THE 5S RNP AND THE REGULATION OF THE TUMOUR SUPPRESSOR, P53

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ABSTRACT

Ribosomes are vital molecular machines involved in catalysing mRNA translation into proteins. Understandably, when ribosome production is disrupted, there are mechanisms available to prevent further cell growth. The main complex involved in this process is the 5S RNP, a ribosome assembly intermediate consisting of the ribosomal proteins RPL5 and RPL11, and the 5S rRNA. The 5S RNP induces the tumour suppressor p53 by interacting with, and inhibiting, MDM2 an ubiquitin ligase responsible for maintaining low levels of p53 in the cell. The 5S RNP-p53 pathway has been implicated in diseases known as ribosomopathies, however the regulation of these pathways have not been fully characterised and there is still much that is unknown. The aim of this project was to identify the components involved in regulating 5S RNP integration into the ribosome and to determine what further components may be required for 5S RNP-mediated p53 activation.

There have been many recent studies elucidating the mechanism by which the 5S RNP is incorporated into the ribosome in yeast, however this mechanism appears to vary in humans. The data presented in this thesis determined that mimicking phosphorylation of RPL5 Y30 prevented 5S RNP incorporation into the ribosome. Furthermore, depletion of RPL7, RPL18, and RPL21 resulted in large subunit biogenesis defects and abrogated 5S RNP incorporation into the ribosome.

Previous studies have suggested that SRSF1 and PRAS40 may be involved in the regulation of p53 via RPL5 and RPL11 respectively. In congruence with this, the data presented here showed SRSF1 interacts with the 5S RNP. Furthermore, depletion of SRSF1 results in reduction in 5S RNP-dependent p53 activation, mis-localisation of RPL11, and reduction in 5S RNP integration. Collectively, these data suggest that SRSF1 may be important for both ribosome production and p53 regulation. The role of PRAS40, however, still remains unclear. Despite not interacting with the 5S RNP, depletion of PRAS40 appeared to abrogate 5S-RNP mediated p53 induction.

In summary, this work has provided a basis for studies into how the 5S RNP is recruited into the ribosome and provides evidence to suggest that SRSF1 is a component of the 5S RNP complex to activate p53.

DECLARATION

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

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TABLE OF CONTENTS

Abstract i

Declaration iii

Acknowledgements iii

Table of contents v

Index of figures xi

Index of tables xv

List of abbreviations xvii

Chapter One: Introduction 1

1.1 The ribosome 1

1.2 Ribosome biogenesis 1

1.2.1 The nucleolus 2

1.2.2 rRNA processing 3

1.2.3 Small and large subunit processing and assembly in yeast 9

1.2.4 Nuclear export 11

1.2.5 The 5S RNP recruitment and rearrangement 11

1.2.6 Quality control of ribosome biogenesis 14

1.3 The tumour suppressor, p53 14

1.3.1 The p53 family of proteins 15

1.3.2 Regulation of p53 via MDM2 and MDMX/MDM4 16

1.3.3 Activation of p53 18

1.3.4 Transcriptional activation via p53 19

1.4 The role of the ribosome in p53 signalling 24

1.4.1 Ribosomal proteins and p53 signalling 24

1.4.2 The 5S RNP and MDM2/p53 26

1.4.3 The 5S RNP and MDMX 28
1.4.4. The 5S RNP and p73 29
1.4.5 The 5S RNP-p53 pathway is linked to the expression of oncogenes and tumour suppressors 30
1.5 Regulation of the 5S RNP-p53 pathway 31
1.5.1 PICT-1 32
1.5.2 SRSF1 33
1.5.3 PRAS40 34
1.5.4 Post-translational modifications 37
1.6 Diseases as a result of aberrant ribosome biogenesis 40
1.6.1 Diamond Blackfan Anaemia 41
1.6.2 5q-syndrome 43
1.6.3 Treacher Collins Syndrome 44
1.6.4 Cancers 45
1.6.5 Other diseases as a result of aberrant ribosome biogenesis 47
1.7 Project aims and objectives 49
Chapter Two: Methods 51
2.1 Reagents 51
2.2 DNA methods 51
2.2.1 Plasmid purification 51
2.2.2 PCR 51
2.2.3 Sequencing 53
2.2.4 Agarose gel electrophoresis 53
2.2.5 Restriction digests 54
2.2.6 Ligation 54
2.2.7 Transformation into *E.coli* 54
2.3 RNA methods 54

2.3.1 RNA extractions 54
2.3.2 Northern blot methods and probes 55
2.3.3 Labelling RNAs for interaction studies 56
2.3.4 Quantification of RNA 57
2.4 Human Cell Culture 58
2.4.1 Human Cell lines 58
2.4.2 RNAi 59
2.4.3 Creation of pcDNA5 cell lines 59
2.4.4 Chemical treatments 61
2.4.5 Whole Cell extracts 61
2.4.6 Immunofluorescence 61
2.4.7 Pulse labelling experiments 62
2.4.8 Glycerol Gradient analysis of 5S RNP components 63
2.4.9 Cell cycle analysis by flow cytometry 63
2.5 Protein Methods 63
2.5.1 SDS-PAGE 63
2.5.2 Western blotting 64
2.5.3 Phosphorylation gels 65
2.5.4 Luciferase assays 65
2.6 Over-expression and purification of GST-tagged proteins in *E.coli* cells 65
2.6.1 Purification of GST-tagged proteins 65
2.6.2 Desalting 66
2.7 Interaction studies 66
2.7.1 Glutathione-sepharose protein-RNA pull downs 66
2.7.2 Immunoprecipitations 66
Chapter Three: Understanding the factors involved in recruiting the 5S RNP into the ribosome 69

3.1 Introduction 69
3.2 Results 73
3.2.1 Depletion of RPL7, RPL10, RPL18 and RPL21 in U2OS cells 73
3.2.2 Depletion of RPL7, RPL18 and RPL21 affects integration of the 5S rRNA into the ribosome 74
3.2.3 RPL7, RPL18 and RPL21 are crucial for ribosome biogenesis and RPL7 and RPL18 knockdowns lead to accumulation of the 36SC precursor 75
3.2.4 Knockdowns of RPL7, RPL18 and RPL21 but not RPL10 induce p53 via the 5S RNP 78
3.2.5 Depletion of RPL7, RPL18 and RPL21 result in an accumulation of cells in G1 in an RPL5-dependent manner. 80
3.2.6 *In vitro* binding assays to show RPL11/28S rRNA interactions 82
3.2.7 RPL11 directly binds helix 83-85 of the 28S rRNA but not the 5S rRNA 85
3.3 Discussion 86
Chapter Four: Investigation into the role of SRSF1 and PRAS40 as potential regulators of the 5S RNP-p53 pathway 93
4.1 Introduction 93
4.2 Results 99
4.2.1 Creation of inducible cell lines expressing FLAG-SRSF1 and FLAG-PRAS40 99
4.2.2 Localisation of FLAG-SRSF1 and PRAS40 100
4.2.3 Expression of FLAG-tagged SRSF1 and PRAS40 increase p53 levels in U2OS cells 102
4.2.4 SRSF1 and PRAS40 over-expression does not affect cell cycle progression 103
4.2.5 SRSF1, but not PRAS40, co-purifies with the 5S rRNA 104
4.2.6 Depletion of PRAS40 increases p53 activity whilst depletion of SRSF1 reduces p53 activity 106
4.2.7 SRSF1 and PRAS40 have opposing effects on ActD treatment 108

4.2.8 Depletion of SRSF1 and PRAS40 counteract the p53 response from RPL5 and RPL11 over-expression.....110

4.2.9 Knockdown of SRSF1 and PRAS40 have effects the localisation of 5S RNP components.....112

4.2.10 Depletion of SRSF1 affects integration of the 5S RNP into the ribosome..113

4.2.11 Knockdowns of SRSF1 and PRAS40 have no significant effect on rRNA processing in pulse-labelling experiments 115

4.2.12 p53 induction from Deguelin treatment is RPL5-dependent. 116

4.3 Discussion 119

Chapter Five: The role of phosphorylation of RPL5 in cell signalling via p53 127

5.1 Introduction.....127

5.2 Results 131

5.2.1 Tyrosines 30, 31 and 44 of RPL5 are located on the interaction surface with the ribosome and, thus, if phosphorylated, may block ribosomal integration. 131

5.2.2 Creation of inducible cell lines expressing FLAG-tagged RPL5 phosphorylation mutants.....133

5.2.3 Mutation of RPL5 tyrosine 30 does not affect the localisation of RPL5. 135

5.2.4 Mutating Y30 to phenylalanine or glutamic acid does not affect RPL5-5S rRNA interactions.....136

5.2.5 Expression of RPL5 Y30E results in increased levels of non-ribosomal RPL5 compared the RPL5 Y30F mutant and wild type RPL5. 137

5.2.6 RPL5 shows evidence of differential phosphorylation between non-ribosomal and ribosomal RPL5 138

5.2.7 FLAG-RPL5 Y30 mutants associate with MDM2 and p53 140

5.2.8 The wild type and mutant RPL5 all increase p53 levels but the Y30F mutation does not increase p53 activity 141

5.2.9 Expression of the wild type RPL5 and the mutants all lead to decreased cellular growth rate. 144

5.2.10 Expression of the wild type, Y30E or Y30F mutants leads to an accumulation of cells in G1/G0 in a p53-dependent manner 145
5.3 Discussion 147
Chapter six: Conclusions and discussion 155
6.1 Introduction 155
6.2 rRNA processing in humans 156
6.3 The recruitment of the 5S RNP into the ribosome 157
6.4 Regulation of the 5S RNP pathway 160
6.4.1 PRAS40 and SRSF1 161
6.4.2 Phosphorylation of RPL5 163
6.4.3 Summary 165
6.5 Future treatments for disease 165
6.6 Conclusions 167
References 169

INDEX OF FIGURES

Figure 1.1 The structure of the eukaryotic ribosome

Figure 1.2 The structure of the nucleolus

Figure 1.3 rRNA processing in *S. cerevisiae*

Figure 1.4 rRNA processing in mammals

Figure 1.5 The domain structure of the p53 family of proteins is highly conserved

Figure 1.6 The structure of the homologous proteins, MDM2 and MDMX

Figure 1.7 Pathways for activating p53

Figure 1.8 The intrinsic apoptosis pathway

Figure 1.9 Cell cycle regulation by p53

Figure 1.10 Ribosomal protein binding sites in MDM2

Figure 1.11 The 5S RNP interacts with MDM2 to promote p53 signalling

Figure 1.12 Regulation of MDMX and MDM2 via the 5S RNP

Figure 1.13 The 5S RNP-p53 pathway

Figure 1.14 The regulation of RPL11-mediated p53 induction via PICT-1

Figure 1.15 Summary of the role of PRAS40 in the Akt/mTOR and p53 pathways

Figure 1.16 Phosphorylation sites reported in RPL5 and RPL11

Figure 1.17 Nucleolar morphology in cancer

Figure 2.1 Schematic representation of the homologous recombination between the pcDNA5 vector and Flp-In T-REX cells

Figure 3.1 The 5S RNP-p53 pathway

Figure 3.2 The 5S RNP 'binding pocket'

Figure 3.3 Treatment of cells with siRNAs for RPL7, RPL10, RPL18 and RPL21 results in a reduction of the relevant mRNA and protein levels

Figure 3.4 RPL7, RPL18 and RPL21 depletion leads to accumulation of ‘free’ 5S rRNA

Figure 3.5 RPL7, RPL18 and RPL21 knockdowns affect ribosome biogenesis

Figure 3.6 Depletion of RPL7, RPL18 and RPL21 induces p53 in a 5S RNP-dependent manner

Figure 3.7 RPL7 and RPL18 knockdowns give an RPL5 dependent accumulation of cells in G1

Figure 3.8 The 28S rRNA contacts RPL11 in the ribosome

Figure 3.9 Protein purifications of RPL5 and RPL11

Figure 3.10 RPL11 directly binds to helix 83-85 of the 28S rRNA

Figure 3.11 rRNA processing defects in ITS1 and ITS2

Figure 3.12 The cryo-EM structure of the human ribosome showing the RNP and the surrounding ribosomal proteins

Figure 3.13 The binding of the 28S rRNA and MDM2 to RPL11 are mutually exclusive

Figure 4.1 The role of the 5S RNP in p53 signalling

Figure 4.2 Summary of the current knowledge of the involvement of PRAS40 and SRSF1 in p53 signalling

Figure 4.3 The role of PRAS40 and SRSF1 in the regulation of translation via mTORC1

Figure 4.4 SRSF1 and PRAS40 expression in cell lines

Figure 4.5 Localisation of FLAG-tagged SRSF1 and PRAS40

Figure 4.6 The effect of SRSF1 and PRAS40 over-expression on p53

Figure 4.7 Cell cycle analysis of SRSF1 and PRAS40 over-expression

Figure 4.8 SRSF1 interacts with the 5S RNP whilst PRAS40 only interacts with RPL11

Figure 4.9 PRAS40 and SRSF1 depletion affects p53 and p21 levels

Figure 4.10 SRSF1 and PRAS40 have opposite effects upon ActD treatment

Figure 4.11	Depletion of SRSF1 or PRAS40 counteracts the p53 response from RPL5 and RPL11 over-expression
Figure 4.12	SRSF1 and PRAS40 depletion affects localisation of RPL5 and RPL11
Figure 4.13	Depletion of SRSF1 affects integration of RPL5 and RPL11 into the ribosome
Figure 4.14	Analysis of the effect of depletion of SRSF1 or PRAS40 on rRNA processing
Figure 4.15	The effect of deguelin, leucine and arginine treatment in U2OS cells
Figure 4.16	A proposed model for SRSF1 in the 5S-RNP mediated activation of p53
Figure 4.17	The mechanism of Deguelin activating p53 via the 5S RNP
Figure 5.1	The 5S RNP activates p53 via interactions with MDM2
Figure 5.2	Reported phosphorylation sites on RPL5
Figure 5.3	RPL5 Y30, Y31 and Y44 are located within the interaction surface with the ribosome and Y30 and Y44 are highly conserved in eukaryotes
Figure 5.4	Phosphorylation of RPL5 in cells
Figure 5.5	Inductions of RPL5 and the mutants in U2OS cell lines
Figure 5.6	Mutation of Y30 does not affect localisation of RPL5
Figure 5.7	Interactions between RPL5 wild type and mutants with the 5S rRNA
Figure 5.8	Mimicking phosphorylation of RPL5 Y30 leads to decreased ribosomal integration of RPL5
Figure 5.9	RPL5 is differentially phosphorylated when incorporated into ribosomes.
Figure 5.10	The RPL5 mutants interact MDM2 and p53
Figure 5.11	Expression of the RPL5 mutants affects p53 protein levels and activity
Figure 5.12	RPL5 expression slows the growth of U2OS cells
Figure 5.13	Expression of RPL5 induces an accumulation of cells in G1

Figure 5.14 Schematic representation of the proposed role of phosphorylation in 5S RNP signalling

Figure 5.15 Figure 5.15. Proposed model for phosphorylation in the regulation of p53 via MDMX

Figure 6.1 Proposed 5S RNP ribosomal integration pathway

Figure 6.2 The proposed model for 5S RNP-p53 signalling via SRSF1 and PRAS40

INDEX OF TABLES

Table 1.1	Diseases of ribosome biogenesis dysfunction
Table 2.1	Primers used for cloning
Table 2.2	The PCR conditions used for cloning
Table 2.3	List of primers used for RT-PCR
Table 2.4	The PCR thermal cycling conditions for Go Taq G2 polymerase
Table 2.5	List of primers used in mutagenesis PCR
Table 2.6	Probes used for northern blot hybridisation
Table 2.7	Conditions for *in vitro* transcription
Table 2.8	Conditions for labelling RNAs
Table 2.9	siRNA sequences used
Table 2.10	Final concentration of chemical treatments used in cell culture
Table 2.11	Antibodies used in immunofluorescence
Table 2.12	List of antibodies used with the dilutions used in Western blotting

LIST OF ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
A. *melanoleuca*	*Ailuropoda melanoleuca*
A. nidulans	*Aspergillus nidulans*
ActD	Actinomycin D
AKT	Protein Kinase B
ALK	Anaplastic Lymphoma Kinase
AML	Acute Myeloid Leukaemia
ARF	ADP ribosylation factor
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Triphosphate
B. Mori	*Bombyx mori*
C	Cysteine
C. elegans	*Caenorhabditis elegans*
CDK	Cyclin Dependent Kinase
cDNA	Complementary Deoxyribonucleic Acid
CHK	Checkpoint Kinase
CK2	Casein Kinase II
CMV	Cytomegalo virus
C-terminal	Carboxyl terminal
DC	Dyskeratosis Congenita
D. discoidium	*Dictyostelium discoideum*
D. melanogaster	*Drosophila melanogaster*
D. rerio	*Danio rerio*
DAPI	4',6-diamidino-2-phenylindole
DBA	Diamond Blackfan Anaemia
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
E	Glutamic Acid
E. coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ETS	External Transcribed Spacer
F	Phenylalanine
FBS	Foetal bovine serum
FRT	Flp Recombination Target
G. gallus	*Gallus gallus*
G1	Gap 1
G2	Gap 2
GST	Glutathione S-Transferase
GTP	Guanosine triphosphate
H. sapiens	*Homo sapiens*
HRP	Horseradish peroxidase
IF	Immunofluorescence

IP	Immunoprecipitation
ITS	Internal Transcribed Spacer
Kb	Kilobase
kDA	KiloDalton
MDM2	Mouse Double Minute 2
MgCl2	Magnesium Chloride
mRNA	Messenger RNA
mTOR	Mammalian Target of Rapamycin
mTORC1	mammalian Target of Rapamycin Complex 1
NaCl	Sodium Chloride
NaH2PO4	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NEDD8	Neural precursor cell expressed developmentally down-regulated protein 8
NES	Nuclear Export Signal
NLS	Nuclear Localisation Signal
N-terminal	Amino-terminal
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIPES	1,4-Piperazinediethanesulphonic acid
PNK	Polynucleotide kinase
pol	Polymerase
PRAS40	Proline-rich AKT substrate of 40 kDa
pre-rRNA	Precursor rRNA
PUMA	p53-upregulated modulator of apoptosis
R. novegicus	*Rattus norvegicus*
rDNA	Ribosomal Deoxyribonucleic Acid
RING	Really Interesting New Gene
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNAi	RNA interference
RNP	Ribonucleoprotein Complex
RP	Ribosomal protein
RPM	Revolutions per minute
rRNA	Ribosomal Ribonucleic Acid
RRP	Ribosomal RNA-processing protein
S	Svedburg unit
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
S-phase	Synthesis Phase
SRSF1	Serine Arginine Rich Splicing Factor 1
T. citricida	*Toxoptera citricida*
T. gondii	*Toxoplasmosis gondii*
TBE	Tris- boric acid- EDTA
Tet	Tetracycline
TFIIIA	Transcription factor 3A

TIP 49	TBP (TATA binding protein) - interacting protein of 49kDa
Tris	Tris (hydroxymethyl)aminomethane
tRNA	Transfer RNA
TSC2	Tuberous Sclerosis 2
U.V.	Ultraviolet
UTP	Uridine triphosphate
v/v	Volume per volume
w/v	Weight per volume
X. laevis	*Xenopus laevis*
Y	Tyrosine
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar

CHAPTER ONE: INTRODUCTION

1.1 The ribosome

Ribosomes are highly conserved complexes involved in translating mRNA into proteins through peptide bond formation. Ribosomes are conserved throughout all domains of life, although their composition varies. The mature eukaryotic ribosome (80S) consists of two subunits, the large (60S) subunit and the small (40S subunit). They are thus named the 80S, 60S and 40S due to their sedimentation coefficient. The structure of the ribosome has been solved in several eukaryotic organisms including *S.cerevisiae*, *H.sapien*, and *T.thermophila*, showing the organisation of the rRNAs and ribosomal proteins (Khatter et al., 2015, Ben-Shem et al., 2010, Klinge et al., 2011). The small and large subunits are composed of ribosomal RNA (rRNA) and ribosomal proteins. The small subunit is involved in the decoding function of the ribosome whilst the large subunit contains the peptidyl transfer centre (Lafontaine and Tollervey, 2001, Jones et al., 2008). The small subunit contains around 32 ribosomal proteins and the 18S ribosomal RNA (rRNA) whilst the large subunit consists of 3 ribosomal RNAs (rRNAs), the 28S (25 in S. cerevisiae), 5.8S and 5S rRNAs as well as approximately 46 ribosomal proteins (Wilson and Doudna Cate, 2012).

The rate of ribosome production dictates cell growth, differentiation and proliferation (Rudra and Warner, 2004, Warner, 1999b, Sulic et al., 2005, Thomas, 2000). Furthermore, ribosome production is down-regulated during differentiation (Villunger et al., 2003) and up-regulated during cancer (Ruggero and Pandolfi, 2003). As ribosome biogenesis is so energy costly to the cell, the regulation of this process is highly important and errors in ribosome biogenesis have been reported in a number of genetic diseases known as ribosomopathies (Danovi et al., 2004).

1.2 Ribosome biogenesis

Eukaryotic ribosome biogenesis is an extremely complex and tightly regulated pathway that involves the coordination of all 3 RNA polymerases as well as more than 200 different ribosome assembly factors (Henras et al., 2008). Ribosome biogenesis is extremely energy consuming. In yeast 60% of the total transcription is dedicated to ribosomal RNA (Warner, 1999a) and thus this process has to be tightly regulated.

1.2.1 The nucleolus

Ribosome biogenesis occurs in the nucleolus, a nuclear sub-compartment formed around the nucleolar organiser regions (NORs) at the end of mitosis. The NORs are stretches of DNA containing the rRNA genes (McStay and Grummt, 2008). There are about 150 copies of the rDNA genes in yeast that are found in tandem repeats on chromosome 12 (Kobayashi et al., 1998). In humans, there are around 400 tandem repeats of the rDNA genes located in NORs on the short arms of acrocentric chromosomes (Chromosomes 13, 14, 15, 21 and 22) (Heliot et al., 1997). In humans, not all of the NORs are active, however those that are not active are believed to be important for nucleolar organisation and chromatin structure (Sanij and Hannan, 2009).

Although the nucleoli are not membrane-bound organelles, they are organised into three different compartments- the fibrillar centres, the dense fibrillar component (DFC), and the granular component (GC) (Derenzini et al., 1990) (Figure 1.1). Depending on the cell type and stage, the arrangement of the nucleoli can vary as their morphology can change in response to altered demands (Hernandez-Verdun, 2006). The FC consists of a network of 4-5nm thick fibrils where components of the transcription machinery are localised whereas the DFCs are composed of 3-5nm thick densely packed fibrils is where rRNA processing occurs (Schwarzacher and Wachtler, 1993). The GC contains 15nm granules and functions as a storage component.

The 47S pre-rRNA precursor is transcribed by RNA polymerase I at the FC/DFC border of mammalian nucleoli (Puvion-Dutilleul et al., 1997, Cheutin et al., 2002) before the RNAs move into the DFC during the elongation process (Cheutin et al., 2002). In the DFC, the initial cleavage steps are thought to occur (Derenzini et al., 1990) before the movement to the GC and subsequent processing takes place (Gerbi and Borovjagin, 1997). Finally, the pre-rRNAs exit the nucleoli and enter the nucleoplasm before being exported to the cytoplasm (Lei and Silver, 2002).

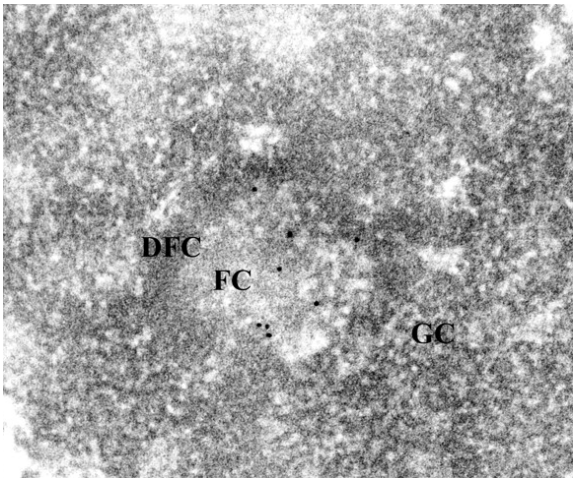

Figure 1.1 The structure of the nucleolus. Immunogold labelling detection of rRNAs from mouse ELT cells.showing the three nucleolar compartments: fibrillar centers (FC), dense fibrillar component (DFC) and granular component (GC). Figure modified from Cheutin et al. (2002).

The nucleolus has many functions including regulation of RNA and RNP biogenesis, organisation of the epigenome, and modulation of cellular stress responses (Quin et al., 2014). Several studies have demonstrated that ribosome biogenesis is up-regulated in cancer tissues, believed to be caused by a loss of tumour suppressor function (As reviewed in Montanaro et al. (2008)). This prompts the notion that the nucleolus may play roles in coordinating the cell cycle and cell death in stress situations, and the formation and maintenance of cancers. Furthermore, expression of dominant negative mutants of the nucleolar protein, BOP1 results in p53-mediated cell cycle arrest (Pestov et al., 2001b).

1.2.2 rRNA processing

The transcription and processing of the ribosomal rRNA is a key step in the formation of ribosomes. In eukaryotes, the 18S, 5.8S and 28S (25S in yeast) rRNAs are transcribed as a single polycistronic precursor by RNA polymerase I in the nucleolus (Leary and Huang, 2001, Nazar, 2004) whilst the 5S rRNA is transcribed by RNA polymerase III in the nucleoplasm. The eukaryotic rRNA precursor is 13kb long and has a sedimentation value of 47S (35S in yeast). The 47S pre-rRNA undergoes a series of processing steps in order to produce the mature ribosomal rRNAs. During transcription of the rDNA, the nascent transcripts emerge from the rDNA template in a “Christmas tree”-like manner known as Miller spreads (Miller and Beatty, 1969). The terminal knobs at the ends of the transcripts correspond to the rRNA processing complexes containing precursors to

the mature ribosomal subunits (Bernstein et al., 2004, Dragon et al., 2002, Grandi et al., 2002). Many of these factors are recruited as part of a large complex called the small subunit (SSU) processome which associates with the pre-rRNA during transcription (Osheim et al., 2004) and is involved in processing, assembly and maturation of the small subunit. These processing steps, whilst similar, vary between organisms.

1.2.2.1 rRNA processing in S. cerevisiae

Most of the current understanding of rRNA processing has been gleaned from studies in yeast and are summarised in Figure 1.2. The sites A_2, A_3 and an internal transcribed spacer (ITS1) are important for separating small subunit rRNA (18S) from the large subunit rRNAs (25S and 5.8S rRNAs).

The 35S pre-rRNA undergoes B_0 cleavage in the 3' ETS by Rnt1 (Kufel et al 1999). This is followed by cleavages at A_0, A_1 and A_2 which requires processing by several snoRNPs (Lafontaine and Tollervey, 1995, Venema and Tollervey, 1995, Morrissey and Tollervey, 1993). Two further proteins have been proposed to be involved in the cleavage of sites A_1 and A_2. Bleichert et al. (2006) suggested that Utp24 may be responsible for the endonucleolytic cleavages at A_1 and A_2 whilst Site A_2 has also been proposed to be cleaved by Rcl1 (Horn et al., 2011).

Removal of the 5' external transcribed spacer (5' ETS1) and cleavage in the internal transcribed spacer, ITS1 is performed by the small subunit (SSU) processome complex (Phipps et al., 2011). The 20S precursor is then transported to the cytoplasm where cleavage at site D by the endonuclease Nob1 then removes the remainder of ITS1 at the 3' end of the 18S rRNA to produce the mature 18S rRNA (Lamanna and Karbstein, 2009, Pertschy et al., 2007).

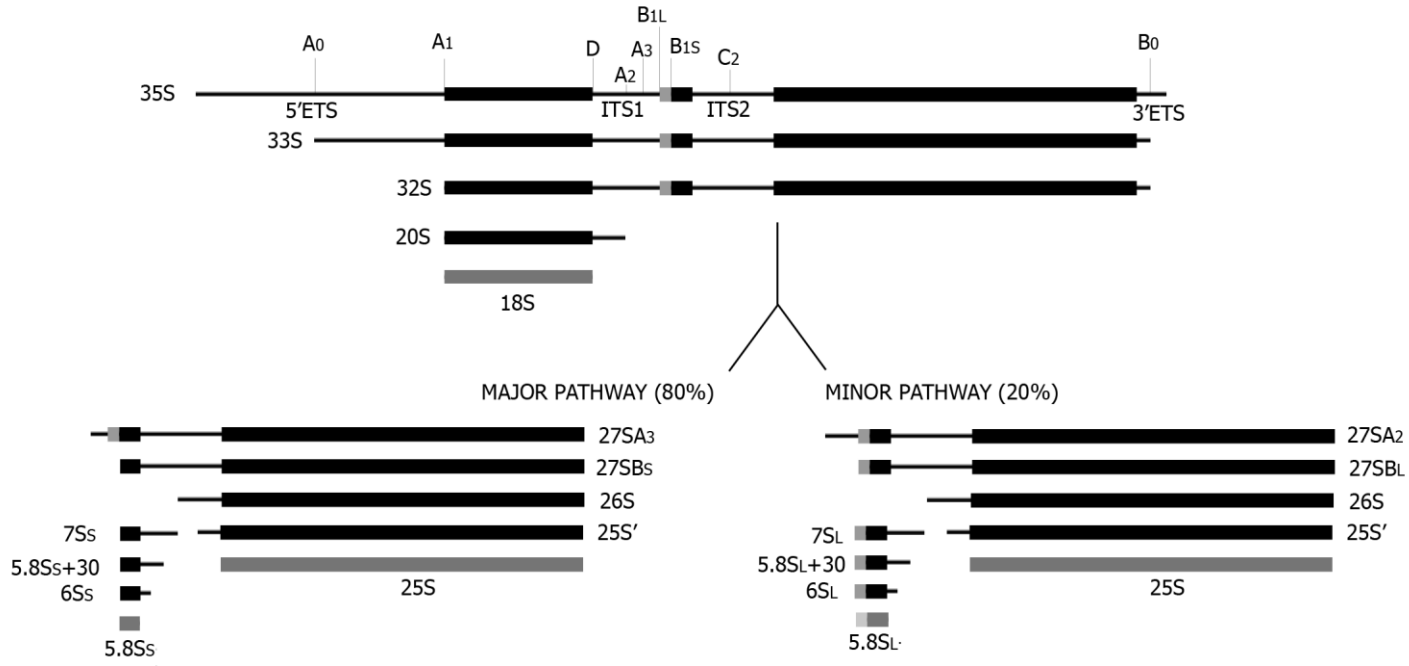

Figure 1.2. rRNA processing in *S. cerevisiae*. Schematic representation of the cleavage events from the 35S precursor to produce the 18S, 5.8S and 25S rRNAs. Bars represent the mature rRNAs and lines represent the transcribed spacers. In yeast, the 25S, 18S and 5.8S rRNAS are transcribed as a single 35S rRNA precursor. The location of the major cleavage sites as well as the internal (ITS1, ITS2) and internal spacer regions (3'ETS, 5'ETS) are shown below the main precusor. Cleavage sites (A_0, A_1 etc) are shown by grey vertical lines with the cleavage site name above. The intermediates produced by the processing events are shown below the full length transcripts. Adapted from Mullineux and Lafontaine (2012)

There are then two different pathways in yeast for the processing of the large subunit rRNAs. In the majority of cases, RNAse MRP, an RNA-protein complex, is required for cleavage at A_3 (Lygerou et al., 1996, Schmitt and Clayton, 1993) along with Nop4 (Sun and Woolford, 1994) and a component of the SSU processome, Rrp5 (Venema and Tollervey, 1996). The 5' to 3' exonucleases, Xrn2/Rat1 (Petfalski et al., 1998) and Rrp17 (at the B_{1S} site) (Oeffinger et al., 2009, Henry et al., 1994) as well as the A_3 cluster of proteins (Granneman et al., 2011, Sahasranaman et al., 2011) are involved in processing the 5' end of the 5.8S rRNA. However, in around 20% of cases, the $27SA_2$ is produced by cleavage at A_2. The B_{1L} site is endonucleolytically processed, by an unknown RNase. The two pathways then follow a similar route, however the minor pathway produces the $5.8S_L$ whilst the major pathway produces the $5.8S_S$ (Henry et al., 1994).

Exonucleolytic cleavage at Site C_2 in the nucleus produces 7S and $27SB_S$/$27SB_L$ (Michot et al., 1999). Exonucleases Rat1 and Rrp17 then process the mature 5' end of the 25S (Oeffinger et al., 2009). The 5.8S containing precursor generated by cleavage at C_2 is processed by the exosome to produce 5.8S with 30 nucleotide 3' extension (5.8S+30). Exonucleases act in both directions following C_2 cleavage and require Las1 (Schillewaert et al., 2012). The 5.8S +30 precursor is cleaved to 6S by Rrp44 and Rrp6 of the exosome (Schillewaert et al., 2012). The mature 3' end of the $5.8S_S$ and $5.8S_L$ are formed in the cytoplasm by Rex1, Rex2 and Ngl2 exonucleases (Faber et al., 2002) The Mature 3' of 25S is produced by exonuclease Rnt1 (Oeffinger et al., 2009).

1.2.2.2 rRNA processing in mammals

In direct contrast to yeast, mammalian ribosome biogenesis is much less studied and thus less is known about the factors involved. The human rRNA precursor transcript is 47S compared to the yeast 35S and has many of the same features including the internal and external spacer regions as shown in Figure 1.3B. Furthermore, the mammalian rRNA processing pathway has multiple different alternative processing pathways (Figure 1.3A).

In mammalian pre-rRNA processing, there are two cleavage steps in the 5' ETS of the 47S transcript at sites A' and A_0 (Hadjiolova et al., 1993, Mullineux and Lafontaine, 2012). The A' cleavage occurs co-transcriptionally and generates the 45S (Mullineux and Lafontaine, 2012). This cleavage relies on UTP-A, XRN2 and MTR4. However, in the

absence of XRN2 but not MTR4, A_0 cleavage becomes the first cleavage in the 5' ETS (Sloan et al., 2014).

