research directions that could be explored to find new therapeutic strategies treating diseases like ribosomopathies and cancer.

Ribosomopathies are a group of diseases caused by genetic mutations in ribosomal proteins and/or ribosome biogenesis factors. For most of these diseases the patients have an increased risk of developing cancers such as Myelodysplastic syndromes and acute myeloid leukaemia (Section 1.4, Chapter 1). Furthermore, there are a few better studied ribosomopathies, such as Diamond Blackfan Anemia, Treacher Collins syndrome and 5q-syndrome, which show elevated p53 levels (Table 1.1). Especially for Diamond Blackfan Anemia, the essential role of 5S RNP in regulating p53 levels has been documented (Jaako *et al.*, 2015). Moreover, the 5S RNP links ribosomal biogenesis and nucleolar stress to p53 regulation (Sloan *et al.*, 2013a). This work investigated roles that 5S RNP play in ribosome biogenesis and p53 regulation. Since ribosome biogenesis and p53 signalling are tied to each other, future research in both aspects are equally important.

Results from this work suggested that phosphorylating threonine 73 of RPL11 may control the turnover of the protein. Although kinases regulating this modification are not yet identified, they could be therapeutic targets to deplete the RPL11 availability in the cell. This strategy could be exploited to reduce free 5S RNP levels since 5S RNP assembly requires recruiting RPL11. This tactics may also be used in combination with anticancer treatments, since ribosome production is a common target for anticancer drugs.

Data presented in this work may also suggest that phosphorylating serine 29 of RPL11 favours the 5S RNP integration into the large ribosomal subunit. If kinases and phosphatases modifying this residue were identified in the future, new therapeutic strategies could be developed deliberately driving 5S RNP integration into ribosomes. This could, in theory, restore functional ribosomes and lower the non-ribosomal 5S RNP levels in terms of treatments for ribosomopathies. In order to identify kinases that are responsible for phosphorylating RPL11, in the future, selective kinase inhibitors could be applied to human cells and their effects on ribosome biogenesis could be used to evaluate their involvement in modifying the protein (Section 5.2.2).

The most interesting evidence highlighted in this work, by comparing recently published structures, is that the MDM2 binding interface in RPL11 co-localises with the region associating with the 28S rRNA in the ribosome (Figure. 4.2.). It provides structural evidence of 5S RNP coupling ribosome biogenesis to the p53 signalling regulation. This work started with proposing that phosphorylation of residues at this binding surface in RPL11 is important to both ribosome biogenesis and regulation of p53 signalling. Data presented in this work indeed suggested that phosphorylating serine 51 of RPL11 blocks 5S RNP integration into the ribosome (Section 4.3.2). However further investigations are needed to elucidate the role of RPL11 phosphorylation in RPL11-MDM2 binding, such as utilising kinase and phosphatase inhibitors in the future studies (Section 5.2.2).

Research in this direction could potentially be beneficial in developing new therapeutic strategies not only for ribosomopathies, but also cancer treatment in the future. As an example, by exploiting the ability of RPL11 to bind to 28S rRNA, short rRNA oligos mimicking the sequence of 28S rRNA could potentially be introduced to cancer cells. In theory these oligos could block the 5S RNP-MDM2 interaction to restore the MDM2 role in negatively regulating p53 levels in cells. As mentioned, previously Loren Gibson from the Watkins Lab demonstrated that a short helix from the 28S rRNA is able to bind to RPL11 in vitro. Therefore, it would be interesting to investigate further to find out whether this helix is stable in human cells and if its association to RPL11 inhibits the RPL11-MDM2 association.

5.2.2 Identify kinase phosphorylating RPL5 (Preliminary data)

During the cause of this PhD project, I also attempted to identify kinase that are involved in RPL5 phosphorylation, however due to time constraints only preliminary data was obtained. Previously Loren Gibson from the Watkins Lab demonstrated that mimicking the phosphorylation at tyrosine 30 of RPL5 stabilises and activates p53, likely by inhibiting the 5S RNP integration into the ribosome (data unpublished).

It was observed that in U2OS cells, from my preliminary results, when expressing phosphorylated mimics of RPL5 at tyrosine 30, the p21 gene expression were upregulated detected using RT-qPCR. This observation was expected and consistent with Loren Gibson's previous finding.

Furthermore, the approach of utilising kinase inhibitor in human tissue cultures was also used to identify kinases involved in the 5S RNP-p53 signalling regulation. One collaborator of the Watkins Lab, using kinase inhibitor screening, highlighted several kinase inhibitor candidates which were associated with p53 regulation. The effects of these candidates on the 5S RNP-mediated p53 activation were subsequently characterised using human cells. Due to confidential terms from the collaborator, name and result details of these tested kinase inhibitors will be remained undisclosed in this work. Preliminary data demonstrated that four kinase inhibitors have inhibiting effects on ribosomal stress-induced p53 activation in a dose dependent manner. Furthermore, preliminary data also showed that three of them inhibit p53 activation specifically through 5S RNP.

These preliminary results may suggest that phosphorylation of RPL5 is important for the 5S RNP-mediated p53 regulation. However, further studies are needed to generate conclusive data and to confirm this hypothesis. As an example, RT-qPCR could also be further in use to gain data of more p53-regulated genes, other than p21, to further confirm how mimicking RPL5 phosphorylation affects p53 activity.

On the other hand, more works are needed to association between kinase inhibitor and RPL5 phosphorylation. Previously Loren Gibson demonstrated that three stable forms of phosphorylated RPL5 are separated and detected from U2OS cells using the PhosTag™ SDS-PAGE (data unpublished). Hence future investigation could focus on whether kinase inhibitors treatment changes the RPL5 phosphorylation status by analysing the protein band intensity detected by the PhosTag™ SDS-PAGE. This could potentially further narrow down the kinase responsible for phosphorylating RPL5.

Finally, because the 5S RNP contains RPL5 and RPL11, and they are both needed in p53 regulation, same strategy could also be used to identify kinase phosphorylating RPL11. To tackle this, kinase inhibitors could be applied to human cells and their involvement in phosphorylating RPL11 / RPL5 could be analysed by on 5S RNP assembly and the 5S RNP integration into ribosome.

5.3 Conclusions

This PhD thesis has investigated the important role of the 5S RNP in both ribosome biogenesis and the 5S RNP-mediated p53 signalling. It has investigated factors involved in 5S RNP assembly, the 5S RNP-p53 signalling and the ribosomal large subunit maturation. In relation to the 5S RNP assembly, this work has demonstrated that RPL11 T73 phosphorylation could be involved in recruiting RPL11 to the 5S RNP. It also extended evidence suggesting SRSF1 acts as a chaperone during the 5S RNP assembly.

In relation to 5S RNP-mediated p53 signalling, this thesis demonstrated that SRSF1 is a potential regulator for 5S RNP-p53 signalling, while HEXIM1, Mybbp1a and NML are not required in this pathway, illustrating the complex nature of regulating the 5S RNP-p53 signalling pathway. Furthermore, this work also provides evidence suggesting RPL11 phosphorylation is involved in large ribosomal subunit biogenesis. Finally, further investigations will be needed to gain a more comprehensive understanding of how protein phosphorylation and protein co-factors contribute to ribosome biogenesis and the regulation of 5S RNP-p53 signalling. Future research may also yield valuable insights in developing new therapeutic strategies treating ribosomopathies and other diseases.

Chapter 6. Reference

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