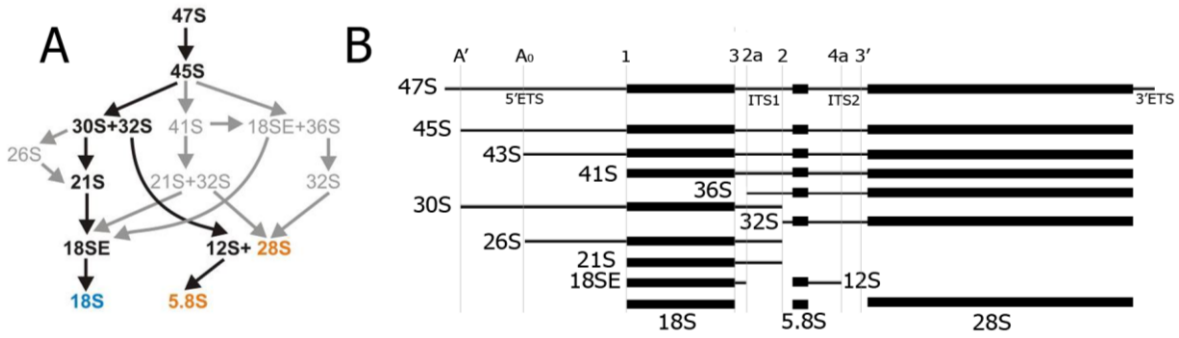

Figure 1.3 rRNA processing in mammals. (A) The rRNA processing pathways in mammals from (Sloan et al., 2013b). The major pathway is represented by black text and arrows whilst the minor pathway is shown with grey text and arrows. The mature rRNAs are shown in orange (large subunit) and blue (small subunit). **(B)** Schematic representation of the rRNA cleavage events in mammals depicting the full length 47S transcript with the key cleavage sites (A', A_0, 1, 3, 2a, 2, 4a and 3') and spacer regions (ITS1, ITS2, 5'ETS, 3'ETS) mapped. The bars represent the rRNAs whilst the lines represent the transcribed spacer regions. The processed intermediates are shown below the full length transcript and are processed into the mature 18S, 5.8S and 28S rRNAs as shown at the bottom.

After A' processing, cleavage at site 2 produces the 30S and 32S precursors and separates the large and small subunit rRNAs. The 5'ETS is removed from 30S to generate 21S. At this stage, there are two potential pathways. Either A_0 and site 1 are cleaved simultaneously, or if A_0 is cleaved before site 1, via a 26S intermediate (Bowman et al., 1981, Hadjiolova et al., 1993). The order at which these steps occur can vary and ITS1 cleavage can occur after cleavages to remove the 5'ETS. This generates the 41S which is cleaved at site 2 to produce the 21S and 32S. ITS2 contains two cleavage sites known as 3' and 4a. Cleavage at the 3'-region of ITS2 results in the generation of the 12S pre-rRNA (Hadjiolova et al., 1993) and further cleavage at site 4a results in a 7S pre-rRNA (Farrar et al., 2008). The mature 3' end of the 5.8S is generated by exonucleolytic processing involving the exosome (Wang and Pestov, 2011). Exonucleolytic processing is required for the formation of the 5' and 3' ends of the 28S.

The production of the 18S in mammals is known to be slightly more complicated than that in yeast. Site 2 cleavage was shown to be the initial cleavage in ITS1 in humans (Mullineux and Lafontaine, 2012) and the efficiency of site 2 cleavage is insensitive to defects in the cleavage of A_0 and site 1 and depends on PES1, BOP1, NOL2, RPL15 and RPL26 (Lapik et al., 2004, Preti et al., 2013, Sloan et al., 2013b).

Furthermore, it has been demonstrated that there are two pathways for the processing of ITS1 in human cells, the major and the minor pathways. In the major pathway, site 2 is cleaved and the 5' end of 5.8S and site 2a is processed by exonucleases to produce the 18SE precursor (Preti et al., 2013, Sloan et al., 2013b). After nuclear export site 3 is processed by NOB1 to produce the mature 18S (Preti et al., 2013, Sloan et al., 2013b). The minor pathway is triggered when 18SE production is blocked, resulting in an endonucleolytic cleavage at sites 2 and 2a. RRP6, a component of the exosome then processes to site 2a (Sloan et al., 2013b). Recently, hUTP24 has been demonstrated to be essential for the processing of the 5'-end of the 18S rRNA (Tomecki et al., 2015).

1.2.2.3 rRNA modifications

In addition to the processing events, pre-rRNAs also undergo co-transcriptional and post-transcriptional covalent modifications (Kos and Tollervey, 2010) which are mediated by small nucleolar RNPs (snoRNPs). There are two different modifications that take place; 2'-O-methylation and the conversion of uridine to pseudouridine (pseudouridylation). Many of the modifications take place in functionally important domains and are thus extremely crucial for ribosome function (Decatur and Fournier, 2002, Ofengand, 2002, Liang et al., 2007, Baudin-Baillieu et al., 2009).

These two modifications are mediated by two different sub-classes of snoRNPs. The first class are BOX C/D snoRNPs that are responsible for 2'-O-methylation whilst the second class are BOC H/ACA snoRNPs involved in pseudouridylation (Watkins and Bohnsack, 2012). 2'-O-methylation is important for stabilising base pairing and RNA folding whilst pseudouridylation is important for maintaining secondary structures and the interactions between rRNA and ribosomal proteins (Helm, 2006, Decatur and Fournier, 2002, Decatur and Fournier, 2003). It has also more recently been shown that snoRNPs may have other important roles such as regulating gene expression by alternative spicing and gene silencing, and mediating stress responses (Bratkovic and Rogelj, 2011, Bratkovic and Rogelj, 2014).

1.2.3 Small and large subunit processing and assembly in yeast

In yeast, after A_2 cleavage to separate the large and small subunits, many of the factors involved in 60S maturation are assembled onto the pre-rRNA (Grandi et al., 2002, Schafer et al., 2003, Tschochner and Hurt, 2003). The 40S particles contain most of the small subunit ribosomal proteins (Ferreira-Cerca et al., 2007) as well as some 40S biogenesis factors. The 40S particle, in conjunction with the biogenesis factors, is exported rapidly to the cytoplasm to complete maturation (Schafer et al., 2003).

Maturation of the 60S, however, requires more processing prior to its export and final maturation in the cytoplasm. There are around 80 factors thought to be involved in the maturation of the 60S subunit. One of the main factors involved in the maturation of the 60S subunit is the A_3 cluster of proteins consisting of Nop7, Erb1, Ytm1, Rlp7, Nop15, Nsa3/Cic1 and Rrp1 (Pestov et al., 2001a, Oeffinger et al., 2002, Oeffinger and Tollervey, 2003, Fatica et al., 2003, Miles et al., 2005, Wu et al., 2001, Granneman et al., 2011, Sahasranaman et al., 2011). Erb1, Nop7 and Ytm1 form a scaffold onto which other A_3-cluster proteins, exonucleases and ribosomal proteins assemble (Sahasranaman et al., 2011, Tang et al., 2008). The A_3 cluster of proteins is linked to the processing of 27S A_3 pre-rRNA which acts to complete the maturation of the 5' end of the 5.8S rRNA. Furthermore, most of the A_3 factors bind within the pre-rRNA at sites close to the 5’ region of the 25S rRNA or the 3’ end of the 5.8S rRNA (Granneman et al., 2011). The release of the A_3 factors is believed to trigger a conformational change within ITS2, triggering further maturation events (Sahasranaman et al., 2011, Granneman et al., 2011).

The dynein-related AAA-ATPase, Rea1 is involved in removing factors from the 60S subunit at many steps as it matures and it contacts the pre-ribosome through its ring structure (Ulbrich et al., 2009, Nissan et al., 2004). Ytm1, Nop7 and Erb1, all A_3 cluster proteins, are removed at this stage (Bassler et al., 2010), however Rsa4 and Rea1 dissociate at a later stage (Ulbrich et al., 2009) after aiding in the recruitment and rearrangement of the 5S RNP (Bassler et al., 2015).

Both the 40S and 60S subunits require transporting to the cytoplasm (see 1.2.3 for detail) to finalise their maturation process. After export, the pre-40S and pre-60S particles undergo a series of final maturation steps. Once in the cytoplasm, the ribosomal protein RPS3 is incorporated into the pre-40S particle whilst Ltv1 and Enp1,

ribosomal biogenesis factors, dissociate (Schafer et al., 2006). Most of the factors involved in the later stages of 40S maturation have had their binding sites identified and it has been proposed that these factors prevent premature association of the translation machinery and joining of the subunits (Strunk et al., 2011, Granneman et al., 2010).

The cleavage of the 20S pre-rRNA to the 18S rRNA is one of the final maturation steps of the 40S precursor and is mediated by Nob1 (Lamanna and Karbstein, 2009). This step is initiated by Fun12, the yeast equivalent of the eIF5b translation initiation factor (Lebaron et al., 2012), and involves the general translation termination factors Rli1 and Dom34 (Strunk et al., 2012).

The maturation of the 60S subunit also involves many factors acting in coordination. When the pre-60S is exported, all of the ribosomal proteins associate but the non-ribosomal assembly factors dissociate and are recycled to the nucleus (Hedges et al., 2005, Hung and Johnson, 2006, Zemp and Kutay, 2007). The AAA-ATPase Drg1 removes the shuttling proteins Nog1 and Rlp24 (Kappel et al., 2012, Pertschy et al., 2007). Removal of the RPL24-like protein rlp24 allows for the incorporation of RPL24 which, in turn, recruits Rei1. Rei1 and Jjj1 promote the release and recycling of factors Arx1 and Alb1 (Demoinet et al., 2007). Arx1 is a protein that is found at the ribosome exit tunnel and acts to inhibit the association of translation factors (Bradatsch et al., 2012) and thus removal of this protein is important for the creation of mature ribosomes.

The removal of Tif6 is also an important step in the maturation of the 60S subunit as Tif6 (eIF6 orthologue) inhibits ribosomal subunit joining (Gartmann et al., 2010). The removal of Tif6 is mediated by Efl1 and Sdo1 and is known to require Arx1 removal (Senger et al., 2001, Menne et al., 2007). In order for Efl1 to be able to remove Tif6, the formation of the 60S stalk is required which requires the incorporation of the ribosomal protein P0 (Lo et al., 2009). The final step is the removal of Nmd3 by Lsg1 and Rpl10 which requires the prior removal of Tif6 (Hedges et al., 2005). The fully matured subunits are then free to join together and translate mRNA into protein.

1.2.4 Nuclear export

For successful export, the subunits have to interact with the hydrophobic channel of the nuclear pore complex. Export of both subunits involves the exportin, Xpo1/Crm1, several nuclear pore components, and the RanGTPase system. In higher eukaryotes, export of the pre-60S complex can occur independently of Crm1 through the export factor, Exportin 5 (Wild et al., 2010).

Crm1 mediates export by recognising molecules with a leucine rich nuclear export signal (NES) and is recruited to the 60S subunit via Nmd3 (Ho et al., 2000). The NES sequences are found in each pre-ribosome on adapter molecules (Fukuda et al., 1997, Zemp and Kutay, 2007). In the nucleus, Crm1 binds to RanGTP and translocates to the cytoplasm through a nuclear pore complex. RanGTP is hydrolysed, causing dissociation and subsequent release of the pre-ribosomal complexes into the cytoplasm (Zemp and Kutay, 2007, Guttler and Gorlich, 2011).

Other factors have also been thought to be implicated in this process, for example, in yeast, Mtr2p, Mex67 and Rrp12 have also been suggested to be involved in the export of both subunits (Faza et al., 2012, Oeffinger et al., 2004, Santos-Rosa et al., 1998) whilst Arx1, Ecm1, Bud20, and Npl3 have been implicated in the export of the 60S subunit (Bassler et al., 2012, Bradatsch et al., 2007, Yao et al., 2010, Hackmann et al., 2011).

1.2.5 The 5S RNP recruitment and rearrangement

One important assembly intermediate of the large subunit is a ribonucleoprotein complex (RNP) called the 5S RNP which consists of the 5S rRNA complexed with two large subunit ribosomal proteins, RPL5 and RPL11.

The 5S rRNA is the smallest ribosomal RNA species, the structure of which is generally highly conserved throughout most organisms and is found in most ribosomes apart from some mitochondrial ribosomes (Bhat et al., 2004). A significant proportion of the 5S rRNA in the cell is not associated with ribosomes (Knight and Darnell, 1967, Sloan et al., 2013a) and 5S is the only rRNA that forms a pre-ribosomal complex with ribosomal proteins prior to integrating into the ribosome (Ciganda and Williams, 2011). The 5S RNP is a major component of the ribosome central protuberance, although the

5S rRNA is not a component of the peptidyltransferase, decoding or elongation factor binding centres and, instead, acts to connect all of these components (Dinman, 2009).

The biosynthesis of the 5S rRNA depends upon interactions with TFIIIA in the cytoplasm to the 5S gene in *Xenopus*, but it has also been demonstrated that 5S rRNA can bind TFIIIA itself (Szymanski et al., 2003, Sloan et al., 2013a). In some organisms, the binding of the 5S rRNA and TFIIIA forms the 7S RNP (Layat et al., 2013). In *Xenopus* oocytes, the 7S RNP is stored in the cytoplasm and TFIIIA acts to protect the rRNA from degradation (Allison et al., 1991). After transcription, the 5S rRNA contains a 2-3 3' nucleotide extension, which is processed and removed to form the mature 5S rRNA. This processing step involves several exonucleases in yeast (Rex1p, Rex2p, and Rex3p) (van Hoof et al., 2000) and, more recently, Sloan et al. (2013a) demonstrated that RPL5 was also essential for this maturation step in humans.

The integration of the 5S RNP into the ribosome is not yet fully understood but is known to require Rrs1 and Rpf2 in yeast. Morita et al. (2002) demonstrated a role for Rpf2 and Rrs1 in yeast ribosome biogenesis by showing interactions between Rpf2 and Rrs1 and also demonstrating that Rpf2 interacted with RPL11 in the pre-60S ribosome particles. Depletion of Rpf2 resulted in a defect in 60S ribosomal subunit assembly based on polysome profiling and it was concluded that Rpf2 and Rrs1 were implicated in the processing of the 27SB to the 25S rRNA.

Furthermore, it was shown that Rrs1 and Rpf2 were responsible for the integration of the 5S RNP into 90S pre-ribosomes containing the 35S pre-rRNA (Zhang et al., 2007). Zhang et al. (2007) further demonstrated that Rpf2, Rrs1, RPL5, RPL11 and the 5S rRNA formed a complex in yeast and that depletion of Rpf2 and Rrs1 resulted in a lack of integration of the 5S RNP into pre-ribosomes. It was also demonstrated that depletion of Rpf2/Rrs1 prevented Rpl10 association with pre-ribosomes. This data suggested an essential role of the Rpf2/Rrs1 complex in 5S RNP recruitment.

Recently, the crystal structure of Rrs1 and Rpf2 complex has been solved in *Aspergillus nidulans* (Asano et al., 2014, Kharde et al., 2015) and yeast (Madru et al., 2015) leading to a greater understanding of the role of the Rpf2/Rrs1 complex in 5S RNP recruitment in these organisms. Kharde et al. (2015) and Madru et al. (2015) demonstrated that Rpf2 interacts with the 5S rRNA but that Rrs1 does not, whilst the whole complex also

interacts with RSA4. This interaction with Rsa4 occurs at the UBL domain, which, crucially, is involved in the recruitment of Rea1, a dynein-related AAA-ATPase (Ulbrich et al., 2009).

RPL5 and RPL11 are delivered to the nucleus and probably assembled onto the 5S rRNA by the symportin, Syo1 (Calvino et al., 2015) to form the 5S RNP. The 5S RNP docks onto helix 84 of the 25S rRNA (Rhodin et al., 2011). When the 5S RNP is recruited into the ribosome, it is locked into conformation by the C-terminal tail of Rpf2, Rsa4 and Nsa2 to prevent any rotation (Kharde et al., 2015, Bassler et al., 2015). Interestingly, the conformation of the 5S RNP in pre-ribosomes is not the same as that found in mature ribosomes (Leidig et al., 2014). Rea1 releases Rsa4 from the 5S RNP and rotates the structure by 180^{o} (Bassler et al., 2015, Leidig et al., 2014). This allows for the correct formation of the central protuberance (Leidig et al., 2014) and is an important step in ribosome formation.

Whilst Rrs1 and Rpf2 are crucial for the recruitment of the 5S RNP into ribosomes in yeast, the role of the human homologues, RRS1 and BXDC1, in human ribosome assembly appears to be different. Sloan et al. (2013a) demonstrated that RRS1 and BXDC1 do not contact the 5S RNP outside of ribosomes and showed that depletion of RRS1 or BXDC1 resulted in a reduction of RPL5 in the nucleolus whilst only depletion of BXDC1 had the same effect on RPL11. However, depletion of both proteins increased the nucleoplasmic localisation of RPL5 and RPL11. Cross-linking experiments revealed that RRS1 and BXDC1 both directly contact the 5S rRNA suggesting that RRS1 and BXDC1 directly contact the 5S RNP complex to regulate its nucleolar localisation. Interestingly, it was also found that depletion of PICT-1 (also known as Glioma tumour suppressor candidate region gene – GLTSRC2), the human homologue of the yeast 60S ribosome biogenesis factor, Nop53, (Thomson and Tollervey, 2005), had a more profound effect than depletion of RRS1 or BXDC1 on 5S rRNA ribosomal recruitment. PICT-1 was demonstrated to directly contact the 5S rRNA and be required for the integration of the 5S rRNA into the ribosome. Unlike RRS1 and BXDC1, PICT-1 had a lesser effect on localisation of RPL5 and RPL11, only increasing the levels of nucleoplasmic RPL11. This suggested that RRS1 and BXDC1 may not be as important for recruitment of the 5S RNP into human ribosomes as they are in yeast, but instead

be an important factor for the localisation of the 5S RNP whilst PICT-1 is an important integration factor.

1.2.6 Quality control of ribosome biogenesis

As ribosome biogenesis is such an energy costly process, ribosome assembly is monitored in order to prevent the formation of defective ribosomes. Aberrant or stalled pre-ribosomes are picked up by a surveillance system and targeted for degradation. It is thought that biogenesis factors are assembled onto the pre-ribosomal complexes much earlier than they are required in order to provide a quality control system. For example, Dim1 is recruited early and its presence, rather than dimethylation activity, is required for A_0 and A_1 cleavages (Lafontaine et al., 1998).

When ribosome biogenesis is disrupted, pre-RNAs do not accumulate very significantly, suggesting that they are unstable and targeted for degradation rapidly (Houseley and Tollervey, 2009). Pre-ribosomes that accumulate in the nucleus are degraded by the exosome complex (Allmang et al., 2000, Mitchell et al., 1997). Furthermore, a co-factor of the exosome, the TRAMP complex, targets the exosome to substrates that are destined for degradation (Dez et al., 2006).

Defective ribosomal subunits that escape the initial degradation and are present in the cytoplasm are also targeted for degradation. Ribosomes with mutations in important sites such as residues within the peptidyl transfer centre of the large subunit or the decoding centre of the small subunit results in the degradation of the RNA components by non-functional rRNA decay (NRD) (Cole et al., 2009, LaRiviere et al., 2006).

1.3 The tumour suppressor, p53

The tumour suppressor p53 is a protein that was first identified in 1979 as a protein linked to the transformation of cells (Linzer and Levine, 1979, DeLeo et al., 1979). It wasn't until ten years later that p53 earned its title as a tumour suppressor gene (Baker et al., 1989, Finlay et al., 1989). It has since become well established for its role in malignancy as it is mutated in the majority of cancers (Muller and Vousden, 2013), and its involvement in malignancy development earned the protein the title, 'guardian of the genome' (Lane, 1992). Introduction of mutated p53, which abrogated cell cycle arrest activity but retained apoptosis activity, into $p53^{-/-}$ mice demonstrated resistance

to spontaneous tumour development compared to $p53^{-/-}$ counterparts (Toledo et al., 2006). However, mice harbouring a P172R mutation in p53 are deficient in p53-driven apoptosis but were still protected from cancer (Liu et al., 2004). This demonstrates the versatility of p53 as a tumour suppressor as it exerts its activity by triggering pathways such as apoptosis and cell cycle arrest.

1.3.1 The p53 family of proteins

p53 belongs to a family of proteins which includes two structural homologs of p53- p63 and p73. The overall domain structure of the three proteins is conserved (Figure 1.4). All three proteins contain a transactivational domain (TAD), proline rich domain (PRD), DNA binding domain (DBD) and the oligomerisation domain (OD) (Levrero et al., 2000). p63 and p73 also contain an additional sterile alpha motif (SAM) domain, although, unlike other SAM domains, these are not involved in homo- or hetero-dimerisation (Chi et al., 1999). As p63 and p73 are structural homologues of p53, they are able to form oligomers, bind DNA, and transactivate p53 responsive genes much like p53 (Zhu et al., 1998, Jost et al., 1997, Yang et al., 1998, De Laurenzi et al., 1998, Melino et al., 2004, Keyes et al., 2005). However, both p63 and p73 also regulate genes that are not targets of p53 (Harms et al., 2004).

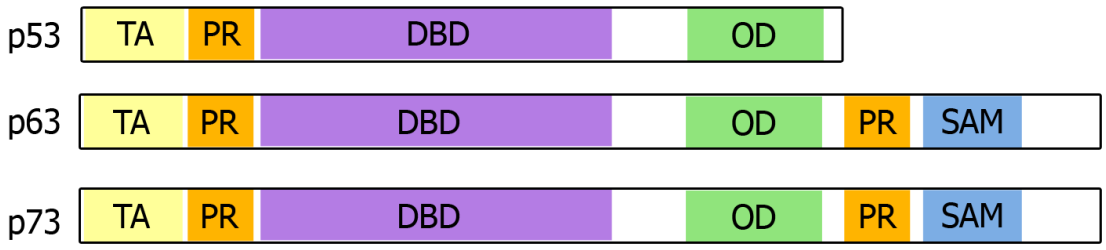

Figure 1.4 The domain structure of the p53 family of proteins is highly conserved. Schematic representation of the domain structure of p53, p63 and p73. All three proteins contain a transactivational domain (TAD), proline rich domain (PRD), DNA binding domain (DBD) and the oligomerisation domain (OD). The longest forms of p63 and p73 also contain an additional PRD and a sterile alpha motif (SAM).

Both the *TP63* and *TP73* genes have two separate promoters, the P1 promoter yields the full length proteins with the TAD. The P2 promoter, located in intron 3, yields N-terminal truncation proteins lacking the TAD, which are referred to as ΔNp63 and ΔNp73 (Yang et al., 1998, Senoo et al., 2001). Furthermore, 5' exon splicing within the P1 transcripts of *TP73* results in additional isoforms of p73 lacking the TAD known as

ΔN'p73, Δex2p73 and Δex2/3p73.(Fillippovich et al., 2001, Stiewe and Putzer, 2002, Ishimoto et al., 2002).

Mutations in *TP63* and *TP73* are rare in cancers (Nomoto et al., 1998, Park et al., 2000). However, ΔN isoforms of p73 and p63 are overexpressed in many different cancer types (Uramoto et al., 2006, Uramoto et al., 2004, Dominguez et al., 2006, Wager et al., 2006, Park et al., 2000). ΔNp63 and ΔNp73 proteins function as dominant-negative inhibitors of the p53 family of proteins by competing for p53 DNA binding sites and forming complexes with p63 and p73 over p53 (Stiewe and Putzer, 2002, Chan et al., 2004, Yang et al., 1998, Grob et al., 2001). Therefore, it is possible that the ΔN isoforms of p63 and p73 act as proto-oncogenes by negatively regulating the p53 family of tumour suppressors.

1.3.2 Regulation of p53 via MDM2 and MDMX/MDM4

The main regulator of p53 is Mouse Double Minute 2 (MDM2- HDM2 in humans), an ubiquitin ligase which promotes the ubiquitination and degradation of p53 by targeting it to the 26S proteasome (Honda and Yasuda, 1999). Loss of function of MDM2 is embryonic lethal in mice due to p53-induced apoptosis (Itahana et al., 2007), thus showing the importance for MDM2 in suppression of p53. Furthermore, MDM2 is also involved in the suppressing the transactivation potential of p63 and p73 without promoting their degradation (Kadakia et al., 2001, Zeng et al., 1999).

In addition to its involvement in the degradation of p53, MDM2 is responsible for the nuclear export of p53 through concealment of its nuclear localisation signal (Boyd et al., 2000) and, furthermore, the binding of MDM2 to p53 also conceals p53's transactivation domain (Kussie et al., 1996). In stressed conditions such as DNA damage or chromosomal aberrations, MDM2 is suppressed and leads to the stabilisation and activation of p53 (Michael and Oren, 2003). Furthermore, MDM2 is regulated at the transcriptional level by a p53 responsive element in its second intron, meaning that MDM2 serves as a negative feedback regulator of p53 (Wu et al., 1993).

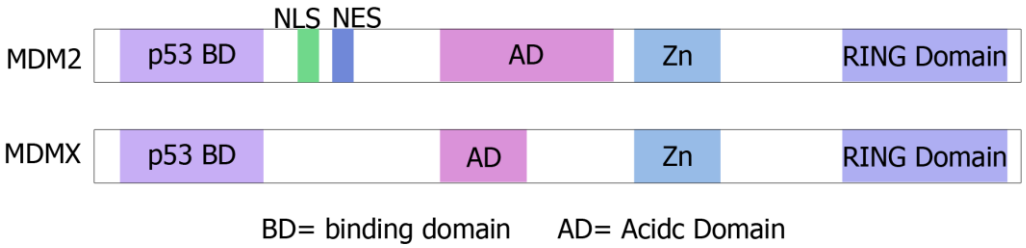

Figure 1.5 The structure of the homologous proteins, MDM2 and MDMX. A schematic representation of the MDM2 and MDMX structure as described in (Wade et al., 2010, Makitie et al., 2004).

MDMX is a protein that was first identified through its ability to bind to p53 from a mouse cDNA library screen (Shvarts et al., 1996). It is homologous to MDM2, most notably in its p53 binding domain where both proteins are able to inhibit p53s transcriptional activation function (Figure 1.5). However, MDMX, most importantly, lacks the E3 ubiquitin ligase activity of MDM2, due to an inactive ring domain and thus cannot target p53 for degradation. This means that MDMX has the ability to prevent p53 activity without promoting p53 degradation (Jackson and Berberich, 2000). Together with the evidence that MDMX-p53 interactions are also far less susceptible to treatment with the small molecule inhibitor Nutlin-3 (which blocks interactions between MDM2 and p53), it was suggested that the two proteins work through different mechanisms (Joseph et al., 2010).

Initially there were two separate models for the suppression of p53 via MDM2 and MDMX; one is whereby the two work in conjunction as a heterodimer, whilst the other states that the two proteins have separate, specific roles. In fact, it appears that the proteins work both alone and in conjunction with one another to suppress p53.

1.3.2.1 MDM2 and MDMX working in conjunction

There is a lot of evidence to suggest that MDM2 and MDMX work together to inhibit p53 and that they are dependent on one another for function. Using siRNA knockdowns of MDM2 and MDMX, it was found that MDM2 is dependent on MDMX for stabilisation whilst MDMX requires MDM2 for shuttling to the nucleus (Gu et al., 2002).

MDM2 and MDMX interact with one another through their RING (Really Interesting New Gene) finger domains (Tanimura et al., 1999) and are able to form heterodimers

as well as homodimers. It was shown that this RING-RING interaction is required for the E3 ubiquitin ligase activity of MDM2, suggesting that MDMX is crucial for MDM2s main mechanism of action against p53 (Kawai et al., 2007).

1.3.2.2 MDM2 and MDMX as distinct mechanisms

MDM2 and MDMX whilst clearly acting in co-operation, have many mechanistic differences. MDM2 inhibits p53 mainly through its E3 ubiquitin ligase activity, targeting p53 for degradation (Honda et al., 1997) whereas MDMX inhibits p53 by binding to its transactivation domain (Danovi et al., 2004).

Whilst both proteins were found to be crucial for development, it was shown that MDM2 deletion in mice lead to apoptosis whilst deletion of MDMX resulted in p53-dependent cell cycle arrest suggesting alternative mechanisms of action of each protein (Chavez-Reyes et al., 2003). There is also a difference between different cell types whereby one can be deleted but not the other (Boesten et al., 2006, Grier et al., 2006).

1.3.3 Activation of p53

Several pathways have been identified that lead to p53 activation as summarised in Figure 1.6. One such pathway involves activation of p53 through DNA damage such as ionising radiation through ataxia telangiectasia mutated (ATM) kinase and the checkpoint kinase, CHK2. CHK2 works downstream of ATM and is able to phosphorylate p53, preventing MDM2 binding and allowing for p53 activation (Vogelstein et al., 2000).

Another pathway is initiated by growth signals produced by the expression of oncogenes such as c-Myc and Alternative Reading Frame (ARF). ARF, which in humans produces a 14kDa protein, $p14^{ARF}$, is a negative regulator of MDM2 and binds to, inactivates and promotes degradation of MDM2, the main suppressor of p53 (Zhang et al., 1998), thus inducing p53. Interestingly, p53 provides a feedback loop and has the ability to inhibit the transcription of the *ARF* gene (Jin and Levine, 2001). Furthermore, ARF is activated by c-Myc, an oncogene frequently translocated in Burkitt Lymphoma and overexpressed in multiple myeloma (Dang, 2012).

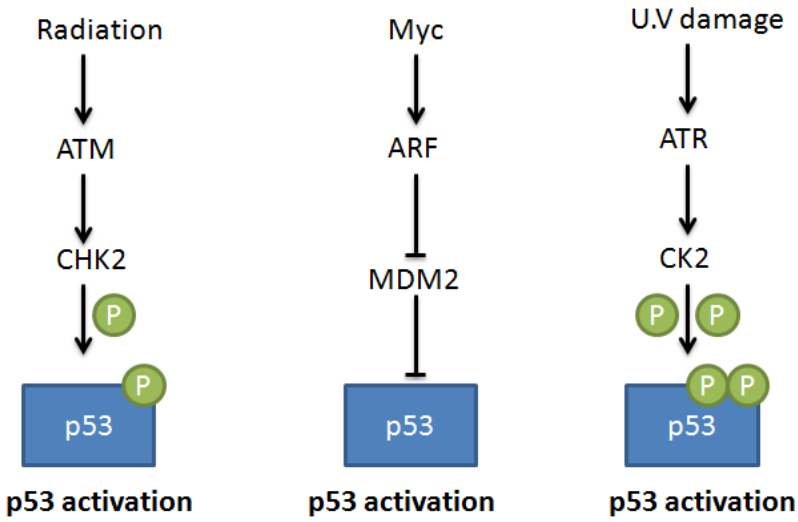

Figure 1.6 Pathways for activating p53. A schematic representation of various pathways involved in the activation of p53. Ionising radiation leads to activation of ATM kinase which phosphorylates and activates CHK2. CHK2, in turn, phosphorylates p53 and activates it. When onocogenes such as Myc or ARF are activated, ARF inactivates MDM2, the negative regulator of p53. This allows p53 to be active. In U.V damage, ATR activates CK2 which phosphorylates p53 at two sites, leading to activation.

The final way that p53 can be activated is by chemotherapeutic drugs and U.V. light through interactions with Ataxia Telangiectasia Related (ATR) and casein kinase II (CK2) through the phosphorylation of serine 37 of p53, enhancing its transcriptional ability (Meek, 1999).

1.3.4 Transcriptional activation via p53

Under cellular stress such as DNA damage, oncogene activation, hypoxia, nucleolar stress or changes in cellular metabolism, p53 is activated and leads to DNA repair, autophagy, senescence, cell cycle arrest or apoptosis through a variety of signalling pathways (Ko and Prives, 1996, Timofeev et al., 2013, Sperka et al., 2012, Sugikawa et al., 1999, Vogelstein et al., 2000, Vousden and Lane, 2007, Vousden and Prives, 2009).

1.3.4.1 p53 in the regulation of apoptosis

One of the main roles that p53 is most commonly known for is its ability to regulate apoptosis in cells. Apoptosis is a process often referred to as 'programmed cell death' and results in cellular death without the loss of membrane integrity (Kerr et al., 1972).

There are two apoptotic pathways- the extrinsic (death receptor pathway) or the intrinsic (mitochondrial pathway) (Elmore, 2007). Both pathways result in the activation of molecules known as caspases. Caspases are selective proteases that

organise all steps of the apoptotic process (Fan et al., 2005). Caspases exist in the cell in an inactive form known as pro-caspases but, when active, break down cellular molecules and DNA, and condense chromatin (Cohen, 1997). p53 has been implicated in the regulation of both pathways.

The extrinsic pathway is activated by Tumour Necrosis Factor (TNF) family of proteins (Locksley et al., 2001) that activate death receptors resulting in the activation of the cell surface Fas receptor (Apo-1 or CD95). Fas interacts with FADD (Fas-associated death domain), forming the DISC complex (Death inducing signalling complex).The DISC complex activates caspases 3 and 7, resulting in apoptosis (Wajant, 2002, Kischkel et al., 1995). The extrinsic pathway can be activated via p53 transcriptional upregulation. p53 initiates transcription of Fas (Muller et al., 1998) and the death receptor gene, DR5 (Wu et al., 1997).

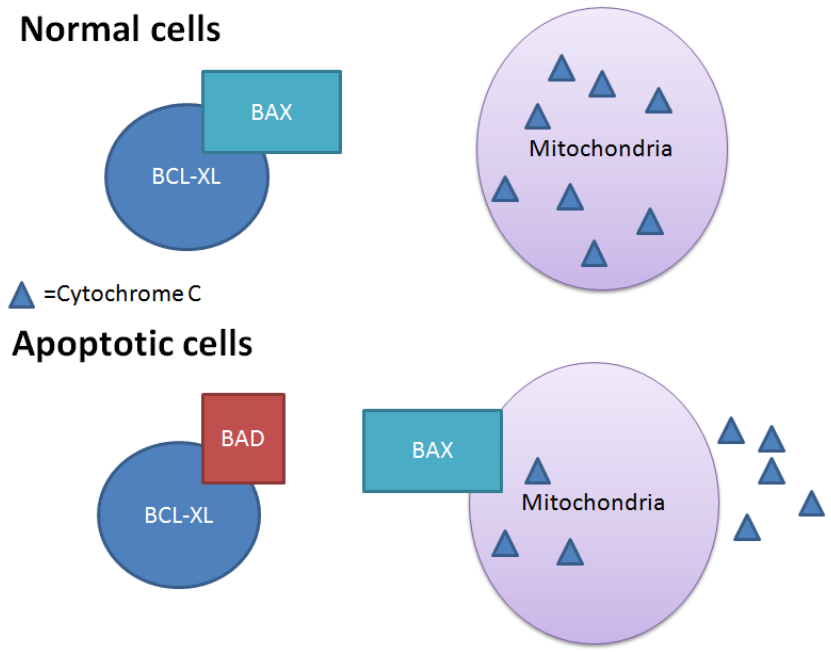

Figure 1.7. The intrinsic apoptosis pathway. In normal cells, the anti-apoptotic gene, BCL-XL binds to the pro-apoptotic gene, BAX and inhibits its activity. During apoptosis, BAD can bind to BCL-XL, displacing BAX. Displaced BAX undergoes a conformational change and binds to the mitochondrial membrane, permeabilising it. This allows for the release of cytochrome C from the mitochondria, triggering the caspase cascade.

The intrinsic pathway is a slightly more complex pathway and is also known as the mitochondrial pathway as it involves cytochrome c release from the mitochondria through the action of the BCL-2 family of proteins. The BCL-2 family of proteins contains both pro-apoptotic and anti-apoptotic members. The anti-apoptotic members

inhibit apoptosis and include proteins such as Bcl-2, Bcl-W, Bcl-XL and Mcl-1. The pro-apoptotic BCL-2 family members can be further subdivided into two groups- the BAX family (e.g. BAX, BAK, BOK) and the BH3-only family (e.g. BID, BAD, BIK, PUMA, NOVA) (Tsujimoto, 1998).

In normal cellular conditions, the anti-apoptotic BCL-2 family members are found in a complex with the pro-apoptotic BAX family proteins in order to inhibit their function and prevent apoptosis. For example, when apoptosis is triggered, BAX (BAX family member) is displaced from Bcl-XL by BAD (BH3-only family member) (Yang et al., 1995). This causes a conformational change in BAX and it is incorporated into the outer membrane of the mitochondria (Liu et al., 2003). Once within the mitochondrial membrane, BAX increases the permeability of the membrane, releasing cytochrome c. This process is summarised in Figure 1.7. Unlike BAX, BAK is always found within the mitochondrial membrane but changes conformation during apoptosis to allow for permeability (Lindsay et al., 2011).

Cytochrome C binds to Apoptotic protease activating factor 1 (APAF-1), forming the apoptosome, a three-dimensional protein structure formed during apoptosis (Acehan et al., 2002). The apoptosome activates procaspase 9 which, in turn, activates caspases 3 and 7, leading to a caspase cascade and apoptosis (Hill et al., 2004).

Stabilised p53 regulates the expression of many of the pro-apoptotic genes involved in both the intrinsic and extrinsic pathways such as BAX (Miyashita and Reed, 1995), NOXA (Oda et al., 2000), PUMA (Nakano and Vousden, 2001), BID (Sax et al., 2002) and APAF-1 (Moroni et al., 2001). Aside from its ability to transcriptionally upregulate important apoptosis regulatory genes, p53 can also directly influence apoptosis. During apoptosis, p53 is also able to migrate to the mitochondrial membrane and can interact with Bcl-XL, freeing BAX from the complex. This allows BAX to relocate to the mitochondria and trigger cytochrome C release (Wolff et al., 2008).

Whilst apoptosis is an important anti-tumourigenic mechanism, it is not the main form of defence. p53-upregulated modulator of apoptosis (PUMA) is a BCl-2 homology domain 3 protein that induces apoptosis through the mitochondrial pathway. PUMA was identified for its ability to be activated by p53 in cells undergoing p53-induced apoptosis (Nakano and Vousden, 2001, Yu et al., 2001). PUMA null mice showed that

PUMA is required for the apoptotic response to p53 in many tissues (Jeffers et al., 2003). However, PUMA null mice do not spontaneously develop cancer (Michalak et al., 2008) supporting the theory that apoptosis is not the main form of defence against cancer and that p53 acts through a diverse range of different pathways to protect cells from transformation.

1.3.4.2 p53 as a cell cycle regulator

One further way that p53 protects the cells from cancer is through the regulation of the cell cycle. Early on it was discovered that p53 had a pivotal role in controlling cell cycle progression by transcriptionally activating the cyclin dependent kinase inhibitor, p21 (el-Deiry, 1998), Growth Arrest and DNA Damage 45 (GADD45) (Carrier et al., 1994) and 14-3-3 sigma (Hermeking et al., 1997) as summarised in Figure 1.8.

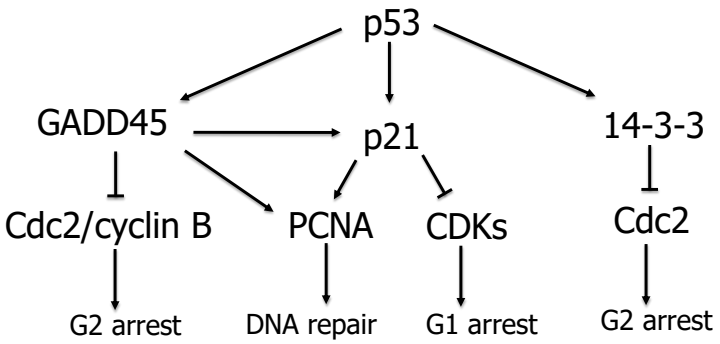

Figure 1.8 Cell cycle regulation by p53. p53 transcriptionally upregulates the genes encoding GAD45, p21 and 14-3-3. GADD45 expression induces G2 arrest by inhibiting Cdc2/cyclin B complexes and also induces G1 arrest by interacting with p21. p21 inhibits CDKs to induce G1 arrest. GADD45 and p21 also bind to PCNA to induce DNA repair pathways. Finally, 14-3-3 induces G2 arrest by inhibiting Cdc2.

The cyclin dependent kinase inhibitor, p21 is regulated at the transcriptional level by both p53 dependent and independent mechanisms (Gartel and Tyner, 1999). p21 expression is induced by low levels of p53 (el-Deiry, 1998, el-Deiry et al., 1993) and p21 is known to play a pivotal role in growth arrest after DNA damage (Dulic et al., 1994, Brugarolas et al., 1995) but is not required for p53-mediated apoptosis (Deng et al., 1995). The p21 promoter contains two binding sites for p53, at least one of which is required for p53 responsiveness after DNA damage (el-Deiry et al., 1995). However, p21 can also be upregulated independently of p53 by proteins such as Signal transducer and activator of transcription 1 (STAT1), Specificity proteins 1 and 3 (Sp1 and Sp3) and activator protein 2 (Ap2) (Chin et al., 1996, Gartel and Tyner, 1999).

Furthermore, MDM2 is able to negatively regulate p21 by targeting it for degradation, independently of p53 (Zhang et al., 2004).

Early on it was shown that over-expression of p21 leads to a G1, G2 or an S-phase arrest (Niculescu et al., 1998, Ogryzko et al., 1997), suggesting a role for p21 in cell cycle progression. p21 is a member of the Cip/Kip family of Cyclin-dependent kinase (CDK) inhibitors which inhibit the action of cyclin dependent kinases required for cell cycle progression. The Cip/Kip family specifically block transition from G1 phase to S-phase (Perez-Sayans et al., 2013). If cell cycle arrest is required, expression of p21 occurs in early to mid G1 and p21 is incorporated into cyclin D-CDK 4/6 complexes to inhibit their activity (Sherr and Roberts, 1999). Furthermore, p21 can bind to Proliferating cell nuclear antigen (PCNA) (Xiong et al., 1992), a protein involved in DNA replication and repair (Warbrick, 2000). The interaction between p21 and PCNA results in a block in DNA synthesis via DNA polymerase δ (Waga et al., 1994, Flores-Rozas et al., 1994) meaning that p53-regulated p21 induction can also block DNA synthesis as well as promote cell cycle arrest.

Furthermore, p53 transcriptionally upregulates GADD45 (Figure 1.8). GADD45 is proposed to have a role in halting the cell cycle arrest at G1 to S-phase transition possibly via p21 (Liebermann and Hoffman, 2008). GADD45 interacts with Cdc2 (CDK1)/Cyclin B complex, causing it to dissociate. The dissociation of the complex halts cell cycle progression (Liebermann and Hoffman, 2008). Furthermore, GADD45 competes with p21 to interact with PCNA to promote DNA repair (Smith et al., 1994, Chen et al., 1995).

p53 has also been shown to interact with the scaffold protein, 14-3-3 (see Figure 1.8) to regulate cell cycle (Hermeking et al., 1997). Activation of 14-3-3 also causes G2 arrest as it prevents the activation of the Cdc2/Cyclin B complex required for G2/M phase transition. The checkpoint protein kinases, CHK1 and CHK2 phosphorylate the cell cycle protein, Cdc25, allowing it to bind to the scaffold protein, 14-3-3. This binding sequesters Cdc2 to the nucleus and prevents G2-M transition (Peng et al., 1997, Furnari et al., 1999). Whilst 14-3-3 is transcriptionally upregulated by p53, 14-3-3 can directly interact with p53 upon DNA damage to enhance its expression and activity via a positive feedback loop (Yang et al., 2003). Furthermore, 14-3-3 over-expression also

blocked MDM2-mediated p53 ubiquitination and thus 14-3-3 also regulates p53 stability (Yang et al., 2003).

1.3.4.3 p53 and autophagy

Finally, p53 also plays a role in the regulation of autophagy in cells. Autophagy is a process by which dysfunctional or old proteins and organelles are delivered to the lysosome for degradation and acts to recycle the cellular components. One way that p53 can positively regulate autophagy is through the inhibition of the mammalian Target of Rapamycin (mTOR).

AMP-activated kinase (AMPK) is a negative regulator of mTOR and is activated by p53 (Feng et al., 2007). AMPK activates a subunit of the tuberous sclerosis complex, Tuberous Sclerosis Complex 2 (TSC2) which negatively regulates mTOR (Inoki et al., 2003). Furthermore, p53 can positively regulate autophagy by transactivating pro-autophagic genes such as the damage-regulated autophagy modulator (DRAM) (Liu et al., 2013). Interestingly, the pro-apoptotic genes upregulated by p53 such as BAX, BAD and PUMA have also been shown to play a role in the upregulation of autophagy (Yee et al., 2009, Maiuri et al., 2007). Conversely, it is also believed that inactivated, cytoplasmic p53 can inhibit autophagy (Tasdemir et al., 2008).

1.4 The role of the ribosome in p53 signalling

As the rate of ribosome biogenesis dictates the proliferation rate of the cell, it is extremely important to tightly regulate this process. Many studies have shown a link between defects in ribosome biogenesis and the activation of p53 (Fumagalli and Thomas, 2011). The dysregulation of p53 has been reported in a number of diseases known as ribosomopathies.

1.4.1 Ribosomal proteins and p53 signalling

Many ribosomal proteins have been shown to bind to MDM2 including RPL23 (Jin et al., 2004), RPL26 (Ofir-Rosenfeld et al., 2008), and RPS7 (Chen et al., 2007). All ribosomal proteins, apart from RPL11, have been shown to interact with the acidic domain of MDM2 as shown in Figure 1.9.

Immunoprecipitations using over-expressed MDM2 revealed interactions between MDM2 and RPL5, RPL11 and RPL23 (Jin et al., 2004). Further to this, it was found that

RPL23 binds to MDM2 in a region distinct to that which binds RPL11, thus suggesting they do not compete with one another and may act in coordination.

It was demonstrated by Chen et al. (2007) that, using *in vitro* pull down assays in yeast, RPS7 could directly bind to MDM2 with the absence of any co-factors and that this binding occurred at amino acids 180-298 of the MDM2 protein. However, this has been debated as the binding site has also been mapped to 273-339 of MDM2 (Zhu et al., 2009). It was shown that over-expression of RPS7 in U2OS cells resulted in p53 stabilisation and activation. Furthermore, Zhu et al. (2009) showed that RPS7 was a target for MDM2 ubiquitination, whilst also being able to inhibit the E3 ligase activity of MDM2. Ubiquitination of RPS7 was demonstrated to be able to prevent MDM2-mediated degradation of p53 and promote apoptosis much more strongly than RPS7 alone. This suggested that RPS7 ubiquitination via MDM2 is an important regulator of p53.

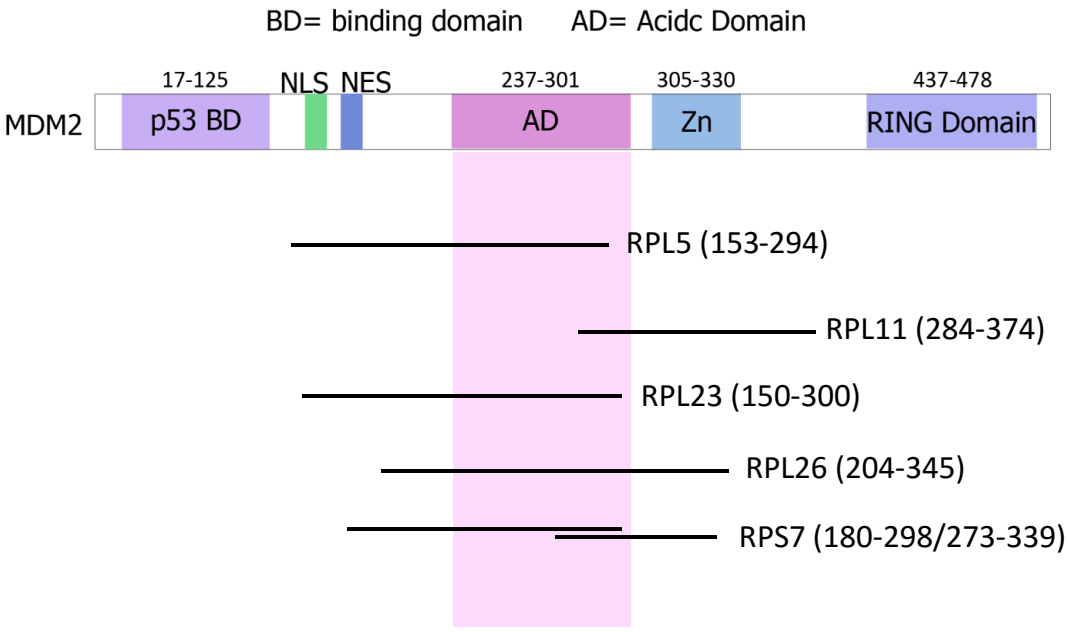

Figure 1.9 Ribosomal protein binding sites in MDM2. Schematic representation of the MDM2 protein domain structure showing the location of the p53 binding domain (p53 BD), the nuclear localisation signal (NLS), the Nuclear export signal (NES), the acidic domain (AD), the Zinc finger domain (Zn) and the RING domain. Ribosomal proteins such as RPL5, RPL23 and RPS7 have been shown to interact with the acidic domain of MDM2 whilst RPL11 interacts with the zinc finger domain. p53 binds to the p53 binding domain whilst MDMX interacts with the RING domain. Figure adapted from Kim et al. (2014).

RPL26 has been shown to function in a slightly different manner. RPL26 binds to the 5' untranslated region of p53 mRNA and is able to control p53 translation after DNA damage (Takagi et al., 2005). Furthermore, Ofir-Rosenfeld et al. (2008) established

that, using a yeast two-hybrid screen and immunoprecipitations from HEK293 cells, RPL26 interacted with MDM2. This MDM2 interaction was found to prevent RPL26 from binding to p53 mRNA and thus inhibit p53. Interestingly, it was also shown that MDM2 was able to polyubiquitinate RPL26 and target it for proteasomal degradation.

It has since been demonstrated that RPS7, RPL26, or RPL23 were not required for p53 responses after Actinomycin D (ActD) treatment but that RPL5 and RPL11 were (Bursac et al., 2012). ActD is a drug that activates p53 by selectively preventing transcriptional elongation of RNA polymerase I (Sobell, 1985) and thus induces ribosomal stress to cells without affecting 5S rRNA transcription. As RPL5 and RPL11 were crucial for this response and not the other ribosomal proteins, this suggests that it is the 5S RNP pathway that is crucial upon ribosomal stress. One reason that many ribosomal proteins interact with MDM2 is that ribosomal proteins are basic in their structure (Warner, 1977) and thus may naturally interact with the acidic domain of MDM2. This may mean that all of the ribosomal proteins seen to interact with MDM2 are not necessarily involved in p53 signalling.

1.4.2 The 5S RNP and MDM2/p53

A link between RPL5 and p53 was first established more than 20 years ago. Marechal et al. (1994) showed in 3T3DM cells, with immunoprecipitations, an interaction between RPL5, MDM2, and p53. Whilst this was an important observation, it was not yet clear what the purpose of this complex was. It soon became clear that RPL5 and RPL11 were binding partners of MDM2 that could suppress the E2 ligase activity and promote p53 activation (Dai and Lu, 2004, Lohrum et al., 2003).

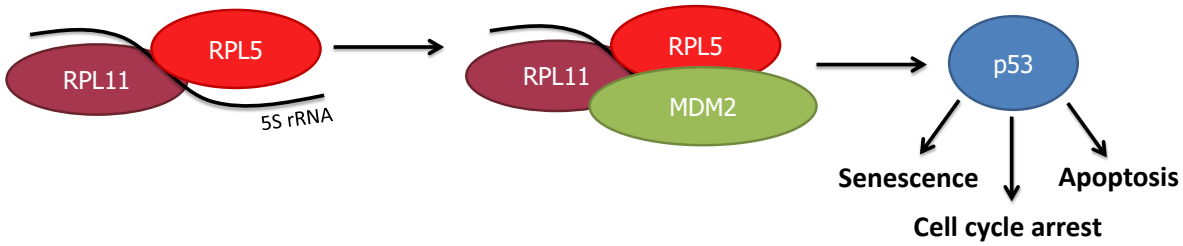

Figure 1.10 The 5S RNP interacts with MDM2 to promote p53 signalling. Schematic representation of the 5S RNP-p53 pathway. The 5S RNP is a complex of RPL11 (maroon), RPL5 (red) and the 5S rRNA (black line). Non-ribosomal 5S RNP binds to MDM2 (green), blocking its E3 ubiquitin ligase activity, activating p53 (blue).

Initial studies demonstrated that over-expression of RPL5 or RPL11 resulted in the stabilisation of p53 (Dai and Lu, 2004, Horn and Vousden, 2008), however it was also suggested that the two proteins could also work in co-ordination to activate p53 (Horn and Vousden, 2008). Furthermore, RPL11 or RPL5 depletions were able to counteract the effects on p53 of ActD treatment to cells (Bhat et al., 2004, Bursac et al., 2012, Dai and Lu, 2004). These studies suggested that RPL5 and RPL11 were important for the activation of p53 in ribosomal stress situations. However, as RPL5 and RPL11 form a sub-complex with the 5S rRNA (the 5S RNP) prior to incorporation into the ribosome (Zhang et al., 2007), it was suggested that the 5S RNP complex, rather than RPL5 and RPL11 alone, may be involved in the stabilisation of p53 (Fumagalli et al., 2012). The 5S rRNA involvement in p53 activation was shown in more recent studies by two independent groups (Donati et al., 2013, Sloan et al., 2013a). Like RPL5 and RPL11, the 5S RNA directly contacts MDM2 and depletion of the 5S RNA was able to reduce the p53 response after ActD treatment (Sloan et al., 2013a). Furthermore, these data showed that when ribosome biogenesis was blocked by depletion of ribosomal proteins or ribosome biogenesis factors, the entire 5S RNP accumulates in the nucleoplasm and binds to MDM2 in order to stabilise p53 as demonstrated in Figure 1.10. Furthermore, it has recently been demonstrated that the 5S RNP is involved in maintaining p53 homeostasis in unstressed cells (Sloan et al., 2013a).

The outcome of 5S-RNP-mediated p53 activation appears to be variable. Depletion of ribosomal proteins, apart from RPL11 and RPL5, in human cell lines results in p53-dependent cell cycle arrest (Fumagalli et al., 2012), presumably through the 5S RNP pathway. Furthermore, RPL5 overexpression in U2OS cells was shown to promote p53-dependent cell cycle arrest (Dai and Lu, 2004), however in p53 null Saos2 cells, RPL5 overexpression induces p73-mediated apoptosis (Zhou et al., 2015). SRSF1-RPL5-MDM2 interactions (described in section 1.5.2) were implicated in inducing cellular senescence (Fregoso et al., 2013). In addition, apoptosis in red blood cells, as a result of ribosome biogenesis defects and p53 activation, have been implicated in disease pathogenesis (described in section 1.6.1)(Danilova et al., 2014). More recently RPL5 and RPL11 have been shown to be involved in coordinating the regulation of nutrient stress with lipid metabolism through p53 signalling (Liu et al., 2014). All these data together show the complexity of the 5S RNP-MDM2-p53 pathway.

1.4.2.1 Interactions between the 5S RNP and MDM2

Recently, the crystal structure of RPL11 in a complex with MDM2 has been solved, showing the binding regions between the two proteins (Zheng et al., 2015). It was revealed that MDM2 binds to RPL11 in the same region that RPL11 binds to the 28S rRNA in the ribosome and that MDM2 interacts with RPL11 through an acidic domain and two zinc fingers. This supports the work by Zhang et al. (2011) who had previously demonstrated that RPL11 binds to distinct regions of the zinc finger domain of MDM2. RPL5, however, binds to the acidic domain of MDM2 (Dai and Lu, 2004).

Furthermore, it has been shown that the 5S rRNA also directly contacts MDM2 but not mutant MDM2 that can no longer interact with RPL5 or RPL11 (Sloan et al., 2013a). In addition, the interaction between the 5S rRNA and MDM2 was significantly stronger when ribosome biogenesis was disrupted. These data together demonstrate that all three components of the 5S RNP directly bind to MDM2 to co-ordinate p53 stabilisation.

1.4.3 The 5S RNP and MDMX

Despite the similarities between MDM2 and MDMX, there have been no reported interactions between MDMX and the 5S RNP complex. However, it was shown that 5S rRNA binds to MDMX via its RING domain and acts as an inhibitor of MDMX degradation by Mdm2 (Li and Gu, 2011).

p53 activation by ribosomal stress involves degradation of MDMX in an MDM2-dependent manner (Gilkes et al., 2006). RPL11-MDM2 interactions result in MDMX ubiquitination and degradation (Gilkes et al., 2006), suggesting a role for RPL11 in regulating p53 via MDMX. In light of recent findings that the 5S RNP binds to MDM2 as a whole (Donati et al., 2013, Sloan et al., 2013a), this suggests a more intricate pathway for regulation of p53 via the 5S RNP involving MDMX as summarised in Figure 1.11.

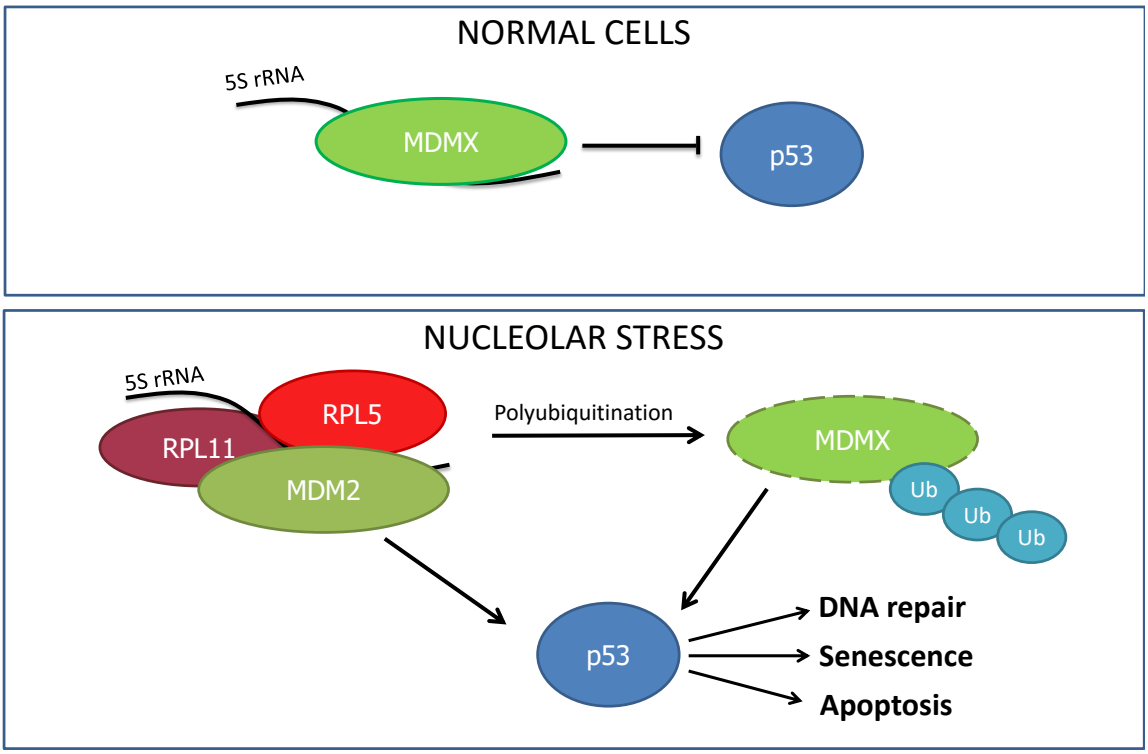

Figure 1.11 Regulation of MDMX and MDM2 via the 5S RNP. In normal cells, the 5S rRNA binds to the RING domain of MDMX and protects it from MDM2-mediated degradation. Upon nucleolar stress, the 5S RNP binds to MDM2 and prevents its inhibition of p53, thus activating p53. Furthermore, the binding of the 5S RNP to MDM2 results in the polyubiquitination of MDMX via MDM2, resulting in the degradation of MDMX. As MDMX is an inhibitor of p53, this activates p53.

1.4.4. The 5S RNP and p73

As MDM2 is also involved in the regulation of the p53 isoforms, p63 and p73 (Kadakia et al., 2001, Zeng et al., 1999), it is unsurprising that the 5S RNP has also been implicated in the activation of p73. However, no interactions between p63 and the 5S RNP have yet been described.

Zhou et al. (2015) demonstrated that RPL5 and RPL11 directly bound to p73 independently of MDM2, preventing MDM2-p73 interactions. Furthermore, the authors showed that depletion of RPL5 and RPL11 resulted in a decrease in p73 activity. As this occurs independently of MDM2, it is unclear whether this involves the 5S RNP complex or RPL5 and RPL11 proteins independently. However, given that the 5S rRNA/RPL5 complex is found in high proportions in the cell (Sloan et al., 2013a) and ribosomal proteins are generally unstable (Lam et al., 2007), these data taken together suggest a role for the 5S RNP in regulating p73.

1.4.5 The 5S RNP-p53 pathway is linked to the expression of oncogenes and tumour suppressors

The 5S RNP-p53 pathway (Figure 1.12) has also been linked to the expression of oncogenes and tumour suppressors such as c-Myc and ARF. ARF is able to down-regulate ribosome biogenesis whilst c-Myc up-regulates it (Stumpf and Ruggero, 2011).

ARF is a 14kDa protein that is induced by the expression of oncogenes (Zindy et al., 1998, Palmero et al., 1998). ARF binds to, and inhibits, MDM2, allowing for p53 activation (Honda and Yasuda, 1999). Furthermore, ARF is able to sequester MDM2 in the nucleolus and inhibits the nucleolar export of both MDM2 and p53 (Tao and Levine, 1999). The region in which RPL11 and RPL5 contact MDM2 is also a region important for ARF binding although the two binding regions do not overlap (Zhang et al., 2011) . This suggested that RPL5 and RPL11 may act in a similar way to ARF. However, ARF has since been shown to bind to RPL11 (Dai et al., 2012) and RPL11 has been shown to be important for ARF-mediated MDM2 suppression. When ARF is induced in U2OS cells, p53 and p21 are induced in a 5S RNP-dependent manner (Sloan et al., 2013a). This suggests that the entire 5S RNP, including the 5S rRNA, is important in ARF signalling to p53.

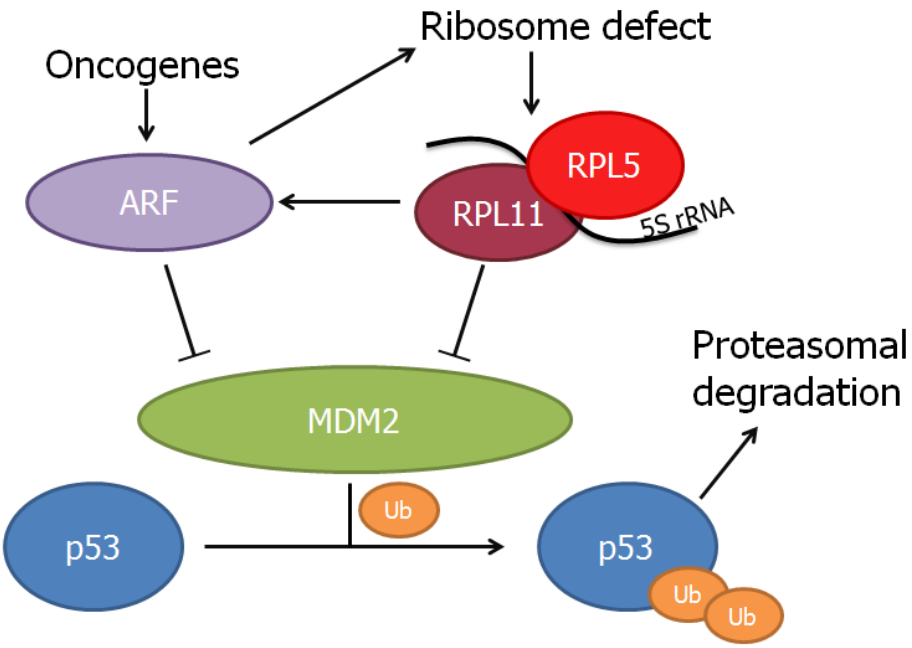

Figure 1.12 The 5S RNP-p53 pathway. A schematic representation of 5S-RNP mediated p53 regulation. When ribosome biogenesis is disrupted, the 5S RNP is not incorporated into the ribosome. It can bind to MDM2 and inhibit its E3 ubiquitin ligase activity, preventing ubiquitination of p53 and causing p53 activation. The 5S RNP can also interact with ARF in order to further inhibit MDM2.

Interestingly, the 5S RNP pathway also has links with the oncogene, c-Myc. When investigating the links between RPL11 and MDM2, one important mutation, MDM2 C305F, was identified from cancer patients, as being able to block RPL11 binding (Lindstrom et al., 2007, Zhang et al., 2011). Disruptions in ribosomal protein (RP)-MDM2 interaction accentuated the p53-mediated response to ribosomal stress and caused an increase in c-Myc induced lymphomagenesis (Macias et al., 2010). This is interesting as it suggests that the 5S RNP is able to inhibit the oncogene c-Myc and that this pathway may also be an alternative protective pathway against c-Myc oncogenesis from the canonical ARF-MDM2-p53 pathway. Interestingly, RPL11 is involved in a feed-back loop with c-Myc. c-Myc is able to upregulate the transcription of RPL11 whilst over-expression of RPL11 inhibits c-Myc induced transcription and prevents proliferation of the cell (Dai et al., 2007). More recently, it has been demonstrated that RPL5 and RPL11 are able to co-operatively inactivate c-Myc via the RNA-induced silencing complex (RISC) (Liao et al., 2014). All these data together implicate the 5S RNP in the repression of c-Myc.

1.5 Regulation of the 5S RNP-p53 pathway

Regulation of the 5S RNP-p53 signalling pathway is not yet fully understood. It has been demonstrated that 5S RNP accumulates in the nucleoplasm upon ribosomal stress and it was suggested that any proteins found to affect 5S RNP formation, localisation or integration into the ribosome may have a significant role in p53 regulation via the 5S RNP (Sloan et al., 2013a).

Several proteins have been shown to interact with either RPL5 or RPL11 and affect their ability to induce p53, but it is not yet understood how this interplays with the fact that the whole 5S RNP is required to activate p53 (Donati et al., 2013, Sloan et al., 2013a). Two proteins have been shown to inhibit p53 signalling via RPL11, PICT-1 (also known as GLTSRC2- Glioma tumour suppressor candidate region gene) and PRAS40 (the proline-rich Akt Substrate of 40kDa) whilst SRSF1 (Serine-Arginine Rich Splicing Factor 1) has been demonstrated to be required for p53 activation via RPL5.

1.5.1 PICT-1

One of the potential candidates for regulating the p53 response to ribosomal stress is PICT-1. PICT-1 is the human homologue of the yeast protein, Nop53 which is a 60S ribosome biogenesis factor (Thomson and Tollervey, 2005). Furthermore, PICT-1 is a nucleolar protein that regulates the phosphorylation and stability of Phosphatase and Tensin Homologue (PTEN) (Okahara et al., 2004). PTEN, like p53, is a tumour suppressor that is frequently altered in cancer and, when depleted in cells, promotes p53 activation (Song et al., 2012). PICT-1 itself has been linked to many different cancers such as glioblastoma (Kim et al., 2008), skin carcinomas (Kim et al., 2013), lung cancer (Okamura et al., 2014) and breast cancer (Moon et al., 2013).

Over-expression of PICT-1 has been demonstrated to induce apoptosis in a caspase and mitochondrial independent manner (Yim et al., 2007). PICT-1 is frequently located in the nucleolus but moves into the nucleoplasm in ribosomal stress situations where it is proposed to stabilise p53. PICT-1 achieves this by binding p53 but, crucially, in an ARF-independent manner (Lee et al., 2012). As ARF is often down-regulated in cancers, this could be an alternative pathway for p53 activation in tumour cells.

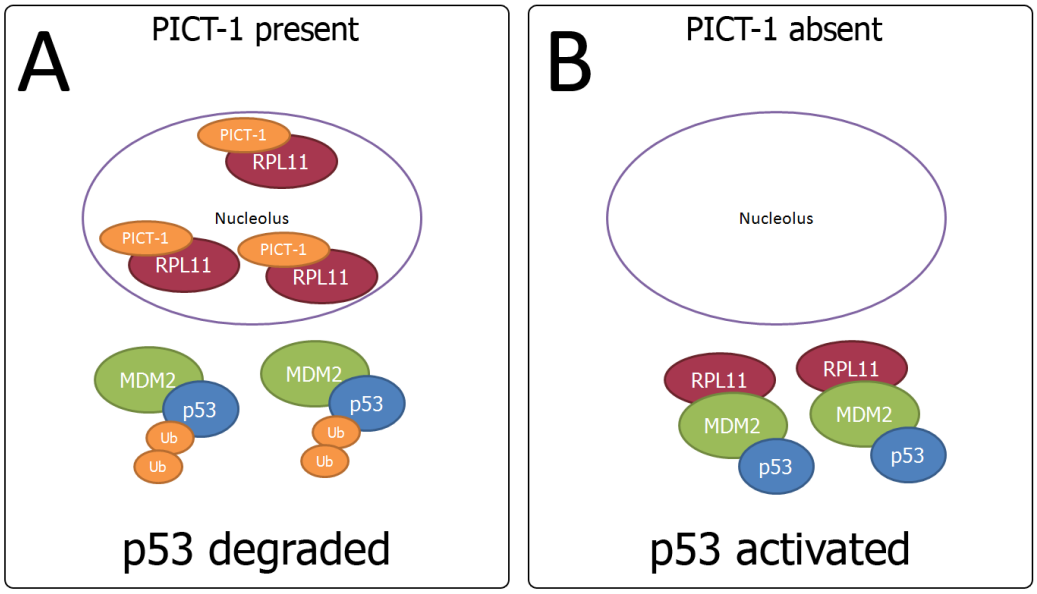

Figure 1.13 The regulation of RPL11-mediated p53 induction via PICT-1. (A) PICT-1 binds to nucleolar RPL11, preventing RPL11's exit from the nucleolus. MDM2 can polyubiquitinate p53 and promote its degradation. **(B)** In the absence of PICT-1, RPL11 can exit the nucleolus and bind to MDM2, activating p53

In contrast, Sasaki et al. (2011) showed that PICT-1 depletion in cells activated p53. PICT-1 was shown to interact with RPL5 and RPL11 but that only RPL11 depletion was

able to counter-act the p53 induction after PICT-1 depletion. This suggested that PICT-1 and RPL11 interaction was important for p53 signalling. PICT-1 depletion also caused loss of RPL11 nucleolar localisation, suggesting that PICT-1 sequesters RPL11 to the nucleolus as summarised in Figure 1.13. Furthermore, (Uchi et al., 2013) found that PICT-1 depletion in a gastric cancer cell line caused p53 activation by preventing p53 ubiquitination. Cells depleted of PICT-1 were found to arrest in the cell cycle at G1 and showed decreased cell growth and an increase in apoptosis markers, all correlating with p53 induction.

As described in Section 1.2.5, it has recently been shown that PICT-1 directly binds to the 5S rRNA and facilitates 5S RNP integration into the ribosome (Sloan et al., 2013a, Donati et al., 2013). Interestingly, Sloan et al. (2013a) also demonstrated that PICT-1 was an essential ribosome biogenesis factor. This, combined with the role of PICT-1 in integration of the 5S RNP into the ribosome may offer an explanation as to why PICT-1 was previously shown to retain RPL11 in the nucleolus.

1.5.2 SRSF1

SRSF1 (also Alternative Splicing Factor- ASF) is a 28kDa member of the SR protein family of splicing factors which are involved in constitutive and alternative splicing as well as being involved in mRNA nuclear export, mRNA translation and nonsense mediated decay, an RNA surveillance pathway to eliminate mRNAs containing premature stop codons (Long and Caceres, 2009).

SRSF1 was shown to interact with translating ribosomes when SRSF1 is hypophosphorylated in the RS domain (Sanford et al., 2004, Sanford et al., 2005) and it has since been shown that SRSF1 can enhance translation via the mammalian Target of Rapamycin (mTOR) signalling. Michlewski et al. (2008) demonstrated that SRSF1 interacts with mTOR and Protein Phosphatase 2 (PP2A), two regulators of the Eukaryotic translation initiation factor 4E binding protein (4E-BP). 4E-BP is a competitive inhibitor of cap-dependent translation (Pause et al., 1994) and thus suppression by SRSF1 via mTOR and PP2A results in increased translation. Furthermore, SRSF1 regulates alternative splicing of the ribosomal protein S6 kinase (S6K). The alternative splicing of S6K produces short isoforms which bind to mTORC1 and enhance 4E-BP phosphorylation, increasing translation initiation (Karni et al., 2007, Ben-Hur et al., 2013). Finally, SRSF1 is able to interact with the mTORC1

complex, increasing the phosphorylation of S6K and 4E-BP to enhance translation efficiency (Michlewski et al., 2008).

SRSF1 is upregulated in many cancers such as breast cancer (Anczukow et al., 2012) which could possibly be attributed to its ability to be upregulated by c-Myc (Das et al., 2012). As c-Myc is often up-regulated in cancers and may lead to apoptosis, it could potentially do this through interaction with SRSF1. Interestingly, c-Myc is also involved in up-regulating ribosome biogenesis by targeting many of the factors involved in ribosome biogenesis (Ruggero and Pandolfi, 2003).

Fregoso et al. (2013) demonstrated a role for SRSF1 in p53 signalling via RPL5. Interactions were shown between RPL5 and SRSF1, whereas there was no evidence of the involvement of RPL11 or the 5S rRNA. SRSF1 was also found to bind to MDM2 and thus it was suggested that RPL5, MDM2 and SRSF1 form a complex to activate p53.

Furthermore, it has been demonstrated that SRSF1 depletion was able to counteract the p53 induction from ActD treatment Fregoso et al. (2013). ActD, at concentrations below 10nM is able to stimulate p53 in a specifically 5S-RNP dependent manner and thus this suggested that SRSF1 was important for p53 induction via ActD. In addition, it was shown that when SRSF1 was over-expressed in human foreskin BJ-TT cells, p53 was induced in an RPL5 dependent manner. This p53 response resulted in senescence.

Donati et al. (2013) and Sloan et al. (2013a) both demonstrated that RPL5, RPL11 and the 5S RNP were all required for p53 activation in response to ribosomal stress, this raises the question as to how SRSF1 is involved. It is possible that the SRSF1/RPL5/MDM2 complex forms a distinct complex free from RPL11 and the 5S rRNA, as suggested by Fregoso et al. (2013), or that SRSF1 forms a part of the 5S-RNP mediated p53 regulation pathway. Further investigation into the role of SRSF1 is required to answer this question.

1.5.3 PRAS40

PRAS40 (also known as AKT1S1) is a protein that was first identified as an Akt substrate that, when phosphorylated by Akt, is able bind to the scaffold protein 14-3-3 to activate mTORC1 (Kovacina et al., 2003). PRAS40 is involved in signalling elicited by the presence of growth factors, as summarised in Figure 1.14.

One of the major pathways that PRAS40 is important for is the regulation of mTORC1. mTORC1 is a complex of mTOR, Raptor and Mammalian lethal with SEC13 protein 8 (mLST8) that promotes cell growth in response to growth signals (Kim et al., 2003, Kim et al., 2002, Fonseca et al., 2007). As cell growth is an important process in cells, regulation of pathways responding to growth factors is extremely important. One way that mTORC1 is regulated is via Rheb, a small GTPase that enables mTORC1 to be in the active configuration (Avruch et al., 2006). Akt promotes mTORC1 by inhibiting TSC2, a GTPase activating protein that regulates Rheb (Huang and Manning, 2009). This inhibition of TSC2 allows Rheb to remain GTP bound and activate mTORC1. mTORC1 activates many downstream pathways including translation by inhibiting the translation inhibitor 4E-BP and activating S6K (Burnett et al., 1998).

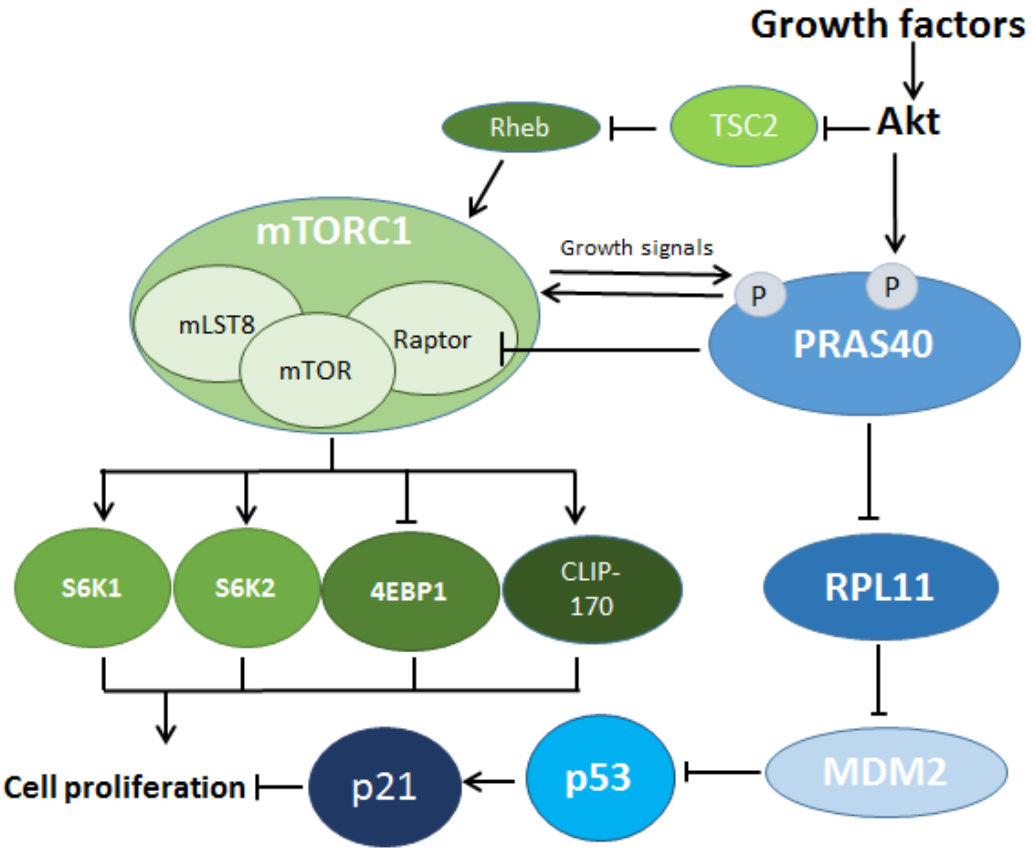

Figure 1.14. Summary of the role of PRAS40 in the Akt/mTOR and p53 pathways. Non-phosphorylated PRAS40 binds to raptor, a component of mTORC1, inhibiting the kinase activity. Upon growth factor stimulation, Akt can inhibit the action of TSC2, allowing for Rheb-mediated activation of mTORC1 leading to increased cell proliferation. Growth signals also promote the phosphorylation of PRAS40 by Akt and mTORC1, dissociating it from raptor, activating mTORC1. Phosphorylated PRAS40 can bind RPL11, inhibiting its interaction with MDM2. MDM2 polyubiquitinates p53 and targets it for degradation, therefore growth arrest is inhibited.

Another regulator of mTOR via Akt is through PRAS40. Non-phosphorylated PRAS40 binds to Raptor, a component of mTORC1, and inhibits the kinase activity of mTORC1. When growth factors are present, PRAS40 is phosphorylated by mTORC1 and Akt. This phosphorylation leads to PRAS40 dissociating from raptor and binding to the scaffold protein, 14-3-3 (Thedieck et al., 2007, Fonseca et al., 2007). This then allows for mTOR signalling to occur (Figure 1.14).

Havel et al. (2015) demonstrated, using immunoprecipitations, that nuclear PRAS40 was able to bind RPL11 and that this binding was dependent upon the phosphorylation of PRAS40 via mTORC1 and AKT at residues S221 and T246. Due to the nuclear nature of this binding, it was suggested that PRAS40-RPL11 complex does not occur within ribosomes.

Further to this, it was shown that PRAS40 depletion in the human osteosarcoma cell line, U2OS, resulted in an increase in p53 protein levels as well as an increase in its downstream targets p21 and BAX. This p53 induction upon PRAS40 depletion was RPL11 dependent. When ActD was used at levels that increase p53 via the 5S-RNP pathway in conjunction with PRAS40 depletion, PRAS40 knockdown was found to have an additive effect suggesting that the two pathways were linked. It was hypothesised that PRAS40 may inhbit RPL11-mediated p53 signalling (Figure 1.14) by blocking RPL11 translocation from the nucleoli to the nucleoplasm and thus prevents its interaction with MDM2 (Havel et al., 2014), similar to what was hypothesised for PICT-1 (Sasaki et al., 2011).

Interestingly, the complex containing RPL11 and PRAS40 was found to have a high molecular weight of between 300-700kDa and thus it was hypothesised that this could contain other unidentified proteins. Questions, therefore, arise as to whether these proteins could be the other 5S RNP components or, indeed, PICT-1 which appears to have a similar role.

1.5.4 Post-translational modifications

1.5.4.1 NEDDylation

Post-translational modifications are important regulators of proteins in Eukaryotic organisms. One such post-translational modification important in regulation of the 5S RNP is NEDDylation. NEDDylation is a process that is similar to ubiquitination (Liakopoulos et al., 1998), and involves the protein, NEDD8, which shares 60% of sequence similarity with ubiquitin (Kumar et al., 1993) and was found, initially, to target cullin proteins. E1, E2 and E3 ligases conjugate NEDD8 to lysine residues on target proteins (Jentsch and Pyrowolakis, 2000, Brown and Jackson, 2015). NEDDylation has been implicated in the regulation of many important cellular processes such as apoptosis, DNA damage responses and cell proliferation (Brown et al., 2015, Chairatvit and Ngamkitidechakul, 2007, Nagano et al., 2012).

In a study into finding targets of NEDD8, 36 ribosomal proteins were identified, including RPL11. It was discovered that in these ribosomal proteins, NEDDylation acts to stabilise the proteins and protect them from degradation and whilst RPL5 was identified in this study initially, it was found to show no evidence of NEDDylation (Xirodimas et al., 2008).. Interestingly, RPL5 has a greater level of stability than the other ribosomal proteins (Lam et al., 2007) and, therefore, may not require this level of protection from NEDDylation.

Further investigations showed a more crucial role in regulation of RPL11 via NEDDylation. Sundqvist et al. (2009) showed that, in addition to protecting RPL11 from destabilisation, NEDDylation was shown to control the localisation and mobility of RPL11 and that prolonged lack of NEDDylation leads to the decrease in RPL11 levels. Interestingly, MDM2 is able to promote NEDDylation of RPL11 and protects RPL11 from destabilisation. ActD treatment was found to decrease NEDDylation of RPL11 and cause relocalisation of RPL11 to allow for interaction with p53.

Further to this, Mahata et al. (2012) demonstrated that RPL11 and NEDD8 were both required for acetylation of K832 of p53, resulting in the activation of p53, upon ribosomal stress. It was demonstrated that RPL11 is recruited to promoter sites of p53 regulated genes upon ribosomal stress and that this requires NEDDylation by NEDD8.

This, together with the previous reports, indicated the importance of NEDDylation in p53 activation via the 5S RNP.

Zhang et al. (2014) showed that lack of NEDDylation of RPS14 reduced stability of the protein and caused mislocalisation, preventing it from interacting with MDM2 and activating p53. The NEDDylation of RPS14 could be inhibited by human coilin-interacting nuclear ATPase protein (hCINAP) which suggested that hCINAP regulated p53 signalling via RPS14. No such regulator has yet been discovered for the NEDDylation of RPL11, but if found it could have a profound impact on diseases caused by 5S RNP signalling defects.

1.5.5.2 Phosphorylation

One other important post-translational modification commonly found in cells is phosphorylation. Phosphorylation plays a key role in the regulation of p53 signalling as described in Section 1.3.4. Furthermore, MDM2 and MDMX are also phosphorylated to mediate p53 signalling. Phosphorylation of MDM2 by ATM and C-Abl inhibits p53 degradation upon DNA damage (Goldberg et al., 2002, Maya et al., 2001). Furthermore, DNA damage results in phosphorylation of MDMX via Chk1, Chk2 and ATM, activating p53 (Jin et al., 2006, Chen et al., 2005). Some ribosomal proteins such as RPS3, RPS6, RPS14 and RPL5 have been found to be phosphorylated (Ruvinsky and Meyuhas, 2006, Kim et al., 2009, Lee et al., 2010, Park and Bae, 1999, Bialik et al., 2008, Ventimiglia and Wool, 1974).

Phosphorylation of RPS6 by the ribosomal protein S6 kinase (S6K) has been shown to regulate glucose metabolism, cell size and is important for the translation of 5' terminal oligopyramidine tract (TOP) mRNAs (Ruvinsky and Meyuhas, 2006). Despite RPS6 being shown to be involved in the activation of p53 (Fumagalli et al., 2009), it has since been demonstrated that depletion of RPS6 results in 40S ribosome biogenesis defects (Fumagalli et al., 2012) and presumably this results in 5S RNP-mediated p53 activation. However, phosphorylation of RPS6 has also been shown to be present in higher levels in pancreatic cancer and mice deficient for RPS6 phosphorylation were found to have increased p53 levels (Khalaileh et al., 2013), suggesting that phosphorylation may play a role in p53 signalling via ribosomal proteins.

Several phosphorylations have been reported in RPL5 and RPL11, according to Phosphosite (http://www.phosphosite.org). RPL5 has 17 reported phosphorylation sites whilst there are 8 reported for RPL11 (Figure 1.15). Park and Bae (1999) demonstrated that RPL5 could be phosphorylated by Casein Kinase 2 (CK2), a highly conserved serine/threonine kinase that is involved in cell cycle control and DNA repair (St-Denis and Litchfield, 2009). Using the yeast two-hybrid system, Park and Bae (1999) demonstrated that RPL5 was able to interact with the β-subunit of CK2, which is responsible for substrate binding. By expressing and purifying human RPL5 in *E.coli*, phosphorylation of RPL5 was observed upon the addition of purified CK2 protein. Further analysis revealed that the phosphorylations by CK2 were exclusively serines in the regions of amino acids 142-200 and 272-297 of the RPL5 protein. Interestingly, RPL5 that had been incubated with CK2 and ATP prior to binding to the 5S rRNA showed a reduced binding efficiency for the 5S rRNA, suggesting that these phosphorylations block the formation of the 5S RNP.

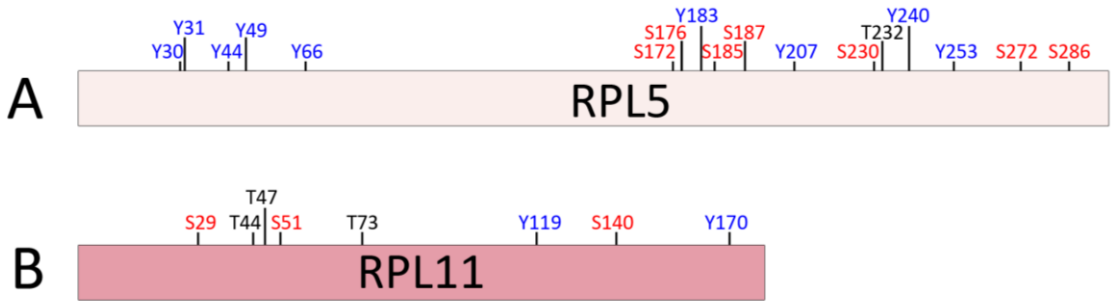

Figure 1.15 Phosphorylation sites reported in RPL5 and RPL11. (A) A schematic representation of the tyrosine (blue), serine (red) and threonine (black) phosphorylation sites reported in RPL5 on Phosphosite (http://www.phosphosite.org). **(B)** As in A, showing the phosphorylation sites reported in RPL11.

RPL5 was also shown to interact with other kinases and phosphatases although their role in the regulation of RPL5 is unclear. RPL5 was demonstrated to interact with Death-associated protein kinase (DAPk) using high throughput screening from HeLa cell extracts (Bialik et al., 2008). Whilst phosphorylations occurred when DAPk and ATP were incubated with RPL5, it was concluded that RPL5 was a weak substrate and thus was not able to be studied further. Furthermore, RPL5 has also been shown to interact with a phosphatase, Protein Phosphatase Type 1 (PPI) in yeast two-hybrid screening (Hirano et al., 1995) although it remains unclear as to the importance of this interaction. As phosphorylation plays a key role in p53 signalling (Meek, 1999,

Goldberg et al., 2002, Maya et al., 2001) (Jin et al., 2006, Chen et al., 2005), it is possible that phosphorylation of RPL5 or RPL11 may play a role in p53 regulation.

1.6 Diseases as a result of aberrant ribosome biogenesis

Defective ribosomes and, consequently, the activation of p53, can lead to diseases known as ribosomopathies which have increased cancer risk levels. The clinical features of ribosomopathies can be variable although many patients exhibit anaemia and a predisposition to cancer.

Disease	Gene defect	Cancer risk	References
Diamond Blackfan Anaemia	RPS19, RPS24, RPS17, RPL36, RPL35A, RPL5, RPL11, RPS7, RPS15, RPS27A	Osteosarcoma, MDS, AML	(Cmejla et al., 2009, Gazda et al., 2008, Delaporta et al., 2014)
5q-syndrome	RPS14	AML	(Barlow et al., 2010, Boultwood et al., 2007)
Schwachman-Diamond Syndrome	SBDS	MDS, AML	(Shammas et al., 2005, Elghetany and Alter, 2002)
Dyskeratosis Congenita	DKC1	AML, Head and neck tumours	(Zhang et al., 2012)
Treacher Collins syndrome	TCOF1	None reported	(Wise et al., 1997, Jones et al., 2008)
Autism	RPL10	None reported	(Klauck et al., 2006, Chiocchetti et al., 2011, Gong et al., 2009)
Hereditary Hypotrichosis simplex	RPL21	None reported	(Zhou et al., 2011)
X-linked microcephaly	RPL10	None reported	(Brooks et al., 2014)
X-linked syndromic intellectual disability	RPL10	None-reported	(Thevenon et al., 2015)

Table 1.1 Diseases of ribosome biogenesis dysfunction

Some examples of well-characterised ribosomopathies include Diamond Blackfan Anaemia, 5q-syndrome and Treacher Collins Syndrome but ribosomal defects not resulting in ribosomopathies have also been implicated in cancer such as acute myeloid leukaemia (AML).

1.6.1 Diamond Blackfan Anaemia

Diamond Blackfan Anaemia (DBA) is characterized by a severe reduction in erythroid precursor cells due to apoptosis or a failure to differentiate (Quarello et al., 2010b). These patients are also predisposed to cancers such as leukaemia, osteogenic sarcoma and myelodysplastic syndrome (Lipton et al., 2001). 40% of DBA patients also present with growth defects and congenital abnormalities and solid tumours (Lipton et al., 2006).

Many of the mutations found in patients with DBA have been in the genes encoding ribosomal proteins, including the ribosomal proteins RPS7, RPS19, RPS27, RPS26, RPL27, RPL5, RPL11 (Smetanina et al., 2015, Wang et al., 2015, Mirabello et al., 2014, Konno et al., 2010). 50-70% of all DBA patients present with a mutation in ribosomal proteins and 25% of all cases have mutations in RPS19 (Horos and von Lindern, 2012, Pereboom et al., 2014).

As the most common ribosomal protein defect found in DBA patients, RPS19 has been studied in detail. RPS19 knockdowns lead to a block in ribosome biogenesis and activation of p53 through the 5S RNP (Choesmel et al., 2007, Flygare et al., 2007). It has also been demonstrated that disruption of ribosomal proteins is associated with decreased translation in many different cell types (Gazda et al., 2006, Avondo et al., 2009), which may account for why the symptoms of the disease can be so variable.

Mutations in RPL5 and RPL11 are associated with distinct symptoms in DBA (Gazda et al., 2008, Quarello et al., 2010b). RPL5 mutations have been linked to cleft palate whilst RPL11 has been linked to thumb abnormalities (Gazda et al., 2008). In one study, half of all of the patients with RPL5 mutations were found to have multiple severe abnormalities, whereas patients with RPL11 or RPS19 mutations were rarely found to have such severe symptoms (Gazda et al., 2008).

The pathogenesis of DBA was originally thought to be due to the fact that red blood cell precursors proliferate rapidly (Lajtha and Oliver, 1961) and thus require rapid production of ribosomes during their proliferation and differentiation. Therefore, if there is a reduction in ribosome synthesis, this will affect the erythroid precursors quite drastically (Zhang et al., 2011). However, since defects in ribosomal proteins or ribosome biogenesis factors result in p53 activation (Sloan et al., 2013a), it has also

been proposed that these defects are due to accumulation of p53 and thus cell-cycle arrest and apoptosis in these erythroid cells (Dai and Lu, 2004, Danilova et al., 2014). Interestingly, Kummalue et al. (2015) found that RPL11 expression in erythroleukaemic cells caused activation of STAT5 and resulted in the cells being able to proliferate in the absence of erythropoietin, a growth factor involved in red blood cell development (Ridley et al., 1994). Signal Transducer and Activator of Transcription 5 (STAT5) is part of the STAT family of proteins and acts as promoter of angiogenesis (Yang and Friedl, 2015) and, subsequently, is associated with a range of cancers (Tan and Nevalainen, 2008). This suggested that RPL11 is able to increase erythroid cell proliferation and may give some explanation as to why deficiencies in ribosomal proteins often result in blood disorders.

There is need for a greater understanding of the pathogenesis of DBA as there are few effective treatments available apart from blood transfusions and bone marrow transplants. One current treatment for DBA is treatment with glucocorticoids (Vlachos et al., 2008). Loss of the glucocorticoid receptor in mice resulted in anaemia (Bauer et al., 1999) and p53 was able to block glucocorticoid-induced proliferation in erythroid precursors, hinting that p53 may play a pivotal role in the effectiveness of this treatment in DBA patients. Recently, Sjogren et al. (2015) showed that in RPS19 deficient mice, glucocorticoid treatment counteracted p53 activation and supported the idea that glucocorticoids act in DBA to dampen down the p53 response and promote proliferation in erythroid cells.

One other promising treatment of DBA is treatment with L-leucine. As DBA results in translational defects (Cmejlova et al., 2006) and L-leucine has been shown to regulate translation through mTOR (Kimball and Jefferson, 2006), L-Leucine was trialled as a potential therapeutic for DBA. Initial tests in zebrafish and mouse models of DBA show improvement in their anaemia upon L-leucine treatment (Boultwood et al., 2013, Jaako et al., 2012, Payne et al., 2012). Furthermore, it was shown in a mouse model for DBA that L-leucine treatment reduced p53 levels (Jaako et al., 2012). In patient trials, it appeared to be an effective treatment and within 6 months the patient tested became transfusion independent and showed increased erythroid differentiation (Pospisilova et al., 2007). This is interesting as leucine is known to trigger the mTOR pathway which has recently been closely linked to the 5S RNP via PRAS40 (Havel et al., 2014).

Investigation of this link may further the understanding of the regulation of the 5S RNP and make treatment of DBA more effective.

1.6.2 5q-syndrome

5q-syndrome is a myelodysplastic syndrome that shares many similarities with DBA such as anaemia, decreased erythroid precursors and a predisposition to AML (Vardiman et al., 2002). Like DBA, 5q syndrome has also been linked with ribosome defects as cells from the bone marrow of patients with 5q syndrome showed rRNA processing defects (Ebert et al., 2008).

5q-syndrome is a very distinct form of myelodysplastic syndrome characterised by deletion of the long arm of chromosome 5 (Van den Berghe et al., 1974). One of the proteins affected by this deletion is RPS14. Boultwood et al. (2007) detected haploinsufficiency of RPS14 in a group of patients with 5q syndrome using micro-array based gene expression profiling of CD34+ bone marrow cells from 5q syndrome patients compared to healthy individuals. This suggested that RPS14 may be an important gene in the pathogenesis of 5q syndrome. Subsequently, depletion of RPS14 in CD34++ haematopoietic stem cells was shown to mirror the defects seen in 5q-syndrome, which could be rescued by over-expression of RPS14 (Ebert et al., 2008). This was supported by the evidence of a mouse model for the disease, whereby RPS14 is disrupted (Barlow et al., 2010).

An effective treatment for 5q-syndrome is the drug Lenalidomide (Messingerova et al., 2015, Wei et al., 2013). Lenalidomide is commonly used as a treatment for multiple myeloma and is known to promote erythroid differentiation (Narla et al., 2011) as well as promoting degradation of p53 (Wei et al., 2013). Lenalidomide has been shown to inhibit two phosphatases involved in G2-M checkpoint control, Protein Phosphatase 2A catalytic subunit α (PP2Acα) and Cell division cycle 25 (CDC25C), which are located on 5q and deleted in many patients with 5q-syndrome (Wei et al., 2009). The ability of Lenalidomide to suppress p53 and specifically target phosphatases already downregulated in 5q syndrome (PP2Acα and CDC25C) explains the selective toxicity seen in just the 5q deleted cells. However, Tehranchi et al. (2010) reviewed 7 patients with 5q syndrome that had responded well to Lenalidomide treatment and found that most patients developed Lenalidomide resistance over time and relapsed. This suggested that new therapies or a combination of therapies were needed.

Similar to Diamond Blackfan Anaemia, treatment with L-leucine has had some promising results in the treatment of 5q-syndrome. In CD34+ cells with RPS14 depletion and cells from 5q syndrome patients, L-leucine treatment resulted in increased differentiation and proliferation of the cells (Yip et al., 2012). Furthermore, zebrafish models of 5q syndrome showed improvement in red blood cell count after treatment with L-leucine (Payne et al., 2012). However, whilst L-leucine treatment has initially proven to be successful in DBA patient trials, its efficacy in treating 5q syndrome in human trials seems less successful. Steensma and Ebert (2013) treated three 5q syndrome patients with L-leucine for 3 months and found that whilst there were no adverse effects, their symptoms were not alleviated. In fact, two of the patients only found relief from blood transfusions after Lenalidomide treatment. Lenalidomide, as previously explained, has some adverse effects and patients taking the drug often relapse. Therefore, it is important that the role of the 5S RNP-signalling, which may provide the crucial link between L-leucine and ribosomopathies, is better understood.

1.6.3 Treacher Collins Syndrome

Treacher Collins syndrome (TCS) is a rare autosomal dominant disease, affecting around 1 in 50,000 births (Fazen et al., 1967, Rovin et al., 1964), which is characterised by aberrant neural crest cell development (Ross and Zarbalis, 2014) resulting in craniofacial defects. Neural crest cells are the cells that give rise to cartilage, nose, and connective tissue in the face and head. They are almost stem-cell like in nature due to their ability to differentiate into a range of different cell types and their self-renewal properties (Ross and Zarbalis, 2014). There are a wide range of developmental abnormalities seen in TCS patients, including alterations to the size, shape and position of the external ears, dental malocclusion, cleft palate and ophthalmic abnormalities (Trainor et al., 2009).

The most common defect found in TCS patients is in the gene Treacher-Collins-Franceschetti syndrome 1 (TCOF1) which encodes for a protein called treacle (Wise et al., 1997). Treacle is a nucleolar phosphoprotein that is involved in rRNA transcription through interactions with the upstream binding factor, UBF and RNA polymerase I (Valdez et al., 2004). Mutations in TCOF1 have been shown to lead to ribosome biogenesis defects, p53 stabilisation and thus cell cycle arrest at G1 (Jones et al., 2008).

Interestingly, mutations have also been found in genes that encode for subunits of the RNA polymerases I and III. Dauwerse et al. (2011) found that patients deficient for TCOF1 mutations often had mutations in the *POLR1D* gene that encodes for a subunit for RNA polymerases I and III. The 5S rRNA is transcribed by RNA polymerase III and the other rRNAs are transcribed as a single precursor by RNA polymerase I, suggesting that all rRNA synthesis is affected in these patients and not just the 28S, 5.8S and 18S as with TCOF1 mutations.

Unlike DBA and 5q syndrome, Treacher Collins syndrome does not present with blood related symptoms or cancer predisposition. The main treatments for TCS have been surgical in order to reconstruct the facial abnormalities (Trainor et al., 2009) and may result in several surgeries throughout the patient's life. A greater understanding of the pathogenesis of ribosomopathies in general may help to lead to more treatment options in the future.

1.6.4 Cancers

As ribosome biogenesis defects result in aberrant p53 signalling, it is not surprising that ribosome biogenesis defects have been linked to cancer as p53 is mutated or lost in around half of cancers (Vogelstein et al., 2000).

Both Diamond Blackfan Anaemia and 5q-syndrome result in a predisposition to cancers such as leukaemia (Khanna-Gupta, 2013). Furthermore, mutations in both RPL5 and RPL10 were described in patients with T-cell acute lymphoblastic leukaemia (T-ALL). Keersmaecker et al. (2013) described a common mutation, R98S, in RPL10 in five patients. Expressing these mutants in yeast resulted in ribosome biogenesis defects and a decrease in proliferation, suggesting a role for RPL10 in ribosome biogenesis. This raised the question as to why this mutation that decreases cellular proliferation could result in cancer which is characterised by an overgrowth of cells. Further investigation into this mutation revealed that the mutation resulted in a late-stage 60S ribosome biogenesis defect resulting in a decrease in proliferation, especially in rapidly dividing cells such as blood cell precursors (Sulima et al., 2014). However, after time, it was seen that the cells were subjected to selective pressure, favouring cells that harboured mutations in proteins involved in ribosome quality control. This resulted in the rapid proliferation of cells with defective ribosomes without quality control mechanisms in place. This research was pivotal in understanding how ribosomopathies

result in cancer and may lead to prevention of cancer in ribosomopathy patients in the future.

Interestingly, mutations in RPL22 have also been described in T cell acute lymphoblastic leukaemia (T-ALL) (Rao et al., 2012). Loss of RPL22 was shown to accelerate the development of lymphoma in a mouse model, suggesting a link between RPL22 and transformation of cells. However, the emergence of a role of ribosomal proteins in cancer is fairly recent and thus very little is currently known about whether targeting these proteins could act as a potential therapeutic.

1.6.4.1 The nucleolus and cancer

Large and abnormal nucleoli were observed very early on in cancer cells by the Italian pathologist Giuseppe Pianese (Pianese, 1896) and since then a strong correlation has been made between nucleolar morphology and cancer (Figure 1.16). In some cases, nucleolar size is used as a marker for clinical outcome- with larger nucleoli indicating a poor prognosis (Derenzini et al., 1986, Derenzini et al., 1989). Furthermore, changes in nucleolar morphology help to indicate a patient's response to chemotherapeutic drugs (Derenzini et al., 1998).

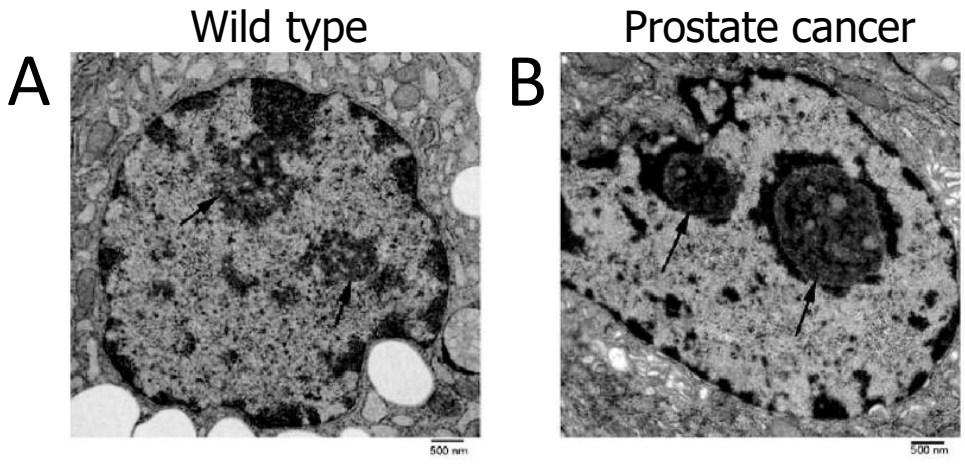

Figure 1.16 Nuclear morphology in cancer. Electron micrographs showing changes in nucleolar morphology in wild type mouse prostate cells **(A)** or in a mouse model for prostate cancer **(B)**. Arrows indicate the nucleoli. Adapted from (Koh et al., 2011).

One nucleolar protein, NPM (nucleophosmin/B23, encoded by the gene *NPM1*) is mutated in several different cancers, including gastric, colon, ovarian and prostate carcinomas (Tanaka et al., 1992, Nozawa et al., 1996, Shields et al., 1997, Subong et al., 1999), Anaplastic Lymphoma Kinase (ALK)+ Anaplastic Large Cell Lymphoma (ALCL),

(Morris et al., 1995) and AML (Falini et al., 2005). NPM has been shown to be involved in ribosome biogenesis, the nucleolar export of the RPL5/5S rRNA complex, protein chaperoning and centrosome duplications (Yu et al., 2006, Feuerstein et al., 1988, Kondo et al., 1997, Okuda et al., 2000, Okuwaki et al., 2001). Furthermore, NPM is involved in p53 signalling. ARF and NPM co-localise to the nucleolus (Gallagher et al., 2006) where NPM protects ARF from degradation (Colombo et al., 2005) and maintains basal ARF levels in the nucleolus (Sherr, 2006). Under cellular stress conditions, NPM and ARF exit the nucleoli and MDM2 and NPM compete for ARF binding. The formation of ARF-MDM2 and NPM-MDM2 results in the activation of p53 (Lee et al., 2005, Kurki et al., 2004). In addition, NPM is able to directly bind to p53 to promote p53 stabilisation and activation (Colombo et al., 2002).

NPM1 mutations exist in around 30% of adult AML cases leading to an increase in mutant cytoplasmic NPM (Falini et al., 2005, Falini et al., 2007b). In a mouse model, overexpression of cytoplasmic mutant NPM resulted in a proliferative advantage of myeloid lineage cells (Cheng et al., 2010), whereas in *D. rerio*, expression of the cytoplasmic mutant NPM resulted in the expansion of hematopoietic cells (Bolli et al., 2010), suggesting that, in AML, *NPM1* mutations contribute to the pathogenesis of the disorder. In 85% of ALK+ ALCL cases, a translocation exists between the *ALK* and *NPM1* genes causing constitutive expression of a the NPM-ALK fusion kinase (Falini et al., 2007a, Morris et al., 1995).

One other nucleolar protein thought to be involved in the activation of p53 is Myb-binding protein 1a (MYBBP1A), a predominantly nucleolar transcription factor involved in p53 acetylation and stabilisation (Kumazawa et al., 2015, Ono et al., 2014). MYBBP1A translocates to the nucleoplasm during ribosomal stress to activate p53 in an RPL5 and RPL11 dependent manner (Kuroda et al., 2011, Ono et al., 2014). This, therefore, suggests a link between the 5S RNP and MYBBP1A.

1.6.5 Other diseases as a result of aberrant ribosome biogenesis

Ribosomal proteins have also been implicated in other diseases such as Schwachman-Diamond Syndrome, Dyskeratosis congenita (DC), Parkinson's disease, Autism spectrum disorder, X linked microcephaly, X-linked syndromic intellectual disability and hereditary hypotrichosis simplex.

Schwachman-Diamond Syndrome is a rare disorder characterised by haematological abnormalities, exocrine pancreatic insufficiency, decreased neutrophils in the blood, and an increased cancer risk (Myers et al., 2013). Approximately 90% of Schwachman-Diamond Syndrome patients have mutations in the Shwachman-Bodian-Diamond syndrome (SBDS) gene. The SBDS protein, which binds to NPM, has been implicated in ribosome biogenesis (Austin et al., 2005, Ganapathi et al., 2007, Finch et al., 2011), suggesting that ribosome biogenesis plays a role in the pathogenesis of the disease. Furthermore, aberrant p53 expression has been demonstrated in Schwachman-Diamond Syndrome patients (Elghetany and Alter, 2002) although no links to the 5S RNP have yet been made.

One other ribosomopathy is DC which is a highly heterogeneous disease characterised by bone marrow failure, predispositions to cancer, reticular skin pigmentation and immune deficiencies (Alter et al., 2009, Mason and Bessler, 2011).There are many different modes of inheritance of DC, the X-linked form of DC is linked to rRNA processing defects and arises from mutations in the DKC1 gene (Heiss et al., 1998, Knight et al., 1998) whilst autosomal dominant DC is linked to defects in the telomerase, TERT (Vulliamy et al., 2001, Basel-Vanagaite et al., 2008). The DKC1 gene encodes dyskerin, a component of the telomerase complex (Brault et al., 2013) and part of the H/ACA snoRNP complex involved in pseudouridylation. The p53 status in DC remains unclear. Mouse models for DC have shown reductions in p53 levels (Bellodi et al., 2010), whereas studies in *D. rerio* have shown that p53 is activated when dyskerin levels are reduced (Ying Zhang et al., 2012). There is much debate about the pathogenesis of DC and it remains unclear whether DC is caused by defects in telomere maintenance or ribosome biogenesis due to the different complexes that dyskerin is involved in. However, due to the phenotypic similarities between DC and ribosomopathies such as DBA and 5q-syndrome, it is likely that ribosome biogenesis defects play a role in the pathogenesis of DC.

Although no ribosome defects have not been reported in Parkinson's disease directly, altered RPS6 expression has been reported in brains of patients with Parkinson's disease (Papapetropoulos et al., 2006). Furthermore, it was shown that the bacterial homologue of the parkinsonism-associated protein DJ-1 was related to translation

defects and resulted in the aggregation of many ribosomal proteins (Kthiri et al., 2010), suggesting a link between ribosomes and Parkinson's disease.

Finally, mutations in the large subunit ribosomal protein, RPL10, have been linked to several neuronal diseases such as autism (Klauck et al., 2006, Chiocchetti et al., 2011, Gong et al., 2009), X-linked microcephaly (Brooks et al., 2014), and X-linked syndromic Intellectual Disability (Thevenon et al., 2015). Furthermore, RPL21 mutations have been reported in hereditary hypotrichosis simplex (Zhou et al., 2011). However, the link to p53 in these diseases has not been characterised.

1.7 Project aims and objectives

The links between the ribosomal proteins and MDM2 have been established for some time. However, it is only recently that it has been elucidated that the 5S RNP-MDM2 interaction is crucial for p53 activation when ribosome biogenesis is disrupted (Donati et al., 2013, Sloan et al., 2013a). Furthermore, it has been shown that the 5S RNP is involved in maintaining normal p53 homeostasis (Sloan et al., 2013a). Ribosome biogenesis defects and subsequent p53 activation has been implicated in a number of diseases known as ribosomopathies (Fumagalli and Thomas, 2011) and, therefore, understanding how the 5S RNP signalling pathway is regulated may lead to novel treatments for these diseases.

As only non-ribosomal 5S RNP can activate p53, integration into the ribosome is a crucial step in the regulation of the 5S RNP. In yeast it is known that Rrs1 and Rpf2 are required for integration of the 5S RNP into the ribosome (Kharde et al., 2015, Morita et al., 2002, Zhang et al., 2007). It has been shown that the 5S RNP docks onto the 25S/28S rRNA and is subsequently rotated 180^{o} in order to form the central protuberance (Leidig et al., 2014). However, it has been recently demonstrated that 5S RNP recruitment may differ in human ribosomes as the human Rpf2/Rrs1 homologues BXDC1/RRS1 do not interact with the 5S RNP outside of pre-ribosomes and were shown to be less important in 5S RNP recruitment (Sloan et al., 2013a). Therefore, it is important to further explore the factors involved in 5S RNP recruitment as it appears to differ between humans and yeast.

One way that the 5S RNP recruitment may be regulated is through post-translational modifications. Post-translational modifications such as ubiquitination, acetylation and

phosphorylation are common in p53 signalling and thus it follows that the 5S RNP may be modified in a similar way. Indeed, RPL11 has been shown to be NEDDylated by MDM2 to protect it from degradation. Furthermore, RPL11 and RPL5 both have multiple phosphorylation sites which may affect 5S RNP-mediated p53 signalling by blocking 5S RNP incorporation into the ribosome.

Finally, two proteins, SRSF1 and PRAS40, were shown to interact with the 5S RNP components to regulate p53 signalling. In light of the recent findings that the 5S RNP is required to activate p53 (Donati et al., 2013, Sloan et al., 2013a), it is possible that these proteins regulate the 5S RNP-mediated p53 pathway. Interestingly, SRSF1 and PRAS40 are both involved with the regulation of the mTOR signalling pathway. One of the successful treatments for the DBA, has been leucine treatment which activates mTOR and thus investigation of SRSF1 and PRAS40 may help to uncover the mechanism by which leucine treatment is successful.

Therefore, this project aimed to:

- Further elucidate the factors involved in 5S RNP recruitment into the ribosome in human cells.
- Determine the role of RPL5 phosphorylation in 5S RNP-mediated p53 signalling.
- Evaluate the role of SRSF1 and PRAS40 in the regulation of p53 signalling via the 5S RNP.

CHAPTER TWO: METHODS

2.1 Reagents

All reagents for buffers were purchased from Sigma Aldrich unless stated otherwise.

2.2 DNA methods

2.2.1 Plasmid purification

Minipreps were carried out using the Promega Wizard® Plus SV Minipreps DNA Purification System according to the manufacturer's instructions and eluted in 50µl H_2O.

2.2.2 PCR

2.2.2.1 PCR and cloning

PCR reactions were used to amplify target sequences from vectors or cDNA. All PCR reactions were carried out using Phusion Polymerase (New England Biolabs) to create blunt-ended PCR products.

Gene	Direction	Restriction site	Sequence
PRAS40	Forward	BamHI	GATCGGATCCATGGCGTCGGGGCGCCCCGAGG
	Reverse	XhoI	GATCCTCGAGTCAATATTTCCGCTTCAGCTTCTGG
SRSF1	Forward	BamHI	GATCGGATCCATGTCGGGAGGTGGTGTGATTCGTGG
	Reverse	XhoI	GATCCTCGAGTTATGTACGAGAGCGAGATCTGC

Table 2.1 Primers used for cloning.

Reactions were carried out using the primers in Table 2.1 using the explained reaction conditions (Table 2.2) using the detailed thermal cycling conditions as per the manufacturer's protocol.

Volume	Component
10 µl	5X Phusion HF Buffer
1 µl	10 mM dNTPs
0.5 µl	100 µM Forward Primer
0.5 µl	100 µM Reverse Primer
1 µl	200 ng/µl Template
0.5 µl	Phusion Polymerase
36.5 µl	Nuclease free water

Table 2.2. The PCR conditions used for cloning.

The PCR products were then cloned into the CloneJET PCR Cloning Kit (Life Technologies) followed by restriction digests to extract the clone of interest for further cloning into the desired vectors.

2.2.2.2 RT-PCR

RNA was treated with Turbo DNAse (Life technologies) for 1 hour at 37^{o}C using the appropriate buffer. 10 pg -500 ng RNA was added to 200µM dNTPs, 2nM of oligo dT primer in order to extend all of the poly-A tailed mRNA sequences. This reaction was heated to 65^{o}C for 5 minutes to anneal the primer (Table 2.3) before placing on ice.

Gene	Primer direction	Sequence
RPL7	Forward	GGTTCCTGCTGTGCCAGAAAC
	Reverse	CCTCAGCATGTTAATCGAAGCC
RPL10	Forward	CAAGCCGTACCCAAAGTCTCG
	Reverse	CTCCTTGTTCTGCAGCTTGGTG
RPL21	Forward	GGAAAGAGGAGAGGCACCCG
	Reverse	GCTGGCGCTTTAGTTGAACC
RPL18	Forward	CTACCTGAGGCTGTTGGTCAAG
	Reverse	CGAAATGCCGGTACACCTCTC

Table 2.3 List of primers used for RT-PCR

The extension of the primer was then performed by adding a final concentration of: 1X First strand buffer (Life technologies), 5mM DTT, 200U SuperScript III reverse transcriptase (Life Technologies) and 30U Recombinant RNasin ribonuclease inhibitor (Promega). The reaction was incubated at 55^{o}C for 1 hour followed by 70^{o}C for 15 minutes to inactivate the enzymes. The PCR reactions were carried out using Go Taq G2 (Promega) with the conditions outlined in the manufacturers protocol using the thermal cycling conditions detailed in Table 2.4.

Step	Temperature (^{o}c)	Time
Initial Denaturation	95	5 minutes
25-35 cycles	95 58-70 72	1 minute 1 minute 1 minute per kb
Final Extension	72	5 minutes
Hold	4	

Table 2.4. The PCR thermal cycling conditions for Go Taq G2 polymerase.

2.2.2.3 Site directed mutagenesis

Stratagene Quikchange site directed mutagenesis kit was utilised to mutate sites in plasmids. The reactions were set up as follows: 1X Pfu buffer, 200 ng DNA, 100 ng of

each primer, 200 nM dNTPs and 2.5U PFU Turbo (Stratagene) made up to 100 µl with water. The PCR reactions conditions were as per the manufacturer's protocol. The resultant PCR products were digested with 1U DpnI (New England Biosciences) for 1 hour at 37^{o}C to digest the parental strand as DpnI digests only methylated DNA. 5 µl was then transformed into DH5α competent cells. The list of primers used is shown in Table 2.5.

Gene	Mutation desired	Primer direction	Sequence
RPL5	Y30F, Y31F	Forward	CGAGAGGGTAAAACTGATTTTTTTGCTCGGAAACGCTTGG
		Reverse	CCAAGCGTTTCCGAGCAAAAAAATCAGTTTTACCCTCTCG
	Y31F	Forward	CGAGAGGGTAAAACTGATTATTTTGCTCGGAAACGCTTGG
		Reverse	CCAAGCGTTTCCGAGCAAAATAATCAGTTTTACCCTCTCG
	Y30F	Forward	CGAGAGGGTAAAACTGATTTTTATGCTCGGAAACGCTTGG
		Reverse	CCAAGCGTTTCCGAGCATAAAAATCAGTTTTACCCTCTCG
	Y30E	Forward	CGAGAGGGTAAAACTGATGAGTATGCTCGGAAACGCTTGG
		Reverse	CCAAGCGTTTCCGAGCATACTCATCAGTTTTACCCTCTCG
	Y44F	Forward	GGTGATACAAGATAAAAATAAATTCAACACACCCAAATACAGG
		Reverse	CCTGTATTTGGGTGTGTTGAATTTATTTTTATCTTGTATCACC
	Y44E	Forward	GGTGATACAAGATAAAAATAAAGAGAACACACCCAAATACAGG
		Reverse	CCTGTATTTGGGTGTGTTCTCTTTATTTTTATCTTGTATCACC

Table 2.5 List of primers used in mutagenesis PCR

2.2.3 Sequencing

To ensure that the site directed mutagenesis and cloning had been successful, restriction digests and agarose gel electrophoresis were used to check size of inserts. This was followed by sequencing by GATC biotech using the appropriate primers to check for correct sequences

2.2.4 Agarose gel electrophoresis

Samples were diluted in 6X DNA loading dye (0.4 % orange G, 0.03 % bromophenol blue, 0.03 % xylene cyanol FF, 15 % Ficoll400, 10 mM Tris-HCl pH 7.5, 50 mM EDTA pH 8.0)) to a final 1X concentration. Typically, 2% agarose (w/v)-TBE (TRIS/Borate/EDTA: 90 mM Tris-HCl, 90 mM Boric acid, 2 mM EDTA, pH 8.0) gels were combined with 1X SYBRSafe (Invitrogen) and run in 1 X TBE buffer at 125v for 30 minutes. Gels were visualised using the SYBRSafe fluorescence setting on the Typhoon FLA 7000 (GE healthcare) or with U.V on the Biorad 1000 transilluminator.

2.2.5 Restriction digests

0.5-2 µg DNA was digested with the 10U of enzymes (Promega) and the manufacturers recommended buffer to a final volume of 20 µl for 1hr at 37°C.

2.2.6 Ligation

PCR products were digested with the appropriate restriction enzymes before the addition of the vector in a 5:1 molar ratio. 0.3U T4 DNA ligase (Promega) and 1X T4 DNA ligase buffer (Promega) were added to a final volume of 10 µl. The reactions were carried out at 18°C for 16 hours.

2.2.7 Transformation into *E.coli*

For the pcDNA5 constructs and cloning, competent DH5α cells were used. RPL5 and RPL11 pGEXgp1 plasmids were kindly provided by Katherine Sloan (Watkins Lab). 50 ng plasmid DNA or 5 µl ligation mixture was added to 50 µl competent cells and incubated on ice for 30 minutes. The mixture was then heat shocked at 42°C for 1 minute before being immediately placed back on ice for 5 minutes. 1 ml Lysogeny Broth (LB) was added and the cells incubated at 37°C for 1hr 30 with gentle agitation. The bacteria was then plated on LB-agar plates containing the relevant selection antibiotic and grown at 37°C overnight. Single colonies were selected and added to 5 ml LB with the relevant antibiotics and rotated overnight ready for plasmid purification.

For the *E.coli* expression vectors, BL21 PlysS cells were utilised to allow high efficiency gene expression whilst maintaining a low level of basal expression. The BL21 PlysS cells were grown overnight on antibiotic-free agar plates. 2-4 colonies of BL21 PlysS cells were resuspended in 120 µl 50mM sterile $CaCl_2$ before the addition of 1 µg of the plasmid and incubation on ice for 15 minutes. The cells were heat shocked at 42°C for 90 seconds before placing back on ice. 120 µl LB was added and incubated at 37°C for 5 minutes with gentle agitation before plating on selective plates.

2.3 RNA methods

2.3.1 RNA extractions

2.3.1.1 Phenol-Chloroform extractions

Phenol:chloroform:isoamylalcohol (25:24:1) solution was added to at a 1:1 ratio with the sample and then vortexed for 1 minute before centrifugation for 5 minutes at 13,000rpm. The upper aqueous phase was transferred to a fresh tube and combined

with NaOAc pH 5.3 was added to a final concentration of 150 mM along with 1 ml ethanol. 50 µg/ml glycogen blue (Ambion) was added to help with the precipitation of the RNA before storage at 20°C overnight. The ethanol was removed by centrifugation for 20 minutes at 13,000 rpm and removing the liquid. The pellet was air-dried and resuspended in 10 µl H_2O.

2.3.1.2 TRI-Reagent extractions

Cell pellets were resuspended in 500 µl TRI-reagent and incubated at room temperature for 5 minutes. 100 µl chloroform was added before shaking vigorously for 15 seconds and then incubated for 2 minutes at room temperature and centrifuged at 13,000rpm for 15 minutes. The upper aqueous phase was separated and combined with 250 µl isopropanol and incubated for 5 minutes at room temperature before centrifugation at 13,000 rpm for 10 minutes to pellet the RNA. The RNA pellets were washed in 500 µl 75% ethanol (v/v) and centrifuged as before for 5 minutes. The ethanol was then removed and the pellet resuspended in 10 µl H_2O prior to being heated for 10 minutes at 55°C.

2.3.2 Northern blot methods and probes

2.3.2.1 Acrylamide Gels for 5S and 5.8S rRNAs

Samples were combined with 2x RNA loading dye (80% formamide, 10 mM EDTA [pH 8.0], 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue) in a 1:1 ratio and separated by electrophoresis using an 8% acrylamide, 7M urea gel in 1x TBE buffer at 350V for 1-2 hours. RNAs were transferred from the gel onto Hybond N membrane (Amersham Biosciences) using a Trans-Blot ® Cell (BIO-RAD) in 0.5x TBE buffer at 65V for 1.5 hours. RNA was cross-linked to the membrane using a Stratalinker UV crosslinker (Stratagene) using the auto-crosslinking setting of 120,000 microjoules/cm^2.

2.3.2.2 Glyoxal Gels for larger RNAs

Glyoxal Loading Buffer (61.2% DMSO (v/v), 20.4% glyoxal (v/v), 12.2% 1x BPTE buffer [30 mM Bis-Tris free acid [pH 7.0], 10 mM PIPES free acid, 1 mM EDTA], 4.8% glycerol (v/v), and 0.02 mg/ml ethidium bromide) was added to RNA samples at a 5:1 ratio. The samples were heated at 55°C for 1 hour.

The samples were loaded onto a gel containing 1.2% agarose / 1x BPTE and run in 1x BPTE for 3 hr 30 at 185 volts. The bands were then visualised using a U.V transilluminator (Biorad). The gel was then washed in order to fix the RNAs with 75mM NaOH for 20 minutes followed by 2x 15 minute washes with Tris-Salt buffer (0.5 M tris pH 7.4, 1.5 M NaCl) and a 20 minute wash in 6X SSC (0.9 M NaCl, 90 mM $Na_3C_6H_5O_7$ pH 7.0).

The RNA was transferred to a Hybond N Membrane (Amersham) overnight using capillary action with 6X SSC buffer. The RNA was cross-linked to the membrane using a Stratalinker U.V crosslinker (Stratagene) as described in section **2.2.2.1**.

2.3.2.3 Hybridisation and probes

Northern blot probes are made as follows. 25-50 ng (approx 1 µl) of PCR template, 1 mM ATP, 1 mM CTP, 1 mM GTP, 0.1 mM UTP, 1U RNasin, 1 µl T7 polymerase (Fermentas), 1x Transcription Buffer (Fermentas) and 2 µl ^{32}P UTP in a 20 µl reaction. Incubated reaction at 37^{o}C for 2 hours before the addition of 1 µl Turbo DNase (Ambion) to the reaction and a further incubation at 37^{o}C for 30 minutes. Water was added to a final volume of 50 µl.

Target	Sequence
ITS1	AGGGGTCTTTAAACCTCCGCGCCGGAACGCGCTAGGTAC
5S	CCGAGATCAGACGAGATCGGGCGCGTTCAGGGTGGTATGG
5.8S	CAATGTGTCCTGCAATTCAC

Table 2.6 Probes used for northern blot hybridisation.

The nylon membranes were pre-hybridised for 30 minutes in 20ml SES1 (0.5 M sodium phosphate pH 7.2, 7 % SDS (w/v), 1 mM EDTA).The labelled probe (Table 2.6) was then added to the pre-hybridisation buffer and incubated at 37^{o}C with rotation overnight. The membranes were then washed twice in 1 x SSC, 0.1 % SDS (w/v) before being dried and exposed to a phosphorimager screen. Signals were then detected using the Typhoon FLA-7000 (GE healthcare).

2.3.3 Labelling RNAs for interaction studies

The 28S and 5S rRNA species were PCR amplified using Go Taq (Promega- see section 2.1.2) from whole-cell cDNA samples using PCR primers containing T7 sites. The PCR products were transcribed into RNA and labelled with ^{32}P UTP by the addition of T7

polymerase (Promega- see Table 2.7). This reaction was incubated at 37°C for 2 hours before the addition of 2U Turbo DNase (Life Technologies) for a further 1 hour at 37°C.

Component	Volume
Template	2 µl
10mM low UTP NTPs	2 µl
^{32}P UTP	4.5 µl
5X T7 buffer	4 µl
1M DTT	2 µl
T7 Polymerase (20U/µl)	0.5 µl
Water	5 µl

Table 2.7. Conditions for *in vitro* transcription.

T4 Polynucleotide kinase was used on the commercially bought RNA species in order to label them with ^{32}P-γ-ATP (see Table 2.8). These were incubated at 37°C for 45 minutes.

Component	Volume used
10µM RNA oligo	1 µl
10X PNK buffer	2 µl
Polynucleotide kinase (1U/µl)	1 µl
^{32}P ATP	4 µl
Water	12 µl

Table 2.8. Conditions for labelling RNAs.

After labelling, both the commercially bought RNA and the transcribed RNA were made up to a final volume of 50 µl with water before spinning in a G50 column (GE healthcare) to remove any unbound label.

2.3.4 Quantification of RNA

Protein quantification was carried out using 1 µl of RNA sample and analysed using Nanodrop (Thermo-Fisher) at 260 nm. Quantification of northern blots was carried out using ImageQuant software (GE healthcare).

2.4 Human Cell Culture

2.4.1 Human Cell lines

U2OS (Human Osteosarcoma), HEK 293 (Human embryonic kidney) and HeLa (cervical cancer) cells were routinely cultured in Dulbecco's Modified Eagles Medium (D5796-Sigma Aldrich) supplemented with 10% (v/v) Foetal Bovine Serum (Sigma) and 100 units/m penicillin and 100 µg/ml streptomycin. MCF-7 (Human breast cancer) cells were routinely cultured in Gibco™ DMEM/F12 media with Glutamax (10565-018- Life Technologies) supplemented with 10% (v/v) Foetal Bovine Serum (Sigma) and 100 units/m penicillin and 100 µg/ml streptomycin. All cells were grown in a monolayer in a 37^{o}C incubator with 5 % CO_2. At 80% confluency, cells were detached using 1x Trypsin EDTA (Sigma Aldrich) in Phosphate Buffered Saline (Sigma Aldrich) and incubated for 5 minutes at 37^{o}C before resuspending in DMEM and reseeding at the required density.

U2OS Flp-In™ T-REX™-cell line had been obtained from Neil Perkins. This cell line contains a Flp Recombinase site contained within a stably integrated pFRT/*lac*Zeo vector site from *S. cerevisiae*. The cell line also contains a pcDNA6/TR vector to allow for Blasticidin S resistance and constitutive expression from a tet-repressor under the control of the human CMV promoter to allow for tetracycline inducible cell lines. Cells were selected using 100 µg/ml hygromycin B at each passage and with 10 µg/ml Blasticidin S every third passage.

U2OS luciferase reporter cell clones were kindly donated by George Schlossmacher. These cells contain the pGL4.38 [*luc2P*/p53 RE/Hygro] (Promega) plasmid allowing for p53-regulated luciferase expression. After transfection with the plasmid, individual colonies were selected and tested for luciferase expression. The cells were selected using 300 µg/ml hygromycin B and routinely cultured with hygromycin B at this concentration at each passage.

All cell lines used were harvested using a swing bucket centrifuge at 900 rpm for 5 minutes to pellet the cells. The media was removed and the pellets were snap frozen in liquid nitrogen and stored at -80^{o}C or used immediately. To make stocks, cells from an 80% confluent flask were resuspended in 2 ml freezing media (DMEM, 10% DMSO, 20% FBS) and cooling to -80^{o}C in a freezing box to obtain gradual cooling.

2.4.2 RNAi

Several proteins of interest were depleted using small interfering RNAs (siRNAs) targeting the specific mRNA sequences (Table 2.9). In all cases, a control siRNA targeting firefly luciferase mRNA (Elbashir et al., 2002) was used as none of the cell lines contain this mRNA.

Target Gene	siRNA Sequence	Source	Reference
GL2	CGUACGCGGAAUACUUCGA	Eurofins	(Elbashir et al., 2002)
RPL5	UACUUUAAGAGAUACCAAG	Eurofins	(Fumagalli et al., 2012)
RPL11	GGUGCGGGAGUAUGAGUUA	Eurofins	(Bursac et al., 2012)
p53	GACUCCAGUGGUAAUCUAC	Eurofins	(Gu et al., 2004)
RPL7	Smartpool	GE Dharmacon	
RPL10	Smartpool	GE Dharmacon	
RPL18	Smartpool	GE Dharmacon	
RPL21	Smartpool	GE Dharmacon	
PRAS40	Smartpool	GE Dharmacon	
SRSF1	Smartpool	GE Dharmacon	

Table 2.9 siRNA sequences used

siRNAs SMARTpools (GE Dharmacon) were diluted to 20 µM in 1x annealing buffer (100 mM KOAc, 2 mM MgOAc, 20 mM HEPES-KOH (pH 7.4)). siRNAs purchased from Eurofins MWG Operon were resuspended in 1x Universal siRNA Buffer (Eurofins MWG Operon) to a concentration of 20 µM.

Chemical siRNA transfection into U2OS cells was performed using Lipofectamine™ RNAiMAX transfection reagent (Invitrogen) using the reverse transfection method. Cells were harvested with trypsin as explained previously and resupended in antibiotic free DMEM and 1.5 x 10^4 cells in 2 ml seeded into each well. For a 6-well plate, 150pmol siRNA was combined with 500 µl Opti-MEM Reduced serum media with L-glutamine (Invitrogen) and 5 µl transfection reagent and incubated at room temperature for 10-15 minutes before addition to the cells. Cells were then usually grown for a further 48 hours before harvesting as described.

2.4.3 Creation of pcDNA5 cell lines

A modified pcDNA5/FRT/TO vector containing a tetracycline regulated CMV/TetO2 promoter, a hygromycin B resistance gene with an FRT site in the 5' coding region and 2 FLAG tags was used to insert the gene of interest. The vector was co-transfected into

Flp-In cells with pOG44, a plasmid encoding Flp recombination, in order to promote homologous recombination between the FRT sites and thus incorporation of the gene of interest and the Hygromycin B resistance into the host cell genome in frame. Cells with such insertions were then selected for by culturing in the presence of Hygromycin B. U2OS Flp-In cells were kindly provided by Neil Perkins.

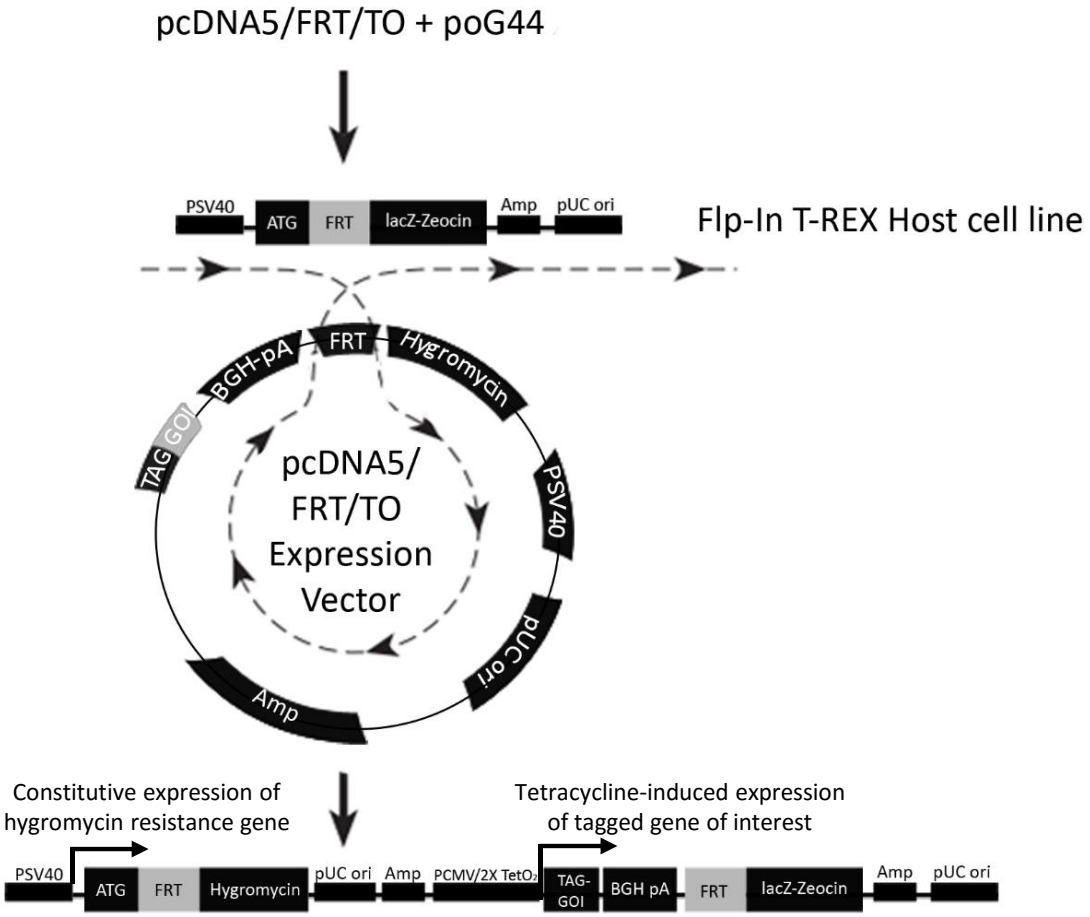

Figure 2.1 Schematic representation of the homologous recombination between the pcDNA5 vector and Flp-In T-REX cells. The insert within the Flp-In host cells is shown at the top. With the addition of the poG44 plasmid, the FRT sites within the pcDNA5 vector (shown in the centre) recombines with the FRT sites (dotted line) within the host cell line to produce the insert as shown at the bottom. Shown are; SV40 promoter (pSV40), start codon (ATG), Flp Recombination sites (FRT), hygromycin resistance gene (hygromycin), origin of replication (pUC ori), Ampicillin resistance gene (Amp), CMV promoter (pCMV), Tetracycline operator sequence (2X TetO2), 2X FLAG Precission protease 6X His Tag (TAG), Gene of interest (GOI), BGH polyadenylation signal (BGH pA) and fusion gene of LacZ with Zeocin resistance gene (lacZ-zeocin). Based on a figure from Invitrogen.

To generate the cell lines, the cells were grown at 37^{o}C in growth medium in 6 well plates until 60% confluent. 9 µl per well of FuGene6 (Promega) was mixed with 91 µl of Opti-mem (Invitrogen) in an microfuge tube and incubated at room temperature for 5

minutes before the addition of 1.8 µg pOG44 and 0.6 µg pcDNA5 and a further 15 minutes of incubation. The mixture was then added to the cells in a drop-wise manner.

Cells were grown for a further 48 hours before the addition of Hygromycin B and Blasticidin S to a final concentration of 100 µg/ml and 10 µg/ml respectively. Colonies were allowed to form for up to a month before resuspension and testing for expression of the tagged proteins with the addition of 0-1mg/ml tetracycline for 48 hours.

2.4.4 Chemical treatments

Human cells were chemically treated as shown in Table 2.10 before being harvested and used for further analysis.

Drug	Concentration used	Length of treatment
ActD	5 nM	18 hours
Nutlin 3A	5 µM	18 hours
Deguelin	100 nM	24 hours
Arginine	10 mM	12-48 hours
L-Leucine	10 mM	12-48 hours

Table 2.10. Final concentration of chemical treatments used in cell culture.

2.4.5 Whole Cell extracts

Human cell lines were cultured as described, harvested with trypsin-EDTA and pelleted by centrifugation before snap-freezing in liquid nitrogen. For immunoprecipitation or gradient analysis, the cell pellets were resuspended in 0.5ml gradient buffer (20 mM HEPES-NaOH pH 8, 150 mM KCl, 0.5 mM EDTA, 1 mM DTT, 5% glycerol) and sonicated for two 20 second intervals at minimum power using a Bandelin Sonopuls HD2070 ultrasonic homogeniser with a 2 mm MS72 titanium microtip. The lysate was centrifuged at 13,000 rpm at 4^{o}c to remove insoluble material. To the lysate, Triton-X100 (0.2% (v/v) was added. For the immunoprecipitations, 1.5 mM $MgCl_2$ was also added and the glycerol concentration increased to 10% (v/v).

2.4.6 Immunofluorescence

HEK293 or U2OS cell lines were grown on 10 mm round coverslips for 48 hours to achieve around 80% confluence prior to immunofluorescence. For the pcDNA5 transfected cell lines, cells were standardly treated with tetracycline for 48 hours prior to immunofluorescence. If RNAi was also required, cells were treated with tetracycline for 24 hours.

Coverslips were washed with PBS before incubation with PBS/4% Paraformaldehyde for 20 minutes at room temperature to fix the cells. Coverslips are then washed with PBS and incubated in PBS/0.1% Triton for 15 minutes at room temperature followed by 4x PBS washes. They were then blocked for 1 hour at room temperature in PBS/10% FBS/0.1% Triton. Primary antibodies were then applied at the appropriate dilution (Table 2.11) in PBS/10%FBS (to prevent non-specific binding) /0.1% Triton for 1 hour at room temperature before 3 x quick and 3x 10 minute washes in PBS. Secondary antibodies were then applied at the appropriate dilution in PBS/10%FBS/0.1% Triton for one hour at room temperature before a repeat of the washes as per the primary antibody, with the addition of DAPI (0.1 µg/ml, Sigma Aldrich) in the penultimate 10 minute wash.

Antibody	Raised in	Dilution for IF	Source
Alexa Fluor 555 anti-mouse	Donkey	1 in 500	Invitrogen A31570
Alexa Fluor 647 anti-rabbit	Donkey	1 in 500	Invitrogen A31573
FLAG	Rabbit	1 in 500	Sigma F7425
Fibrillarin	Mouse	1 in 50	Michael Pollard/Ger Pruijn

Table 2.11 Antibodies used in immunofluorescence

Coverslips were then immersed into water and ethanol before being dried and mounted using Moviol onto glass slides. Cells were analysed using a Zeiss Axiovert 200M inverted microscope with a Plan-Apochromat, 100 x / 1.40 oil DIC, ∞ / 0.17 objective (Zeiss). The Zeiss filter sets used were: 02 (DAPI); 20 (Cy3); 26 (Cy5).

2.4.7 Pulse labelling experiments

After the desired treatment of the cells, the media was removed and replaced with phosphate free media (Sigma-Aldrich), supplemented with 10% dialysed FBS for 1 hour. The phosphate free media was then removed and replaced with phosphate free DMEM supplemented with 10% dialysed FBS and 15µ Ci/ml ^{32}P labelled inorganic phosphate for 1 hour. The cells were washed with FBS and the media replaced with normal DMEM containing 10% FBS for 3 hours. 6 hours was used for gradient analysis for studying 5S RNA incorporation. RNA was extracted from the cells using TRI-reagent and analysed by agarose-glyoxal gel electrophoresis as previously described. The membrane is then left to expose to a phosphorimager screen. Signals were detected using a Typhoon FLA 7000 (GE healthcare).

2.4.8 Glycerol Gradient analysis of 5S RNP components

Gradients were used to separate 'free' ribosomal proteins from those integrated into the ribosome. Gradients were prepared by layering 2 ml of 40% glycerol solution (40% glycerol (v/v), 0.2% Triton-X100 (v/v), 1.5 mM $MgCl_2$, 20 mM HEPES-NaOH [pH 7.9], 150 mM KCl, 0.5 mM EDTA, 1mM DTT) underneath 10% glycerol solution (10% glycerol (v/v), 0.2% Triton-X100 (v/v), 1.5 mM $MgCl_2$, 20 mM HEPES-NaOH [pH 7.9], 150 mM KCl, 0.5 mM EDTA, 1 mM DTT) in 4ml 11x60mm Ultra-clear tubes (Beckman) and spinning at 22 rpm, 83^o for 70 seconds using a Gradient Master (BioComp). Gradients were then incubated at 4^{o}C for 1 hour. Whole cell extracts were prepared as previously described and 400µl added to the top of the gradient after the removal of 400µl gradient mix. 100µl of the whole cell extract is stored for the total. Gradients were centrifuged in a Beckman Optima L8-80 ultracentrifuge using a SW60Ti rotor for 1.5 hours at 52,000 rpm with slow acceleration and no brakes at 4^{o}C. Gradients were fractionated into 200µl aliquots and snap-frozen in liquid nitrogen.

2.4.9 Cell cycle analysis by flow cytometry

Cells were harvested by centrifugation at 300g for 5 minutes before washing with PBS. The cell pellet was then resuspended in cold 70% ethanol while vortexing to ensure complete fixation of cells. Cells were either stored in ethanol at 4^{o}C for a maximum of 48 hours or used immediately. Cells were pelleted by centrifugation at 300g and followed by a PBS wash and a further centrifugation at 3000g to ensure maximal cell recovery. The cell pellet was then resuspended in 50 µl 100 µg/ml RNase A followed by 200 µl Propidium Iodide (50 µg/ml). Cells were analysed for cell cycle using the FACSCanto II flow cytometer (BD Biosciences).

2.5 Protein Methods

2.5.1 SDS-PAGE

Cells were harvested and pelleted by centrifugation before being heated to 95^{o}c for 3 minutes in protein loading dye (74 mM Tris-HCl [pH6.8], 1.25 mM EDTA, 20% glycerol, 2.5% SDS, 0.125% bromophenol blue, 50mM DTT). The samples were vortexed before being loaded onto a denaturing 13% acrylamide SDS-PAGE gel with a 4% stacking gel. Gels were run for 45 minutes at 200V using the BioRad gel system in 1X Running buffer (25 mM Tris-HCl pH 8.3, 250 mM glycine, 0.1 % SDS (w/v)). For standard SDS-PAGE without western blot, proteins were visualised using Coomassie Blue Stain (0.1%

Coomassie Blue (w/v), 40% methanol (v/v), 10% aceitic acid (v/v)) for 16 hours followed by destaining in destain (40% methanol (v/v), 10% aceitic acid (v/v)).

2.5.2 Western blotting

Western blot was carried out after separation of proteins by SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane (Protran, GE healthcare) in transfer buffer (25 mM Tris-HCl, 150 mM glycine, 10% methanol [pH 8.3]) using a BioRad Mini Trans-Blot® Cell western blotting transfer tank for 1.5 hours at 65V. The membranes were then stained using Ponceau S solution to check for the presence of proteins before blocking for one hour in blocking solution (PBS, 0.1% Triton X100 (v/v), 2% Marvel skimmed milk powder (w/v)) to prevent non-specific binding.

Antibody	Raised in	Dilution for WB	Source
FLAG	Rabbit	1 in 10,000	Sigma Aldrich- F725
Goat	Donkey	1 in 10,000	Santa Cruz- sc-2020
Karyopherin	Rabbit	1 in 500	Santa Cruz (H-300)
MDM2	Mouse	1 in 100	Cal Biochem (Ab-1) OP46
Mouse	Donkey	1 in 10,000	Santa Cruz- sc-2314
p21	Rabbit	1 in 1000	Santa Cruz- sc-397
p53	Rabbit	1 in 1000	Santa Cruz- (FL-393)
PRAS40	Rabbit	1 in 1000	Bethyl- A301-201A
Rabbit	Donkey	1 in 10,000	Santa Cruz- sc-2313
RPL10	Rabbit	1 in 100	Abcam- ab128978
RPL11	Goat	1 in 2000	Santa Cruz- sc-25931
RPL5	Rabbit	1 in 5000	Bethyl-A303-933A
RPL7	Rabbit	1 in 2000	Abcam- ab72550
SRSF1	Mouse	1 in 1000	Santa Cruz- sc-33652
α-Tubulin	Mouse	1 in 10,000	Cell Signalling (DM1A)

Table 2.12. List of antibodies used with the dilutions used in Western blotting.

Antibodies were made up in blocking solution to the desired concentration (Table 2.12) and incubated overnight at 4^{o}C with gentle agitation. The membranes were washed (3 x 5 minutes) in wash buffer (PBS, 0.1% Triton X100 (v/v)) before the addition of the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody diluted in blocking solution (Table 2.12). Membranes were incubated at room temperature in secondary antibody for 2-3 hours before washing (3x 5 minutes) with wash buffer. The western blots were then developed by enhanced chemiluminescence (ECL) solution (Pierce) and exposure to Hyperfilm (GE Healthcare) according to the

manufacturer's instructions. Quantification of western blots was carried out using ImageJ software.

2.5.3 Phosphorylation gels

Phos-tag™ Acrylamide (Wako) gels were made as per the manufacturers protocol with a 10% acrylamide gel for comparison. After running, the phos-tag gels were washed in transfer buffer (25 mM Tris-HCl, 150 mM glycine, 10% methanol [pH 8.3]) containing 1 mmol/L EDTA for 10 minutes before a second wash in standard transfer buffer without EDTA. The western blot was then treated as normal.

2.5.4 Luciferase assays

Cells expressing luciferase were plated at $2x10^5$ cells per well and incubated in growth medium at 37^{o}C. After 48 hours the cells were harvested using the Promega luciferase assay kit per the manufacturer's specifications with Cell culture lysis buffer (Promega). The luciferase measurements were taken using a Lumat 100 luminometer (Berthold).

2.6 Over-expression and purification of GST-tagged proteins in *E.coli* cells

2.6.1 Purification of GST-tagged proteins

GST tagged proteins were expressed in E. coli BL21 cells and used to inoculate a litre of 2X YT broth with the selective antibiotics. These cultures were grown at 37^{o}c until an $O.D_{600}$ of around 0.3. A final concentration of 1mM IPTG was added and the cultures grown for a further 3-18 hours at either 37^{o}C or 18^{o}C. The cells were harvested by centrifugation at 4,000 rpm, 4^{o}C for 30 minutes. The supernatant was removed and the pellets resuspended in GST buffer (20 mM Tris-HCl pH 8, 5 mM $MgCl_2$, 300 mM KCl, 0.1% Tween 20, 10% glycerol) before adding ½ of a protease inhibitor tablet and THP to a final concentration of 0.5 mM. The samples were sonicated on ice for 3 minutes/5 cycles/90% power to lyse the cells. The lysate was then collected by centrifugation at 18,500 rpm for 45 minutes at 4^{o}C for 45 minutes. The pellet was discarded. 1 ml glutathione-sepharose beads were equilibrated in GST buffer before being added to the clarified lysate. These were rotated at 4^{o}C for 2 hours. The beads were pelleted by centrifugation at 4,000 rpm at 4^{o}C for 3 minutes before discarding the supernatant and resuspending in 50ml GST buffer and washing. After discarding the supernatant, the bead pellet was resuspended in 1.5ml GST buffer with 50 mM glutathione before

placing back on the wheel for 1hr at 4°C. The beads were pelleted by centrifugation before the supernatant containing the eluted GST-tagged proteins was taken and snap-frozen in liquid nitrogen.

2.6.2 Desalting

To remove glutathione from the GST-tagged proteins, the purified proteins were passed through a HiTrap desalting column (GE) on the AKTA basic (GE life sciences). The column was equilibrated using GST buffer and samples were loaded via a loop. The resulting fractions were then collected and snap frozen in liquid nitrogen. To separate RNA from protein, the samples were loaded onto a 0.5ml injection loop and run through the AKTA basic using a Q-sepharose column, running a gradient from 100 mM NaCl to 1 M NaCl.

2.7 Interaction studies

2.7.1 Glutathione-sepharose protein-RNA pull downs

Glutathione-sepharose beads were equilibrated in pull-down buffer (20 mM Tris-HCl pH 8, 5 mM MgCl2, 150 mM KCl, 0.1% triton, 10% glycerol, 0.5 mM DTT). Appropriate volumes of GST-tagged protein were added accordingly to the pelleted beads and the solution made up to 0.5ml with pull-down buffer. These were rotated at 4°C for 1 hour to bind the proteins. The beads were pelleted at 3,000 rpm for 3 minutes at 4°C before discarding the supernatant and washing in pull-down buffer. 0.5 ml pull-down buffer, 1 µl tRNA and 1 µl ^{32}P labelled RNA were added to the pelleted beads and rotated at 4°C for 1 hour. The beads were pelleted and washed before adding 0.5ml homogenisation buffer (1% SDS, 50 mM Tris HCl pH 7.6, 60 mM NaCl, 0.5 mM EDTA) to elute the bound protein-RNA complexes. A phenol-chloroform extraction and ethanol precipitation were performed to extract the RNA before running on 8% or 12% acrylamide urea gels at 160V for 40 minutes. The gel was fixed in 10% aceitic acid, 40% methanol before drying using a gel drier. A phosphorimager screen was then placed over the gel and left to expose overnight before scanning on the Typhoon FLA phosphorimager (GE).

2.7.2 Immunoprecipitations

10 µl protein G-Sepharose beads or ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich A2220) per immunoprecipitation were washed thrice in IP Buffer (20 mM HEPES-NaOH pH 8.0, 150 mM NaCl, 3 mM $MgCl_2$, 0.5 mM DTT, 10% glycerol (v/v), 0.1% Triton-X100 (v/v)) to

equilibrate the beads. The ANTI-FLAG® M2 Affinity gel was used directly whereas the protein G-sepharose beads were resuspended in IP buffer with 5 µl mouse anti-FLAG (Sigma- F3165) antibody or a bead-only control and gently rotated on a wheel at 4°C overnight before washing thrice in IP buffer. Whole cell extracts (as previously described in section 2.2.5) were added to the prepared resins after taking a 10% total of 100µl and rotated at 4°C for 2 hours before centrifugation and washing in IP buffer to remove un-bound proteins. RNA extractions were carried out with phenol-chloroform as described previously.

For western blots, the resin was resuspended in loading buffer without DTT (74 mM Tris-HCl [pH6.8], 1.25 mM EDTA, 20% glycerol, 2.5% SDS, 0.125% bromophenol blue) and heated to 95°C for 5 minutes. A final concentration of 50 mM DTT was added and western blots carried out as previously described.

CHAPTER THREE: UNDERSTANDING THE FACTORS INVOLVED IN RECRUITING THE 5S RNP INTO THE RIBOSOME

3.1 Introduction

The ribosome is a crucial complex within both eukaryotic and prokaryotic organisms. The production of ribosomes dictates cellular proliferation (Donati et al., 2012) and requires over 200 trans-acting factors and the coordination of all three RNA polymerases (Henras et al., 2008). Ribosome biogenesis occurs in the nucleolus, a nuclear body only present within the interphase nucleus. RNA polymerase I transcribes the 5.8S, 18S and 28S rRNA in a single precursor (47S) in the nucleolus which is then processed and modified into the three separate rRNAs through multiple cleavage events whereas the 5S rRNA is transcribed by RNA polymerase III in the nucleoplasm (Leary and Huang, 2001, Nazar, 2004). The 5S rRNA forms a complex with RPL5 and RPL11 known as the 5S RNP and is the only rRNA that forms a pre-ribosomal complex with ribosomal proteins prior to integrating into the ribosome (Ciganda and Williams, 2011).

Recently, ribosome biogenesis dysfunction has been closely linked to the activation of p53. The tumour suppressor, p53, is often referred to as the 'guardian of the genome' and is deleted or inactivated in the majority of cancers. In cellular stress situations, p53 is activated and coordinates with a variety of signalling pathways that result in DNA repair, apoptosis, senescence or G1 cell cycle arrest (Ko and Prives, 1996, Timofeev et al., 2013, Sperka et al., 2012, Sugikawa et al., 1999). The main regulator of p53 is MDM2 which harbours E3 ubiquitin ligase activity, promoting the ubiquitination and degradation of p53 by targeting it to the 26S proteasome (Honda and Yasuda, 1999). In situations of cellular stress such as DNA damage or chromosomal aberrations, MDM2 is suppressed and leads to the stabilisation and activation of p53 (Michael and Oren, 2003). Many ribosomal proteins have been seen to interact with MDM2 such as RPL23, RPS7, RPL5, and RPL11 (Jin et al., 2004, Marechal et al., 1994, Lohrum et al., 2003, Chen et al., 2007) although it has been shown by two independent groups that the 5S RNP (consisting of RPL5, RPL11 and the 5S rRNA) is the critical factor in the maintenance of p53 homeostasis as well as being involved in the induction of p53

under ribosomal stress conditions (Sloan et al., 2013a, Donati et al., 2013) as summarised in Figure 3.1.

Disruptions in translation or ribosome biogenesis have been implicated in many diseases known as ribosomopathies such as DBA and 5q-syndrome. Many of these diseases have links to p53 signalling as a result of a disruption in ribosome production (Fumagalli and Thomas, 2011).

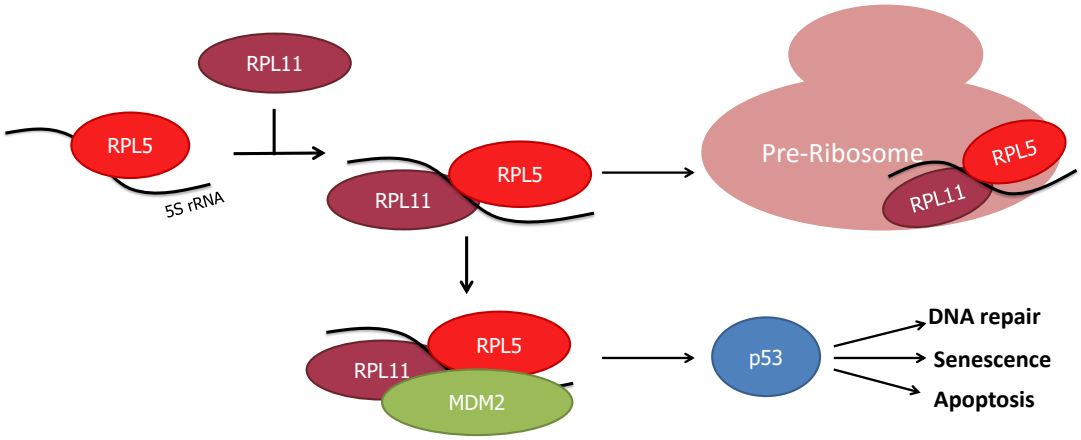

Figure 3.1. The 5S RNP-p53 pathway. In normal cells, p53 is targeted for proteasomal degradation by the E3 ubiquitin ligase, MDM2. The 5S RNP, consisting of RPL5, RPL11 and the 5S rRNA is incorporated into the pre-60S subunit which then forms part of the mature 80S ribosome. When ribosome biogenesis is disrupted, the 5S RNP is not incorporated into ribosomes and is able to bind to MDM2 and activate p53. p53 signals to various pathways inducing those involved in senescence, DNA repair or apoptosis.

DBA and 5q-syndrome share many of the same symptoms such as anaemia, decreased erythroid precursors and a predisposition to cancers such as leukaemia (Quarello et al., 2010b, Vardiman et al., 2002). Almost half of all DBA patients have mutations in genes encoding ribosomal proteins, including RPL11 and RPL5, and the pathogenesis of DBA is partly attributed to the rapid proliferation of red blood cell precursors requiring a high rate of ribosome production (Zhang et al., 2011). Furthermore, defects in ribosomal proteins may lead to accumulation of p53 and thus cell-cycle arrest and apoptosis in these erythroid cells (Dai and Lu, 2004). 5q-syndrome is a myelodysplastic syndrome characterised by the deletion of the long arm of chromosome 5 (Van den Berghe et al., 1974). This deleted region contains the gene encoding the ribosomal protein RPS14, a small subunit ribosomal protein (Ebert et al., 2008). Haploinsufficiency of RPS14 results in ribosome biogenesis defects (Cazzola, 2008, Galili et al., 2009) and activation of p53 (Barlow et al., 2010). Based on more recent findings that the 5S RNP regulates p53 upon disruption of ribosome biogenesis (Donati

et al., 2013, Sloan et al., 2013a), it is presumed that the 5S RNP contributes to the p53 activation in 5q-syndrome.

The regulation of the 5S RNP is therefore crucial to understanding the pathogenesis of these diseases and for the invention of novel treatment therapies. Sloan et al. (2013a) proposed that any proteins involved in the regulation of 5S RNP formation, localisation or integration were central in regulating MDM2 activity and, thus, p53 levels in the cell. However, the factors involved in 5S RNP recruitment into the ribosome are not fully understood. In yeast, RPL5 and RPL11 are delivered to the nucleus and probably assembled onto the 5S rRNA by Syo1, forming the 5S RNP. Rpf2 and Rrs1 form a complex with the 5S RNP and are crucial for the recruitment of the 5S RNP into the 90S pre-ribosomes (Zhang et al., 2007, Morita et al., 2002). Upon joining the pre-ribosome, the 5S RNP docks onto helix 84 of the 25S rRNA (Rhodin et al., 2011) and is locked into conformation by the C-terminal tail of Rpf2, Rsa4 and Nsa2 to prevent any rotation (Kharde et al., 2015, Bassler et al., 2015). Rsa4 is then released from the 5S RNP by Rea1 and the 5S RNP structure is rotated by 180^{o} in order to form the central protuberance (Bassler et al., 2015, Leidig et al., 2014).

The human homologues of Rrs1 and Rpf2, RRS1 and BXDC1, are not as crucial for 5S RNP integration, however, depletion of both proteins increased the nucleoplasmic localisation of RPL5 and RPL11 (Sloan et al., 2013a) and thus it was suggested that in humans, these proteins were involved in localisation of the 5S RNP. Interestingly, it was also found that the nucleolar protein, PICT-1, directly contacted the 5S rRNA and depletion of PICT-1 had a more profound effect on than depletion of RRS1 or BXDC1 on 5S rRNA ribosomal recruitment. This suggested that PICT-1 is a crucial factor for the 5S RNP recruitment into the ribosome in human cells. The differences between yeast and human 5S RNP ribosomal integration means that there could be many more factors involved in humans that have not yet been identified.

The eukaryotic *T. thermophila* structure of the large subunit of the ribosome (Klinge et al., 2011) showed that several ribosomal proteins surround the 5S RNP within the ribosome (Figure 3.2A and B). These proteins were RPL7, RPL10, RPL18 and RPL21 and, as they appeared to form a 'binding pocket' for the 5S RNP, may be involved in the recruitment of the 5S RNP into the ribosome.

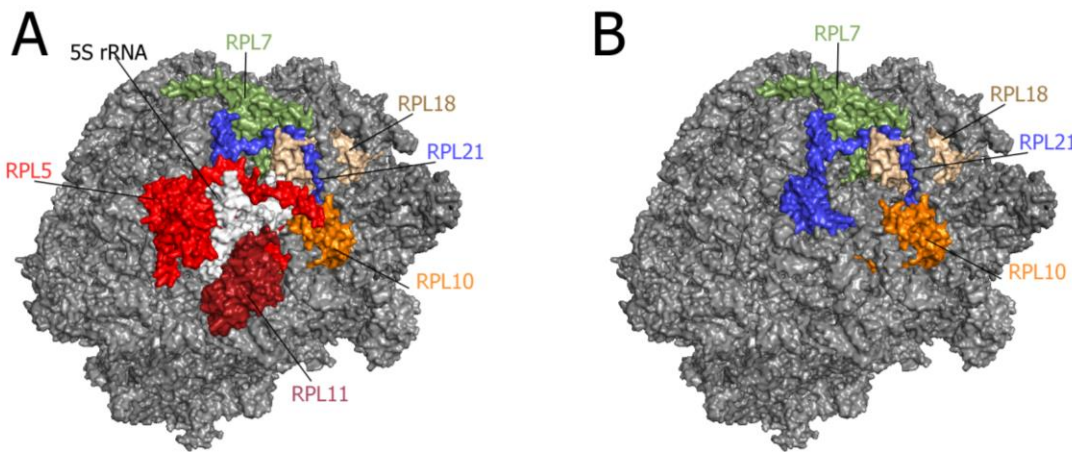

Figure 3.2. The 5S RNP 'binding pocket'. An image of the top of the *T. thermophila* crystal structure of the large ribosomal subunit (Klinge et al., 2011) showing the position of the RPL7 (green), RPL18 (brown), RPL10 (orange), RPL21 (blue) with (A) and without (B) the 5S RNP *in situ*. The grey represents the rRNA and other ribosomal proteins. The proteins surround the 5S RNP and form a 'pocket' that may be important for the recruitment of the 5S RNP.

Interestingly, two of these proteins, RPL10 and RPL21 have been shown to mutated in disease. Mutations in RPL10 have been described in patients with T-cell acute lymphoblastic leukaemia (T-ALL) (Keersmaecker et al., 2013). Interestingly, the RPL10 mutation found in T-ALL patients, Arg98Ser, appeared to cause ribosome biogenesis and proliferation defects in mammalian lymphoid cells. RPL10 mutations have also been linked to several neuronal diseases such as autism (Klauck et al., 2006, Chiocchetti et al., 2011, Gong et al., 2009), X-linked microcephaly (Brooks et al., 2014), and X-linked syndromic Intellectual Disability (Thevenon et al., 2015). RPL21 mutations have been reported in hereditary hypotrichosis simplex (Zhou et al., 2011). However, the link to p53 in these diseases has not been characterised.

Currently, ways to combat ribosomopathies are limited, a more in-depth understanding of 5S RNP-p53 signalling and how the 5S RNP is integrated into the ribosome could unlock new targeted ways to treat patients. This chapter aimed to identify factors that may affect integration of the 5S RNP into the ribosome and thus p53 signalling through studying interactions between ribosomal proteins, rRNA and the 5S RNP.

3.2 Results

3.2.1 Depletion of RPL7, RPL10, RPL18 and RPL21 in U2OS cells

As RPL7, RPL18 and RPL21 appeared to form a binding pocket for the 5S RNP based on the *T. thermophila* crystal structure (Klinge et al., 2011) it was important to investigate whether depletion of these proteins would result in a block in 5S RNP recruitment. It was necessary to first establish that siRNA transfection was successful at reducing mRNA and protein levels in cells. Therefore, U2OS cells were cultured in growth medium at 37°C post-transfection with siRNA duplexes targeting the four protein's mRNA sequences (20 µM RPL7 and RPL10 and 2 µM RPL18 and RPL21), or a control luciferase siRNA (20 µM GL2) for 48 hours.

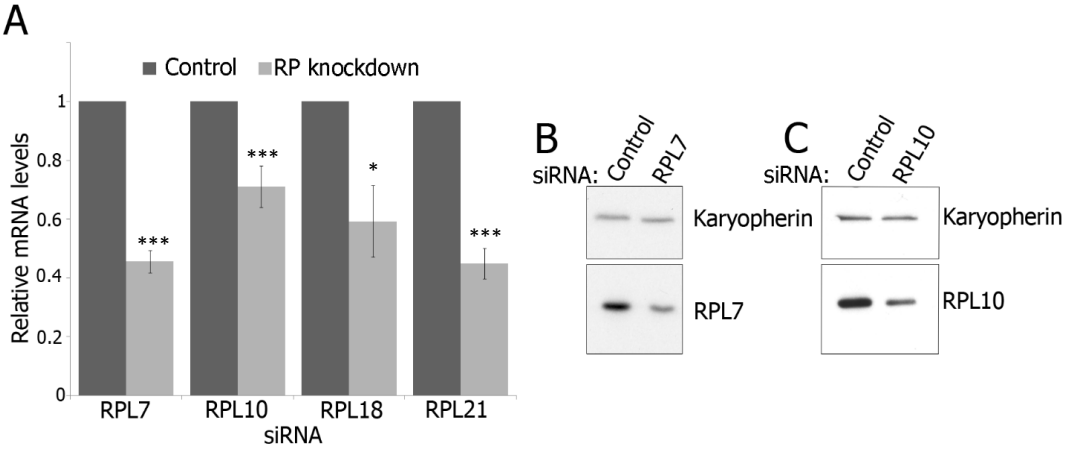

Figure 3.3. Treatment of cells with siRNAs for RPL7, RPL10, RPL18 and RPL21 results in a **reduction of the relevant mRNA and protein levels. (A)** U2OS cells were incubated at 37°C in growth medium post-transfection with siRNA duplexes targeting RPL7, RPL10 (20 µM), RPL18 and RPL21 (2 µM) for 48 hours prior to harvesting, RNA extraction and RT-PCR using primers specific to the 4 ribosomal proteins and a housekeeping gene, GAPDH. The resultant PCR products were separated via agarose gel electrophoresis containing DNA stain and the cDNA signals visualised using the fluorescence setting on a Typhoon FLA imager. The signals were quantified using ImageQuant and the levels of each cDNA in both the control and the knockdowns was normalised to the GAPDH. **(B+C)** U2OS cells were depleted of RPL7 and RPL10 as in A. Whole cell extracts were separated by SDS-PAGE followed by western blotting using RPL7 and RPL10 antibodies alongside karyopherin as a loading control. Bars in A represent the mean ± the standard deviation of the mean, n=3. Statistical analysis determined by paired t-test. NS= non-significant, *= P ≤ 0.05, **= p≤ 0.01, ***= p≤ 0.001.

The RNA was extracted from the cells and reverse transcribed followed by PCR amplification using primers annealing to RPL7, RPL10, RPL18 or RPL21. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) primers were used as a control. The mRNA of each protein was reduced in the knockdowns compared to the control (Figure 3.3A), suggesting that the knockdown had been successful. However, it should be noted that the RPL10 siRNA appeared to have less of an effect on its target mRNA levels than the other siRNAs. It was not possible to obtain antibodies for all of the

proteins, however RPL7 and RPL10 antibodies were used to show the level of knockdown at the protein level. U2OS cells were incubated in growth medium at 37^{o}C post-transfection with 20 µM siRNA duplexes targeting RPL7, RPL10 or the control GL2 for 48 hours prior to harvesting. Whole cell extracts were separated by SDS-PAGE and western blotted using karyopherin (loading control), RPL7, or RPL10 antibodies (Figure 3.3B). The siRNA treated cells showed a reduction in signal compared to the control when using the appropriate antibodies suggesting that the knockdowns had been successful.

3.2.2 Depletion of RPL7, RPL18 and RPL21 affects integration of the 5S rRNA into the ribosome.

In order to determine the importance RPL7, RPL10, RPL18 and RPL21 on the recruitment of the 5S RNP into the ribosome, the proteins were depleted using RNAi and the 5S rRNA was analysed using glycerol gradients and northern blots.

U2OS cells were incubated in growth medium at 37^{o}C post-transfection with 20 µM siRNA duplexes targeting RPL11, RPL7 or RPL10 or 2 µM siRNA duplexes targeting RPL18 or RPL21 for 48 hours. In order to visualise just the newly synthesised 5S rRNA, the cells were depleted of phosphate by incubating in phosphate-free medium for 1 hour and labelled using ^{32}P orthophosphate-supplemented medium, followed by a 6 hour chase with normal medium. The 6 hour chase period was selected for this experiment as 5S rRNA recruitment into the ribosome is slow (Sloan et al., 2013a) and thus to visualise recruitment, a longer time is required.

After depletion of the ribosomal proteins for 48 hours and pulse-labelling, cells were lysed and applied to a 10-40% glycerol gradient in order to separate the large and small complexes. After centrifugation, 20 fractions were taken. Based upon published data, (Sloan et al., 2013a) fractions 1-7 were combined for the smaller complexes that should contain the 'free' 5S rRNA whilst fractions 9-20 were pooled for the ribosome bound 5S rRNA. RNA was extracted from the pooled fractions and separated using an 8% acrylamide-urea gel before transferring to a nylon membrane and exposing to a phosphorimager screen (Figure 3.4A).

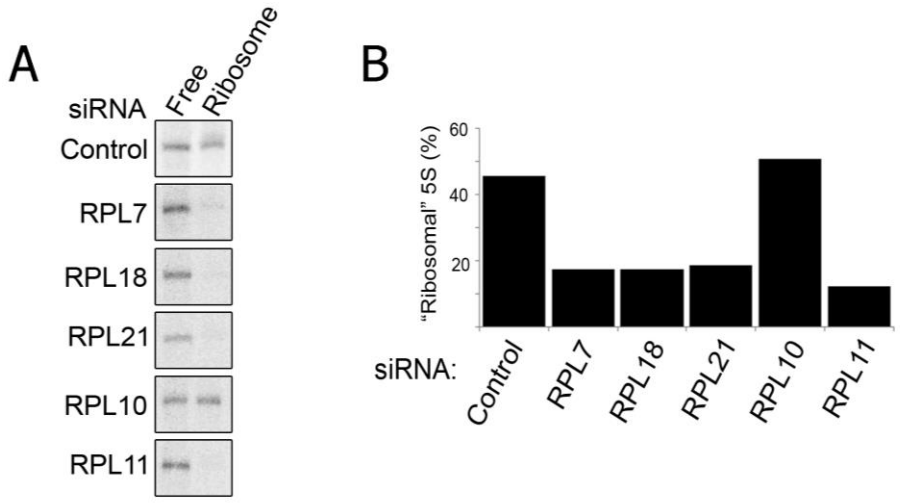

Figure 3.4. RPL7, RPL18 and RPL21 depletion results in accumulation of 'free' 5S rRNA. (A) Gradient centrifugation of siRNA treated cells followed by northern blot with a 5S rRNA probe to reveal 'free' and 'ribosome' bound 5S rRNA. U2OS cells were incubated in growth medium post-transfection with siRNA duplexes targeting RPL7, RPL10 (20µM), RPL18 and RPL21 (2µM) for 48 hours prior to phosphate starving the cells in phosphate free medium. Cells were then incubated in ^{32}P orthophosphate labelled medium for 1 hour before returning the cells to normal growth medium for 6 hours at 37°C. All incubations were carried out at 37°C. Cells were harvested and RNA extracted. The RNA was separated by acrylamide gel electrophoresis before transferring to a nylon membrane and exposing to a phosphorimager screen. **(B)** Quantification of A using densitometry analysis to show the percentage of 5S from the ribosome fractions. n=1.

In control cells, 45% of the 5S rRNA was shown in the 'ribosomal' fractions. Depletion of RPL11 resulted in a decrease in ribosomal 5S rRNA to less than 20%. Depletion of RPL7, RPL18 and RPL21 resulted in a similar reduction in 5S rRNA integration, with around 18-20% of the 5S rRNA in the ribosome fractions (Figure 3.4B). Depletion of RPL10, however, showed no reduction in 5S rRNA in the ribosome fractions. As the effect of depletion of RPL7, RPL18 and RPL21 was similar to that of RPL11, which is crucial for the 5S RNP recruitment into the ribosome (Sloan et al., 2013a), this indicated that RPL7, RPL18 and RPL21 may be required for integration of the 5S rRNA into the ribosome, but that potentially RPL10 is not.

3.2.3 RPL7, RPL18 and RPL21 are crucial for ribosome biogenesis and RPL7 and RPL18 knockdowns lead to accumulation of the 36SC precursor

Depletion of proteins that affect 5S RNP incorporation into the ribosome such as PICT-1 have been shown to also result in ribosome biogenesis defects (Sloan et al., 2013a). As RPL7, RPL18 and RPL21 had reduced 5S rRNA incorporation into the ribosomes, the next step was to investigate whether they were also important for ribosome biogenesis.

To investigate the role of these proteins in pre-rRNA processing, pulse-labelling was utilised whereby cells were phosphate starved with phosphate free medium before the addition of ^{32}P orthophosphate labelled medium and a 3 hour chase period with normal phosphate medium. This method allows for the specific evaluation of newly synthesised rRNAs. The 3 hour chase period ensures that there is enough time for the rRNAs to be processed so that each precursor can be studied.

U2OS cells were incubated at 37^{o}C for 48 hours in growth medium post-transfection with 20 µM (RPL5, RPL7, RPL10) or 2 µM (RPL18, RPL21) siRNA duplexes. The cells were then grown in phosphate free medium for 1 hour, followed by 1 hour incubation with labelled medium. After a three hour chase with normal growth medium, RNA was extracted from the cells and separated by glyoxal-agarose gel electrophoresis (Figure 3.5A- top panel) to separate the large rRNA precursors and acrylamide-urea gel electrophoresis (bottom panel) to separate the 5S and 5.8S rRNAs. The 18S rRNA from the U.V scan of the glyoxal-agarose gel (middle panel) was used as a loading control.

In agreement with previously published data (Sloan et al., 2013a), depletion of RPL5 resulted in a severe reduction in levels of the 28S rRNA. Depletion of RPL7 showed a slight reduction in 5S production but resulted in an almost complete loss of 28S as well as reductions in 32S, 47/45S and 5.8S. Similar, but more extreme, results were observed with the RPL18 and RPL21 knockdowns. The depletion of RPL18 resulted in an almost complete loss of 47/45S, 32S, 28S and 5.8S, with an additional reduction in 18S production. RPL21 depletion resulted in reduction in 28S and 5.8S and an accumulation of 32S, suggesting a defect in cleavage of the 32S precursor into the 28S and 5.8S rRNAs.

Furthermore, rRNA precursors were studied using cell extracts and northern blotting to see whether any further defects could be detected (Figure 3.5B). RNA from knockdown cells was extracted and separated via glyoxal-agarose gels before transferring to a nylon membrane. The northern blots were probed using an Internal Transcribed Spacer 1 (ITS1) probe (Figure 3.5C), which hybridises to small subunit precursors and early large subunit precursors. In all of the northern blot analysis, the 18S RNA, visible from U.V scanning of the gel prior to transfer, was used as a loading control.

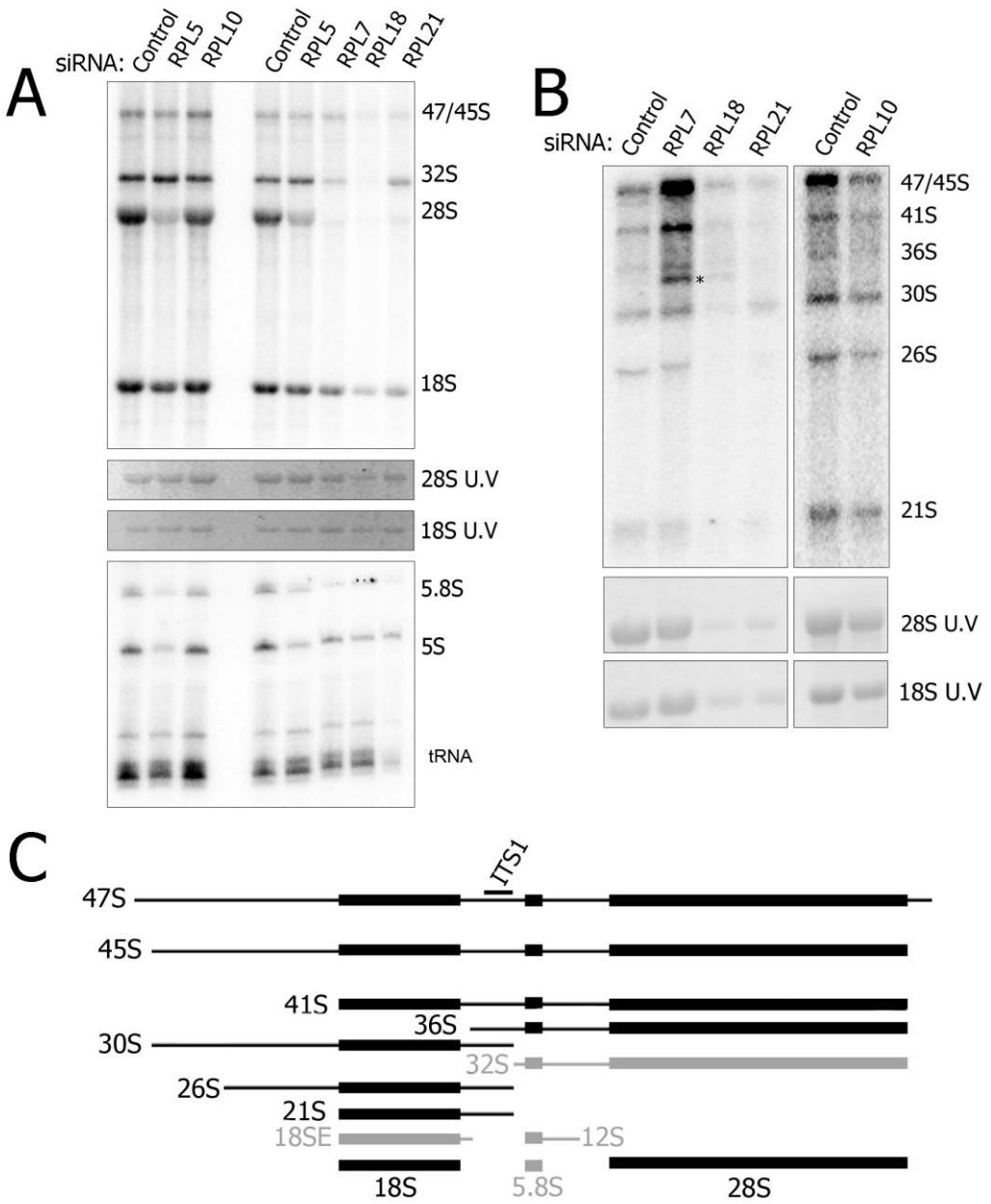

Figure 3.5. RPL7, RPL18 and RPL21 knockdowns affect ribosome biogenesis. (A) Pulse-labelling of knockdowns. U2OS cells were incubated in growth medium containing RPL5, RPL7, RPL10 (20µM), RPL18 and RPL21 siRNA duplexes for 48 hours at 37°C before the addition of phosphate free medium for 1 hour. ^{32}P orthophosphate labelled medium was then introduced into the cells for 1 hour before a 3 hour chase with normal media to allow for rRNA processing to occur. RNA was extracted and separated via Agarose-glyoxal (top panels) and acrylamide-urea (bottom panel) gels and transferred to nylon membranes. The membranes were exposed to phosphorimager screens and visualised via phopshorimaging. **(B)** Northern blot showing the effect of RPL7, RPL10, RPL18 and RPL10 knockdowns in U2OS cells. Cells were in treated with siRNAs for 48 hours prior to harvesting, RNA extraction and separation of the RNAs with a glyoxal-agarose gel. The RNAs were transferred to a nylon membrane via northern blotting and incubated with the ITS1 probe to look at the large subunit rRNA precursors. **(C)** Schematic representation of the rRNA precursors showing the location of the where the ITS1 probe hybridises. The precursors that ITS1 probe hybridises to, seen in A are shown in black. N=3

The RPL18 and RPL21 knockdowns appeared to severely affect the cells, leading to reduced cell numbers and thus it was difficult to get RNA from these cells. Taking into account differences in loading, depletion of RPL7, RPL18 and RPL21 resulted in accumulation of the early precursors (47/45S, 41S and 36S). Interestingly, the RPL21 knockdown showed a great increase in the 30S precursor whereas depletion of the other proteins did not. RPL7 and RPL18 depletion resulted in an accumulation of the 36SC precursor, suggesting that the 36S is not properly cleaved into the 32S in the absence of RPL7 and RPL18. These results, taken in conjunction with the pulse-labelling data, imply that these proteins are required for rRNA processing.

3.2.4 Knockdowns of RPL7, RPL18 and RPL21 but not RPL10 induce p53 via the 5S RNP

The data presented in 3.2.3 highlighted the possibility that knockdown of RPL7, RPL18, or RPL21 may prevent integration of the 5S RNP into the ribosome. When the 5S RNP is not incorporated into ribosomes, it is free to contact MDM2 and thus stabilise p53, therefore the effect of knockdowns of the ribosomal proteins were investigated to determine whether they resulted in accumulation of p53 in a 5S RNP-mediated manner.

U2OS cells were transfected with GL2, RPL7, RPL10, (20μM) RPL18 or RPL21 (2μM) siRNA duplexes alone or in combination with an RPL5 siRNA duplex. The cells were harvested after 48 hours, separated via SDS-PAGE and then western blotted (Figure 3.6A). Antibodies raised against p53, p21, and MDM2 were utilised, and an α-Tubulin antibody was used as a loading control. The western blots revealed that depletion of RPL7, RPL18, or RPL21 increased the levels of p53 and its downstream target, p21 as well as MDM2. These inductions were abolished upon co-depletion of RPL5, suggesting that this p53 induction was 5S RNP dependent. RPL10 depletion showed little change in p53 levels.

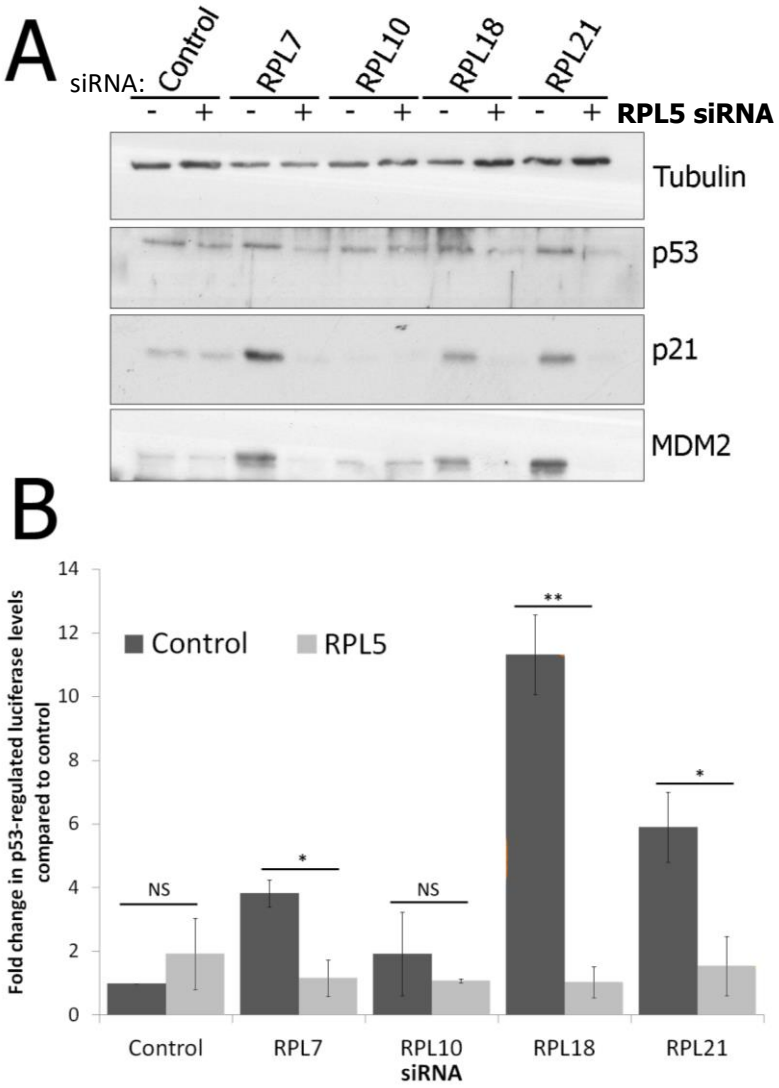

Figure 3.6. Depletion of RPL7, RPL18 and RPL21 induces p53 in a 5S RNP-dependent manner. (A) Representative western blot from three independent experiments. U2OS cells were incubated in growth medium at 37°C post-transfection with RPL7, RPL10 (20 µM), RPL18 and RPL21 (2 µM) siRNAs alone or in conjunction with a 20µM RPL5 siRNA for 48 hours. Whole cell extracts were separated by SDS-PAGE before western blot analysis. α-Tubulin was used as a loading control. **(B)** U2OS cells containing a p53-regulated luciferase reporter were treated with siRNAs as in A before assaying for firefly luciferase reporters. Bars represent the means ± the standard deviation of the means, n=3. Statistical analysis determined by paired t-test. NS= non-significant, *= P ≤ 0.05, **= p≤ 0.01, ***= p≤ 0.001.

Whilst the increase in p21 levels suggested an increase in p53 transcriptional activity, it was decided to analyse this further by directly measuring p53 transcriptional activity as p21 can be activated independently of p53 (Macleod et al., 1995). Therefore, U2OS cells stably expressing a p53-regulated luciferase reporter were utilised. As in Figure 3.6A, the siRNA duplexes alone or in combination with RPL5 were transfected into U2OS cells. The cells were harvested after 48 hours, lysed, and analysed for luciferase activity (Figure 3.6B). As seen with the western blots, depletion of RPL7, RPL18, or RPL21 resulted in an increase in luciferase levels, which was decreased to near-basal

levels upon knockdown of RPL5, indicating that depletion of RPL7, RPL18, or RPL21 resulted in an increase in p53 activity in an RPL5 dependent manner. RPL10 showed no significant increase in luciferase and thus did not result in an increase in p53 activity, thus suggesting that RPL10 depletion does not induce p53 via the 5S RNP.

3.2.5 Depletion of RPL7, RPL18 and RPL21 result in an accumulation of cells in G1 in an RPL5-dependent manner.

As RPL5-dependent p53 induction and activation of p21 upon knockdown of RPL7, RPL18 and RPL21 was observed, it was next important to establish whether this affected cell cycle progression. p21, also known as cyclin-dependent kinase inhibitor 1, is a cell cycle regulator that, when activated, often results in G1 arrest (Deng et al., 1995, Waldman et al., 1995). RPL21 depletion resulted in too few cells for flow cytometric analysis and thus only RPL7 and RPL18 depletions were analysed.

U2OS cells were incubated in growth medium post-transfection with 20 μM (RPL5, RPL7) or 2 μM (RPL18) siRNA duplexes before fixing in ice cold ethanol, staining with propidium iodide and analysing by flow cytometry, gating on the live population (Figure 3.7 A-F). Unfortunately, the RPL5 depletion and the RPL5/RPL18 co-depletion were toxic to cells and thus resulted in very low cell numbers (figure 3.7B and F). However, they resulted in consistent results over 3 experiments.

The number of cells at each stage in the cell cycle were taken as a percentage and were represented in a graph (Figure 3.7G) which revealed that RPL7 and RPL18 depletion resulted in an accumulation of cells at G0/G1. Interestingly, RPL5 depletion gave a small, but significant accumulation of cells in G2 (t test, $p= 0.0281$). When RPL5 was co-depleted with RPL7 and RPL18, it was revealed that RPL5 depletion counteracted the G0/G1 arrest seen in the single knockdowns and, instead, resulted in a slight accumulation of cells in G2. This suggested that RPL5 was required for the accumulation of cells in G1 seen with the RPL7 and RPL18 depletions.

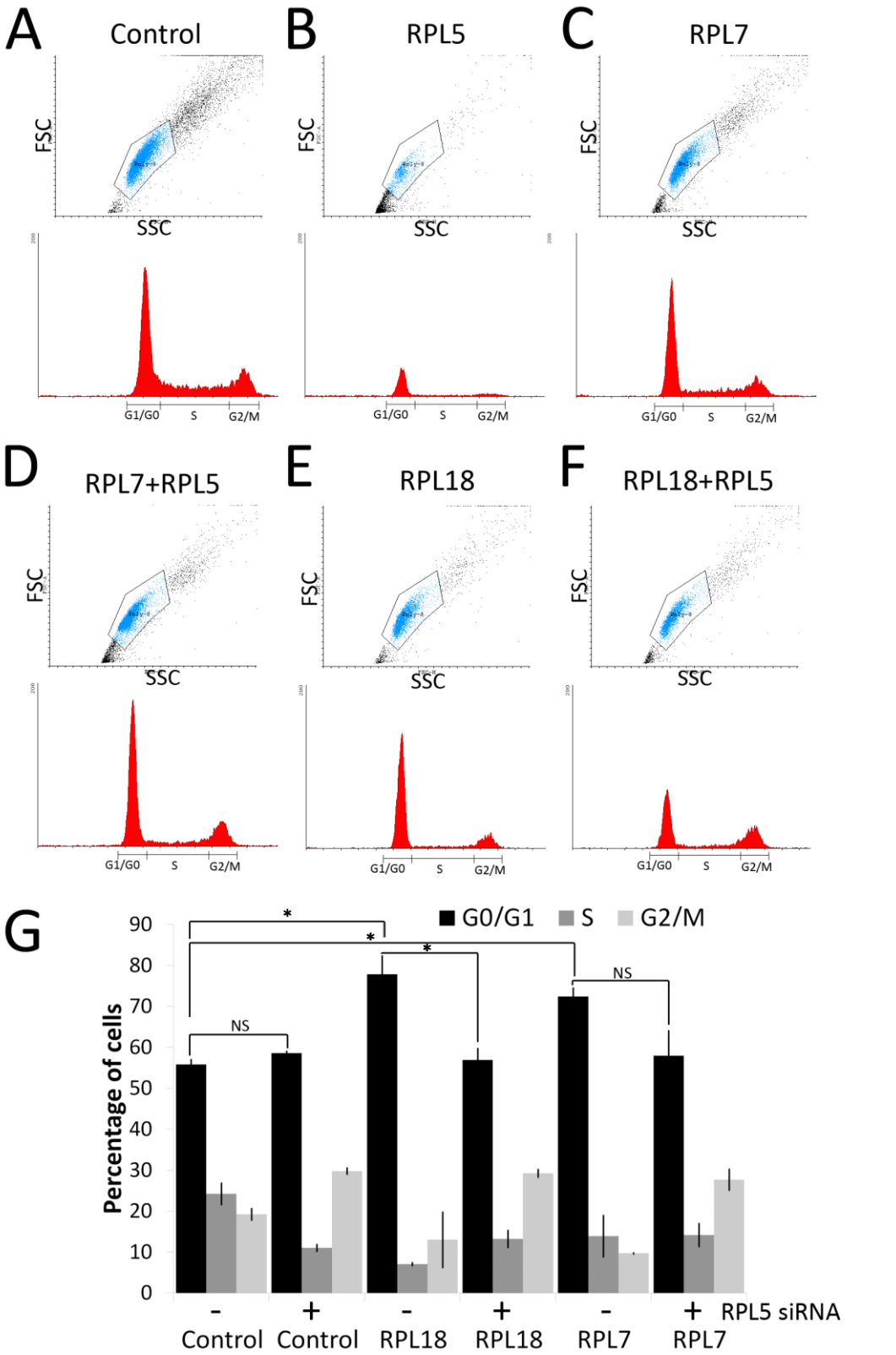

Figure 3.7. RPL7 and RPL18 knockdowns give an RPL5 dependent accumulation of cells in G1. (A-F) Cell cycle analysis using propidium iodide staining and flow cytometry in cells depleted of the ribosomal proteins.U2OS cells were incubated in growth medium containing 20µM (RPL5, RPL7) or 2µM (RPL18) siRNA duplexes at 37°C for 48 hours. The cells were harvested and fixed in ice cold ethanol before the addition of propidium iodide and RNAse before flow cytometric analysis. The forward scatter (FSC) was plotted against the side scatter (SSC) and the live population of cells was gated on as shown in blue. **(G)** Graphical representation of the proportion of cells at each stage of the cell cycle from E-F. Bars in G represent the means ± the standard deviation of the means, n=3. NS= non-significant, *= P ≤ 0.05, **= p≤ 0.01, ***= p≤ 0.001.

3.2.6 *In vitro* binding assays to show RPL11/28S rRNA interactions

Integration of the 5S RNP into the ribosome in yeast requires the 5S RNP to dock onto helix 84 of the 25S rRNA (28S in humans) (Rhodin et al., 2011). From investigation into the crystal structure of *T. thermophila (Klinge et al., 2011)*, it appeared that the region where RPL11 and 28S rRNA interaction occurred overlapped with the site of MDM2 and RPL11 interactions. This raised questions as to whether RPL11 binding to this region of the 28S rRNA could block interactions between RPL11 and MDM2. Therefore, recombinant RPL11 and RPL5 were expressed in purified from *E. coli* cells and 28S rRNA fragments were transcribed *in vitro* in order to test the interactions between them.

3.2.6.1 Creation of rRNA fragments

There were two different rRNA species that were tested for binding to RPL5 and RPL11. As the 5S rRNA is part of the 5S RNP complex with RPL5 and RPL11, the 5S rRNA was used as a control rRNA. A full length transcript of the 5S rRNA was transcribed, using cDNA extracts from U2OS cells as a template, and labelled with ^{32}P for use in pull-down experiments.

The area of the 28S that appeared to contact RPL11 in the same region as MDM2 consisted of helices 83-85 (Figure 3.8A). In order to test binding to RPL11, two different fragments of the helix 83-85 of the 28S rRNA were created (Figure 3.8B). One fragment contained helix 84, a small 20bp fragment (Figure 3.8-green), whilst the other was a larger 57bp fragment consisting of helices 83-85 (Figure 3.8- purple). Both fragments were transcribed in vitro using cDNA reverse transcribed, using U2OS cell RNA extracts as a template, and labelled with ^{32}P for use in interaction studies.

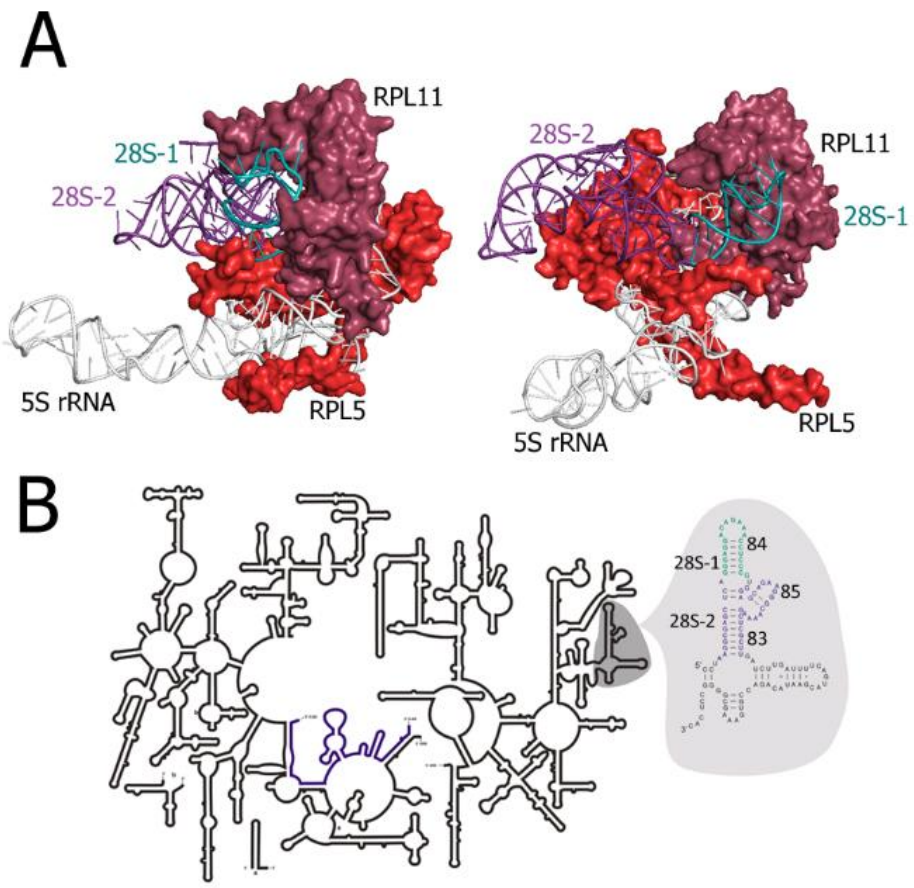

Figure 3.8. The 28S rRNA contacts RPL11 in the ribosome. (A) Cryo-EM structures from the human ribosome (Khatter et al., 2015) showing the helix 83-85 region of the 28S rRNA in contact with the 5S RNP. The two 28S fragments, 28S-1 and 28S-2 are denoted in green and purple, respectively. **(B)** A diagrammatic representation of the 28S rRNA secondary structure denoting the 28S region of interest.

3.2.6.2 Purification of RPL5 and RPL11 proteins to use in pull-down experiments

E. coli cells were transfected with pGEX6p1 plasmids encoding GST-tagged RPL5 and RPL11 and grown in 2X YT broth at 37°C until an OD_{600} of 0.3. After the cells had grown to the desired density, a final concentration of 1mM IPTG was added for 18 hours at 18°C. The cells were harvested, lysed and then purified using glutathione-sepharose beads. It was consistently found that RPL5 was more difficult to purify and generally gave a much less concentrated protein than RPL11 (Figure 3.9A). A much larger volume of culture was therefore required to purify usable amounts of RPL5 than RPL11.

Anion-exchange chromatography was used to further purify the two proteins. Both proteins did not bind to the resin and were found in the flow-through as shown for RPL5 (Figure 3.9B) as the first peak. For RPL5, a second peak was seen, eluting at approximately 500 mM NaCl. As the 260nm level was greater than the 280nm level, it

was presumed that this peak was RNA and suggested that RPL5 purified bound to *E. coli* RNA. RPL5 could not be suitably purified using this approach and was found to be unstable after being run through the anion exchange column (Figure 3.9D). It was previously reported that little or no RNA co-purified with *X. laevis* RPL5 over-expressed in *E. coli* when a much shorter induction time was used (Scripture and Huber, 1995). Therefore, *E. coli* cells expressing RPL5 were induced for 3 hours at 37°C before harvesting and purifying proteins. Anion exchange chromatography of the purified proteins revealed that RPL5 not retained on the column and eluted as the first peak.

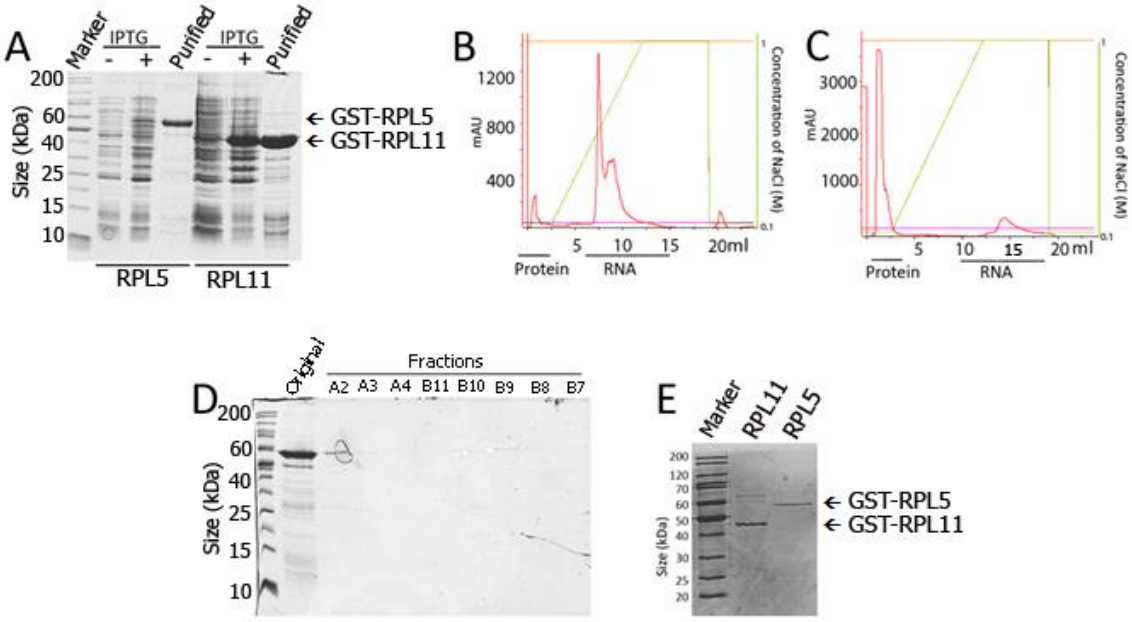

Figure 3.9. Protein purifications of RPL5 and RPL11. (A) *E.coli* BL21 cells transformed with plasmids for GST-tagged RPL5 and RPL11 were induced with 1 mM IPTG for 18 hours at 18°c before harvesting the cells. The cells were lysed and the lysates incubated with glutathione sepharose to purify the GST-tagged proteins. Samples were taken pre- and post-IPTG induction and run on a 13% SDS-PAGE gel together with the final, purified protein. **(B)** The chromatograph (254nm) of RPL5 from an anion exchange column. *E. coli* cells were grown and the proteins purified as in (A). The purified protein samples were applied to an anion exchange column. A large RNA peak can be seen with a fairly small protein peak. **(C)** As in B but the *E. coli* cells were grown for 3 hours at 37°c post-induction with IPTG. **(D)** Analysis of protein containing fractions from D on a coomassie stained SDS-PAGE gel. Fractions A2-A4 represent the first peak whilst B11-B7 represent the second. **(E)** The final purified products of RPL5 and RPL11 for use in interaction studies after anion exchange and then applying the sample to a desalting column.

Furthermore, the second peak, eluting at approximately 500 mM NaCl was significantly reduced,(Figure 3.9C). This implied that significantly less RNA co-purified with RPL5 and that this was a better approach for obtaining RNA-free RPL5. Therefore, these methods were employed for purification of GST-RPL5 for further experimentation. The final resultant purified GST-RPL5 and GST-RPL11 can be seen in Figure 3.9E.

3.2.7 RPL11 directly binds helix 83-85 of the 28S rRNA but not the 5S rRNA

In order to investigate whether RPL11 was able to directly bind the 5S rRNA, equal amounts of the purified GST-tagged RPL5 or RPL11 were bound to glutathione-sepharose beads before the addition of the ^{32}P labelled 5S rRNA, 28S-1 (helix 84) or 28S-2 (helix 83-85) fragments.

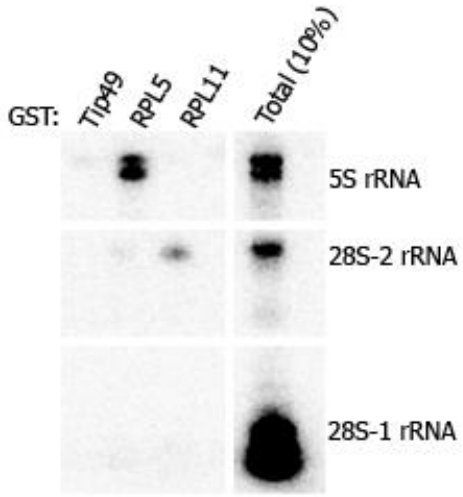

Figure 3.10. RPL11 directly binds to helix 83-85 of the 28S rRNA. Protein-RNA interaction study using recombinant human proteins purified from *E. coli*. Tip49, RPL5 or RPL11-GST plasmids were transfected into E. coli BL21 cells. The cells were grown and induced with IPTG to induce expression of the GST-tagged proteins. After lysis, glutathione sepharose was incubated with the lysate to bind the GST-tagged proteins. The proteins underwent further purification via anion exchange and desalting. The purified proteins were bound to glutathione-sepharose. Three rRNA species (5S, 28S-1 and 28S-2) were transcribed *in vitro* and labelled with ^{32}P before incubating with the bead and protein mixture. Any unbound RNA was washed off before eluting the bound protein/RNA followed by RNA extraction. Denaturing acrylamide-urea gels were utilised to separate the proteins before drying the gels and exposing to phosphorimager screens.

TATA binding protein-interacting protein of 49kDa (Tip49) protein extracts were provided by Dr. Katherine Sloan and used as a control protein as Tip49 was previously demonstrated to not bind RNA (McKeegan et al., 2009). Bound RNAs were isolated and then analysed by polyacrylamide gel electrophoresis (8% acrylamide-urea gels (28S-2 and 5S) or 12% acrylamide-urea gels (28S-1). The gels were fixed, dried and exposed to a phosphorimager screen.

Tip49 did not bind the 5S rRNA, suggesting specificity of binding. RPL5 consistently showed direct binding to the 5S rRNA (Figure 3.5). However, RPL11 did not bind to the 5S rRNA, suggesting that it is not able to directly bind to the 5S rRNA. This suggests

that there may be an induced fit change in the structure of the 5S rRNA upon RPL5 binding that then allows RPL11 to bind.

RPL11 was bound to the full helix 83-85 (as described in Figure. 3.8) 28S-2, the larger of the two fragments but not the smaller (helix 84) fragment (Figure 3.5). This implied that either helix 84 did not contain the region for binding to RPL11 or that the small rRNA fragment did not fold correctly for binding- although it was not clear which.

3.3 Discussion

Understanding how the 5S RNP is recruited into the ribosome is vital to understanding p53 regulation. This chapter aimed to address this issue by looking at 4 ribosomal proteins and a fragment of the 28S rRNA to see how they interact with the 5S RNP and, in the case of the ribosomal proteins, how this affects p53 signalling in U2OS cells.

Based on the crystal structure of the *T. thermophila* ribosome, RPL7, RPL10, RPL18, and RPL21 were identified as proteins that may be involved in 5S RNP recruitment into the ribosome due to their close proximity to the 5S RNP. Ribosomal proteins are known to be required for many different stages of rRNA processing (O'Donohue et al., 2010) although at present very little information is available on the roles of RPL10, RPL18 and RPL21 in ribosome biogenesis. However, in *S. cerevisiae*, depletion of RPL7 showed a decrease in the levels of 28S with an accumulation of the 45S and 41S rRNA precursors. Furthermore, it is thought that RPL7, along with RPL8, might be necessary for the association and function of the A_3 assembly factors in yeast (Jakovljevic et al., 2012). Therefore, in this chapter, knockdowns of RPL7, RPL10, RPL18, and RPL21 were performed and analysed by northern blot analysis and pulse-chase labelling to determine the rRNA processing defects and whether the same defects were shown as described previously. The data showed that depletion of RPL7, RPL18 and RPL21 resulted in ribosome biogenesis defects (Figure 3.5). RPL7 and RPL18 depletion resulted in the accumulation of the 36SC precursor in the RPL7 and RPL18 knockdowns (Figure 3.5B). This precursor is not usually found in cells and indicates a defect in site 2 cleavage (Sloan et al., 2013b), as shown in Figure 3.11. As site 2 in humans is thought to be equivalent to the A_3 cleavage site in yeast (Sloan et al., 2013b), this supports previous findings that RPL7 is required for the functioning of the A_3 factors (Jakovljevic et al., 2012). Furthermore, the RPL21 knockdowns resulted in reduction in 28S and

5.8S but an accumulation in 32S (Figure 3.5). This could be attributed to a defect in ITS2 cleavage, resulting in a lack of cleavage between the 5.8S and 28S rRNAs (as shown in Figure 3.11). This supports recent findings in yeast that RPL7 and RPL18 are both involved early in ribosome biogenesis whilst RPL21 is a later acting factor (Gamalinda et al., 2014). Interestingly, depletion of RPL10 showed no rRNA processing defects. However, it was previously reported that the RPL10 mutation, R98S, caused ribosome biogenesis and proliferation defects in mammalian lymphoid cells (Keersmaecker et al., 2013). Therefore, further experimentation is required to fully understand the role of RPL10 in ribosome biogenesis.

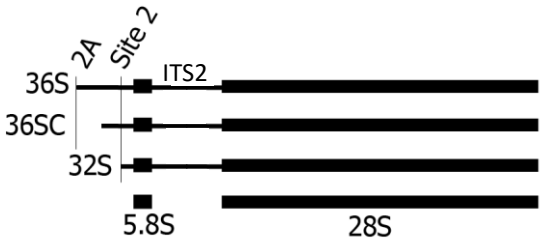

Figure 3.11. rRNA processing defects in ITS1 and ITS2. Schematic representation of the rRNA precursors showing defects in cleavage of site 2 results in the 36SC precursor instead of the 32S precursor. Defects in ITS2 cleavage result in accumulation of the 32S precursor.

These data, taken together, suggest that RPL7 and RPL18 are required for site 2 cleavage in ITS1 to separate the large and small subunit rRNAs, whilst RPL21 is required for cleavages in ITS2 to separate the 5.8S and 28S rRNAs. This data provides a novel insight into the role of these ribosomal proteins in large subunit rRNA processing, helping to unravel the complicated human rRNA processing pathway.

5S RNA incorporation into the ribosomes is an important step as disruptions to this process result in the activation of p53 (Sloan et al., 2013a). Currently it is known in yeast that Syo1 transports RPL5 and RPL11 into the nucleus and acts as an assembly platform for the 5S RNP (Calvino et al., 2015). Rpf2 and Rrs1 then chaperone the complete 5S RNP complex into the ribosome (Kharde et al., 2015, Madru et al., 2015, Morita et al., 2002, Zhang et al., 2007) where RPL11 docks onto the 25S rRNA (Rhodin et al., 2011) and the 5S RNP is locked into conformation by Rpf2, Rsa4 and Nsa2 to prevent any rotation (Kharde et al., 2015, Bassler et al., 2015). The final stage of 5S RNP incorporation relies on the removal of Rsa4 from the 5S RNP which allows for the rotation of the structure by 180^{o} to produce the 5S RNP conformation found in mature

ribosomes (Bassler et al., 2015, Leidig et al., 2014). However, in humans the homologues of Rrs1 and Rpf2, RRS1 and BXDC1, are not associated with the 5S RNP outside of pre-ribosomes and depletion of RRS1 and BXDC1 has little effect on 5S RNP recruitment (Sloan et al., 2013a). However it was shown that depletion of the nucleolar protein, PICT-1 had a much greater effect on 5S RNP recruitment (Sloan et al., 2013a). Therefore, this suggests that the 5S RNP recruitment into the ribosome differs between yeast and ribosomes and thus it was important to investigate this further. The data presented in this chapter offers the possibility that RPL7, RPL18 and RPL21, but not RPL10, are required for integration of the 5S rRNA into the ribosome (Figure 3.4). As these factors severely affect large subunit production (Figure 3.5), it was unsurprising that they resulted in a defect in 5S RNP recruitment into the ribosome. However, it had been previously demonstrated that factors involved in large subunit production do not all have the same effect on 5S rRNA incorporation into the ribosome. Depletion of PICT-1, RRS1, or RPL11 had a similar effects on large subunit production but depletion of RPL11 and PICT-1 had a much stronger influence on 5S rRNA integration into the ribosome than that of RRS1 (Sloan et al., 2013a). As the data presented in this chapter showed that depletion of RPL7, RPL18, or RPL21 have a profound effect on large subunit production and have a similar effect on 5S rRNA incorporation into the ribosomes to that of RPL11, this suggests that these proteins may be affecting 5S RNP integration directly. Therefore, it is possible that the 5S RNP incorporation into the ribosome requires interactions with RPL7, RPL18, RPL21 and the 28S rRNA as a binding pocket.

Depletion of RPL7, RPL18 and RPL21, but not RPL10 resulted in RPL5-dependent p53 accumulation in the cells. This suggests that accumulation of ‘free’ 5S RNP, as a result of the depletion of RPL7, RPL18 and RPL21, results in p53 activation. Furthermore, RPL7 and RPL18 depletion resulted in an accumulation of cells in G1/G0 which is consistent with p53 activation (Lin et al, 1992). The data in this chapter, therefore, suggests that depletion of RPL7, RPL18 and RPL21, by decreasing 5S RNP incorporation into the ribosome (Figure 3.4) and affecting ribosome biogenesis (Figure 3.5), induce p53, leading to a cell cycle defect with cells accumulating in G1.

Interestingly, RPL5 depletion resulted in an accumulation of cells in G2. It was previously shown in yeast that depletion of ribosomal proteins leads to either G1 or G2

arrest (Thapa et al, 2013) whilst in mouse embryonic stem cells, haploinsufficiency of RPL5 resulted in delayed G2 progression (Singh et al., 2014). However this is conflicting with Teng et al. (2013) who demonstrated that depletion of RPL5 had no effect on cell cycle progression. The data in this study (Figure 3.7) is in agreement with Singh et al. (2014) that a reduction in RPL5 leads to delayed G2 progression, although the mechanisms behind this are, at current, unknown. Investigation of the factors involved in this G2 arrest could help to uncover new treatments for patients with mutations in RPL5 and RPL11.

Whilst depletion of RPL7, RPL18, and RPL21 all affected 5S RNP integration into the ribosome and induced p53, depletion of RPL10 did not. RPL10 has been shown to be to be a late-acting ribosome biogenesis factor that is recruited in the cytoplasm in yeast (Johnson et al., 2002, Nguyen et al., 1998, Gamalinda et al., 2014) and it was demonstrated that depletion of Rpf2/Rrs1 prevented RPL10 association with pre-ribosomes (Zhang et al., 2007) suggesting that RPL10 recruitment occurs after 5S RNP integration, which may offer an explanation for why RPL10 did not affect 5S RNP integration. Furthermore, the RPL10 knockdown (Figure 3.3) did not appear to be as efficient as the knockdown for RPL7, RPL18, and RPL21 and thus it must be taken into consideration that this may affect the downstream results. Therefore, if a more efficient knockdown could be attained and the same results were seen, this would help to support the findings in this chapter.

The human ribosome structure has been solved during the course of this project (Khatter et al, 2015) and shows some differences in the binding pocket (Figure 3.12). In human ribosomes, RPL7 makes less contact with the 5S RNP, which may explain why the RPL7 depletions had less of an impact on p53 signalling than the RPL21 or RPL18 depletions. RPL18 makes the most contact around the 5S RNP, possibly contributing to the larger impact on p53 levels. As it was also shown that, in yeast, the 5S RNP rotates upon incorporation into the ribosome (Leidig et al., 2014) , this suggests that the position of the complex in the mature ribosome is not the same at the time of incorporation. However, despite this, the data presented in this chapter suggests that RPL7, RPL18 and RPL21 may play a role in recruitment of the 5S RNP, therefore suggesting that the 5S RNP requires contact with RPL7, RPL18, RPL21 and the 28S rRNA for its integration into the ribosome. It would be beneficial in the future to be able to

investigate whether RPL7, RPL18 or RPL21 are mutated in any diseases as this may implicate p53 in the pathogenesis of these conditions. Therefore, published array data could be analysed to detect any changes in the levels of those proteins in various conditions.

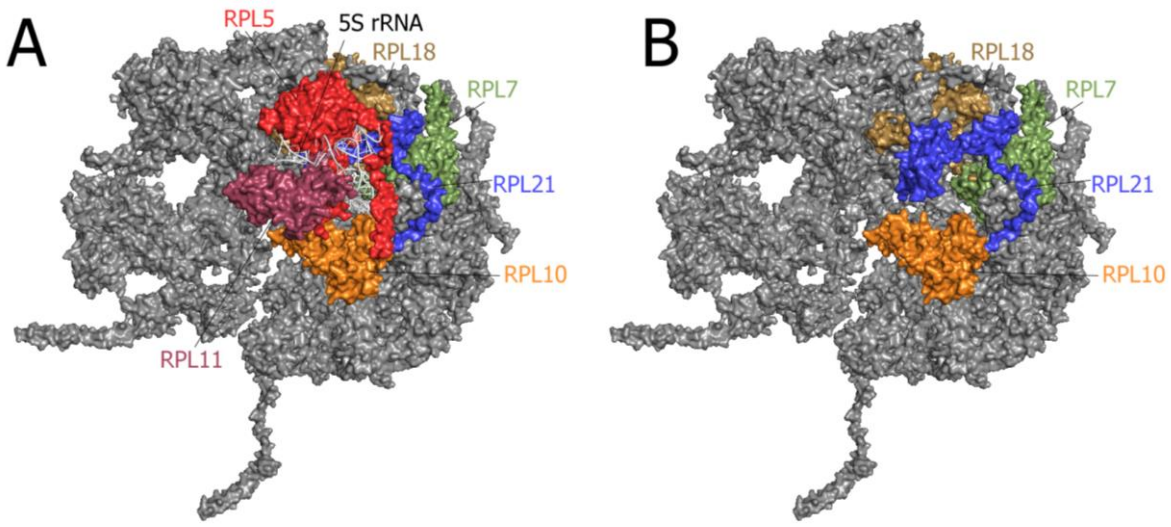

Figure 3.12 The cryo-EM structure of the human ribosome showing the RNP and the surrounding ribosomal proteins. Top view of the human ribosome structure showing the 4 ribosomal proteins, RPL7, RPL10, RPL18 and RPL21 with **(A),** or without **(B),** the 5S RNP. The 5S rRNA is shown in white, RPL5 in red and RPL11 in purple. RPL18 is brown, RPL7 is green, RPL21 is blue, RPL10 is orange and the 28S rRNA is in grey.

The second part of this chapter investigated the ability of RPL11 to bind to the 28S rRNA. As previously described, the 5S RNP docks onto helix 84 of the 25S/28S rRNA upon integration into the ribosome (Rhodin et al., 2011). By analysis of the *T. thermophila* ribosome crystal structure (Klinge et al., 2011) it was noted that the 28S appeared to contact RPL11 in the same region that MDM2 binds (Zhang et al., 2011). Upon investigation, it was found that helices 83-85 of the 28S rRNA (Figure 3.8) bind directly to GST-RPL11 (Figure 3.10). Helix 84 alone did not bind to GST-RPL11, implying that it is either missing the crucial binding region or that it does not fold correctly. This could be an important finding as helix 83-85 could potentially be used therapeutically to block 5S RNP-mediated p53 induction. During the course of this project, the structure of the RPL11-MDM2 complex was solved (Zheng et al., 2015) and the authors demonstrated that the binding of the 28S rRNA to RPL11 and MDM2 was mutually exclusive (Figure 3.13), further supporting the data in this body of research.

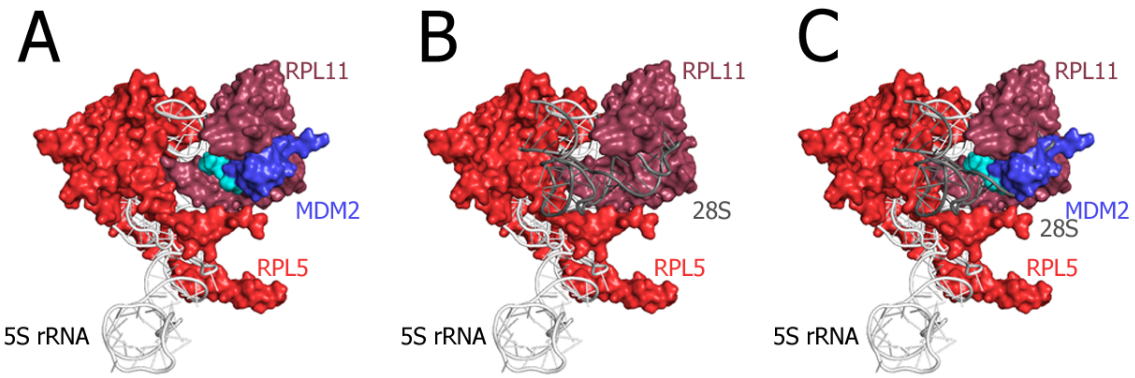

Figure 3.13 The binding of the 28S rRNA and MDM2 to RPL11 are mutually exclusive. (A) Interaction between the 5S RNP and MDM2. Cryo-EM structure from the human ribosome (Khatter et al., 2015) super-imposed with the MDM2-RPL11 structure (Zheng et al., 2015). RPL5 is shown in red, RPL11 in purple and the 5S rRNA in white. MDM2 is shown in blue with the lighter shade representing the zinc finger domain and the dark blue the acidic domain. **(B)** As in A but with the 28S rRNA-5S RNP interaction in the ribosome. The 28S rRNA is shown in grey. **(C)** A combination of A+B to show overlapping regions.

An interesting observation from this study was that GST-RPL11, under the conditions used, appeared to be unable to bind the 5S rRNA. However, GST-RPL5 was shown to bind the 5S rRNA. This suggested that, as RPL5 and RPL11 are both known to bind the 5S rRNA in the RNP but not each other, RPL11 may require RPL5 binding first in order to bind the complex. This could be an important factor for the formation of the 5S RNP as there may be mechanisms in place to regulate these binding steps. Furthermore, it suggested that RPL5 may induce a conformational change in 5S upon binding that then allows for the binding of RPL11. Interestingly, it was demonstrated in *X. laevis* that the binding of RPL5 to the 5S rRNA caused structural changes in the region of the rRNA that binds RPL11 (Scripture and Huber, 2011) and thus my data may support these findings.

This work has revealed many insights into how the 5S RNP is recruited into the ribosome and has laid a foundation for future research. It was shown that the helix 83-85 of the 28S rRNA is able to bind to RPL11 and thus it would be interesting to test the hypothesis that this rRNA fragment can prevent RPL11 binding to MDM2 *in vitro*. In addition, introduction of this rRNA fragment into cells to attempt to block MDM2/RPL11 interactions could provide a novel way to prevent p53 induction in cells. Furthermore, the data presented in this chapter has shown that RPL7, RPL18 and RPL21 are ribosome biogenesis factors, involved in ITS1 and ITS2 processing respectively, that are required for integration of the 5S RNP into the ribosome.

CHAPTER FOUR: INVESTIGATION INTO THE ROLE OF SRSF1 AND PRAS40 AS POTENTIAL REGULATORS OF THE 5S RNP-P53 PATHWAY

4.1 Introduction

Ribosomes are essential for the translation of mRNA into proteins and the production of ribosomes dictates cell growth, proliferation and differentiation (Rudra and Warner, 2004, Warner, 1999b, Sulic et al., 2005, Thomas, 2000). As ribosomes are crucial to the survival of cells, ribosome biogenesis is tightly regulated. When ribosome biogenesis is disrupted, one mechanism the cell has to protect itself is activation of the tumour suppressor, p53. Induction of p53 triggers pathways involved in DNA damage repair, cell cycle arrest, senescence, and apoptosis (Ko and Prives, 1996, Timofeev et al., 2013, Sperka et al., 2012, Sugikawa et al., 1999). In normal, unstressed cells, MDM2 polyubiquitinates p53 and targets it for the degradation by the 26S proteasome (Honda and Yasuda, 1999). Furthermore, p53 is regulated by ARF which binds to, inactivates, and promotes degradation of MDM2 (Zhang et al., 1998), thus inducing p53.

One component of the large subunit, the 5S RNP (Figure 4.1A), has been shown to play a crucial role in the regulation of p53 in cells. The 5S RNP has been shown to activate p53 through interaction with MDM2 and ARF, the process of which is summarised in Figure 4.1B. Many ribosomal proteins have been shown to bind to MDM2 (Zhang and Lu, 2009). However, it has since been established that the 5S RNP-MDM2 interactions are crucial for p53 regulation. Depletion of any of the 3 components of the 5S RNP were able to counteract the effects on p53 as a result of Actinomycin D (ActD) treatment to cells (Bhat et al., 2004, Dai and Lu, 2004, Sloan et al., 2013a, Bursac et al., 2012, Donati et al., 2013). ActD is a drug that activates p53 by selectively inhibiting Pol I transcription, preventing transcriptional elongation (Sobell, 1985) and thus induces ribosomal stress to cells as RNA Pol I transcribes the 5.8S, 18S and 28S ribosomal RNAs but, crucially, not the 5S rRNA. As the 5S RNP components were crucial for ActD-induced p53 activation, this suggested that the 5S RNP is involved in activating p53 in ribosomal stress situations. Furthermore, it has recently been demonstrated that the

5S RNP is crucial for the normal homeostasis of p53 (Sloan et al., 2013a) as well as for the activation of p53 during ribosomal stress (Donati et al., 2013, Sloan et al., 2013a).

In addition to activating p53 by interacting with MDM2, one other way that the 5S RNP regulates p53 signalling is through the tumour suppressor, ARF. ARF binds to and inhibits MDM2, allowing for p53 activation (Honda and Yasuda, 1999) and it has been recently shown that 5S RNP is essential for the full activity of ARF (Sloan et al., 2013a). As ARF expression inhibits ribosome biogenesis (Bertwistle et al., 2004, Itahana et al., 2003), this suggested that ARF is able to activate p53 by both directly binding to MDM2 and also by increasing the amount of non-ribosomal 5S RNP by disrupting ribosome biogenesis. All of these data taken together showed the 5S RNP to be a vital link between cell proliferation and ribosome production.

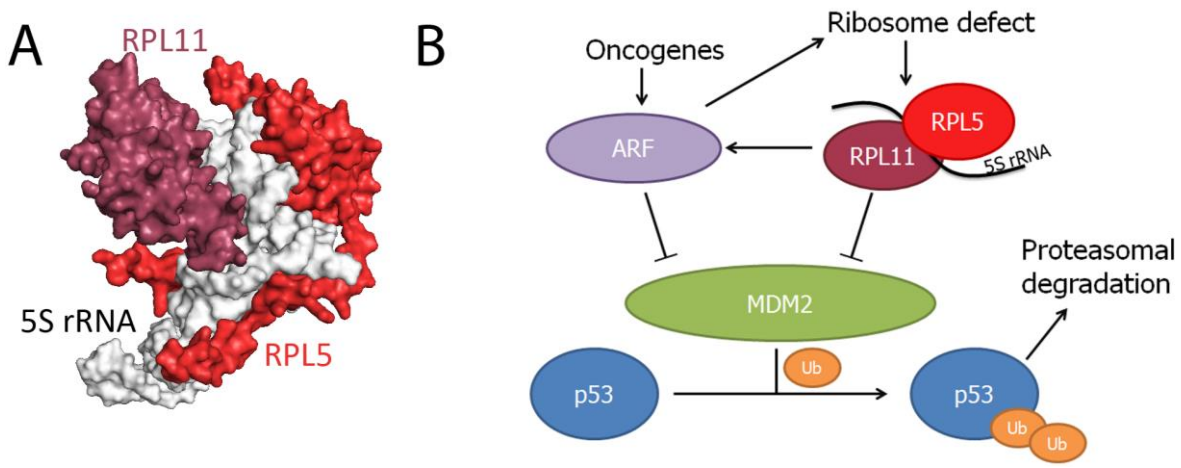

Figure 4.1 The role of the 5S RNP in p53 signalling (A) The surface view of the structure of the 5S RNP from the human ribosome cryo-EM structure (Khatter et al., 2015). The 5S RNP is composed of the 5S rRNA (white) with the ribosomal proteins RPL5 (red) and RPL11 (purple). (B) The current proposed pathway for the interactions between the 5S RNP and p53. Ribosome defects lead to accumulation of the 5S RNP which can bind to MDM2. MDM2 is a negative regulator of p53 but in the presence of the 5S RNP, the suppression is lost and p53 activated. ARF is a suppressor of MDM2 that can also be activated by the 5S RNP to further activate p53.

Defective ribosome production and, consequently, the activation of p53, can lead to diseases known as ribosomopathies and so a greater understanding of 5S RNP signalling may lead to potential new therapies for these diseases. One ribosomopathy is Diamond Blackfan Anaemia (DBA) which is characterized by a severe reduction in erythroid precursor cells due to apoptosis or a failure to differentiate (Quarello et al., 2010a). Almost half of all DBA patients have haploinsufficiency mutations in the ribosomal protein genes of which some of these are in RPL11 or L5. 70% of patients

with physical malformations carry RPL5 and RPL11 mutations. Generally, RPL5 mutations are linked to very severe forms of DBA and can lead to thumb defects, cleft lip and/or palate which are not seen with other DBA mutations (Quarello et al., 2010a, Gazda et al., 2008).

One explanation for the pathogenesis of DBA is that defects in ribosomal proteins may lead to accumulation of p53 and thus cell-cycle arrest and apoptosis in erythroid cells (Dai and Lu, 2004). However, the mechanisms behind the pathogenesis of DBA with patients harbouring RPL5 and RPL11 mutations is less understood. It has been demonstrated that RPL11 expression in erythroleukaemic cells caused activation of STAT5 which acts as a promoter of angiogenesis (Kummalue et al., 2015). This suggested that RPL11 may be involved in the regulation of erythroid cell proliferation. However there is a need for a greater understanding of the pathogenesis of DBA as there are few effective treatments available apart from blood transfusions and bone marrow transplants.

One further treatment of DBA is with L-leucine. L-leucine was suggested due to the links with ribosome production defects and translational inefficacy in DBA patients as amino acids have been shown to regulate translation through mTOR signalling (Kimball and Jefferson, 2006). Initial tests in zebrafish and mice models of DBA resulted in an improvement in their anaemia upon L-leucine treatment (Boultwood et al., 2013, Jaako et al., 2012, Payne et al., 2012). In patient trials within 6 months the patients became transfusion independent and showed increased erythroid differentiation (Pospisilova et al., 2007). L-leucine is able to trigger the mTOR pathway (Lynch, 2001, Gran and Cameron-Smith, 2011) which has recently been closely linked to the 5S RNP via the proline-rich AKT Substrate of 40kDa (PRAS40) (Havel et al., 2014). Investigation of this link may further the understanding of the regulation of the 5S RNP and make treatment of DBA more effective.

PRAS40, also known as AKT1S1, is a protein that was first identified as AKT substrate that, when phosphorylated by AKT, is able bind to the scaffold protein, 14-3-3 (Kovacina et al., 2003). One of the major pathways that PRAS40 is important for is the regulation of mTORC1. mTORC1 is a complex of the mammalian Target of Rapamycin (mTOR), Raptor and mammalian lethal with SEC13 protein 8 (mLST8) that promotes cell growth in response to growth signals (Kim et al., 2003, Kim et al., 2002, Fonseca et

al., 2007). One way that mTORC1 is regulated via AKT is through PRAS40. When PRAS40 is non-phosphorylated, it binds to Raptor and inhibits mTORC1s kinase activity. In the presence of growth factors, PRAS40 is phosphorylated by mTORC1 and AKT, dissociates from Raptor and results in the activation of mTORC1 (Thedieck et al., 2007, Fonseca et al., 2007).

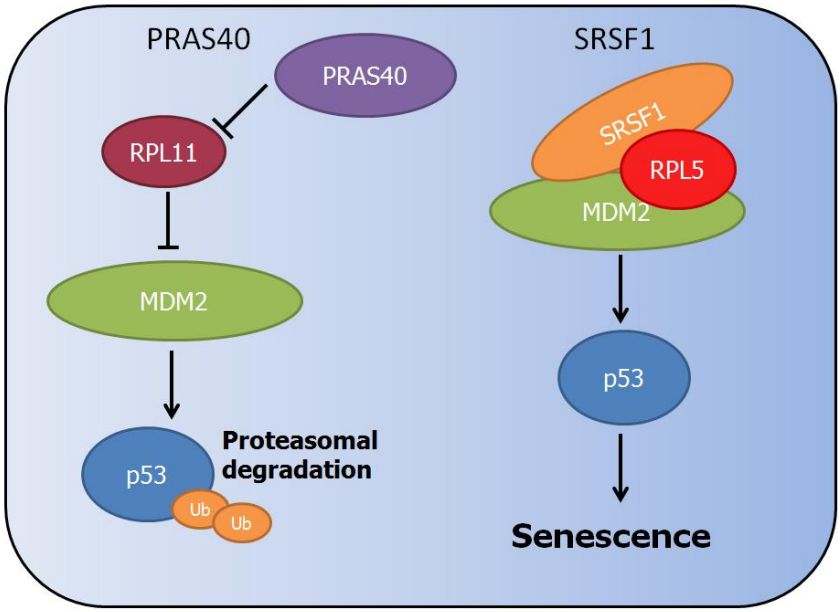

Figure 4.2 Summary of the current knowledge of the involvement of PRAS40 and SRSF1 in p53 signalling. PRAS40 is known to interact with RPL11, preventing its interaction with MDM2. MDM2 polyubiquitinates p53 and targets it for proteasomal degradation, thus inactivating p53. SRSF1 interacts with RPL5 and MDM2 to block the ubiquitin ligase activity of MDM2, thus activating p53 and triggering senescence.

Havel et al. (2014) demonstrated, by immunoprecipitation, that nuclear PRAS40 was able to bind RPL11 and that this binding was dependent upon the phosphorylation of PRAS40 via mTORC1 and AKT at residues S221 and T246. Due to the nuclear nature of this binding, it was suggested that PRAS40-RPL11 complex does not occur within ribosomes. Further to this, it was shown that PRAS40 depletion in U2OS cells lead to an increase in p53 protein levels in an RPL11 dependent manner. When ActD was used at levels that increase p53 via the 5S-RNP pathway in conjunction with PRAS40 depletion, PRAS40 knockdown was found to have an additive effect suggesting that the two pathways were linked. It was hypothesised that PRAS40 may block RPL11 translocation from the nucleoli to the nucleoplasm and thus prevents its interaction with MDM2 as summarised in Figure 4.2. Interestingly, the complex containing RPL11 and PRAS40 was found to have a high molecular weight of between 300-700kDa and thus it was

hypothesised that this could contain other unidentified proteins. Questions, therefore, arise as to whether these proteins could be the other 5S RNP components.

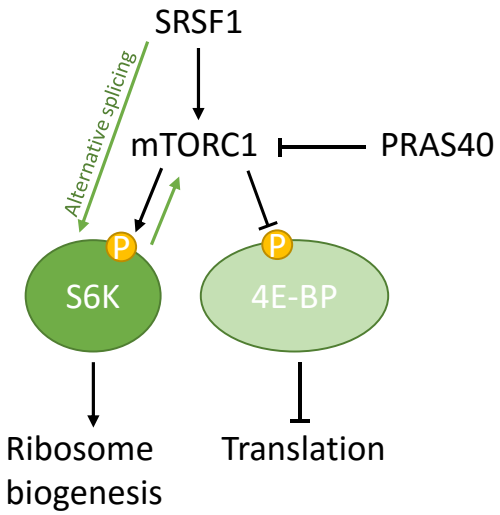

Figure 4.3 The role of PRAS40 and SRSF1 in the regulation of translation via mTORC1. Schematic representation of the two pathways SRSF1 regulates translation. The first pathway is indicated by black arrows. SRSF1 interacts with mTORC1 and results in phosphorylation of S6K and 4E-BP. Phosphorylation of S6K promotes ribosome biogenesis whilst phosphorylation of 4E-BP results in enhanced translation. The second pathway is indicated by green arrows. SRSF1 results in the generation of an alternatively spliced S6K variant that is able to upregulate mTOR and increase translation through the inhibition of 4E-BP. PRAS40 binds to and inhibits mTORC1, resulting in decreased ribosome biogenesis and translation.

Whilst PRAS40 may be suppressing the 5S RNP pathway, a potential activator of the pathway has also been identified. Serine/Arginine rich Splicing Factor 1 (SRSF1, also known as Alternative Splicing Factor- ASF) was shown to interact with RPL5 and induce p53. SRSF1 is an alternative splicing factor involved in a variety of different cellular processes such as chromatin remodelling (Loomis et al., 2009), translation (Michlewski et al., 2008), and mRNA surveillance (Zhang and Krainer, 2004). SRSF1 is upregulated in cancers such as breast cancer and over-expression of SRSF1 is able to trigger oncogenic transformation in mouse and human cells (Anczukow et al., 2012). Furthermore, SRSF1 interacts with translating ribosomes (Sanford et al., 2005, Sanford et al., 2004). SRSF1 regulates alternative splicing of the ribosomal protein S6 kinase (S6K), producing S6K short isoforms which bind to mTORC1 and enhance 4E-BP phosphorylation, increasing translation initiation (Karni et al., 2007, Ben-Hur et al., 2013). Like PRAS40, SRSF1 is also able to interact with mTORC1 and increases the phosphorylation of S6K and 4E-BP, enhancing translation efficiency (Michlewski et al., 2008) as summarised in Figure 4.3.

Fregoso et al. (2013) demonstrated a role for SRSF1 in p53 signalling via RPL5. Interactions were identified between RPL5 and SRSF1, whereas there was no evidence of the involvement of RPL11 or the 5S rRNA. SRSF1 depletion was able to counteract the p53 induction from ActD treatment. As Act D, at concentrations below 10ng/ml, is able to stimulate p53 in a specifically 5S-RNP dependent manner, this suggested that SRSF1 was important for p53 induction via Actinomycin D. In addition, it was shown that when SRSF1 was over-expressed in BJ-TT cells, p53 was induced in an RPL5 dependent manner. This p53 response resulted in senescence. SRSF1 was also found to bind to MDM2 and thus it was suggested that RPL5, MDM2 and SRSF1 form a distinct complex as summarised in Figure 4.2. This contradicts the findings that all 3 components of the 5S RNP are required to activate p53 (Sloan et al., 2013a, Donati et al., 2013) and so it is currently unclear as to whether the 5S RNP is involved or whether RPL5 is acting independently.

SRSF1 was shown to assist in RPL5-dependent p53 activation, whilst PRAS40 was demonstrated as an inhibitor of RPL11-dependent p53 activation. Furthermore, as SRSF1 enhances mTOR activity whilst PRAS40 negatively regulates the pathway (Figure 4.3), these data together suggest that the SRSF1 and PRAS40 may antagonise one another. As mTOR is activated by L-leucine, this could be an important step to understanding the role of L-leucine treatment in patients with DBA.

The aims of this chapter were to establish whether SRSF1 and PRAS40 interact with the 5S RNP as a whole and investigate how this affects ribosome biogenesis and 5S RNP-p53 signalling in U2OS cells. During this chapter I investigated the effect of both over-expression and depletion of SRSF1 and PRAS40 on the 5S RNP signalling pathway through investigating the 5S RNP integration into the ribosome, p53 signalling, localisation of RPL5 and RPL11, and cell cycle progression.

4.2 Results

4.2.1 Creation of inducible cell lines expressing FLAG-SRSF1 and FLAG-PRAS40

In order to study over-expression of SRSF1 and PRAS40 in cells, cell lines were created expressing FLAG-tagged SRSF1 and PRAS40. Furthermore, the effect of depletion of SRSF1 and PRAS40 was investigated. As previous experiments into SRSF1 and PRAS40 interacting with the 5S RNP had been carried out in different cell lines (Fregoso et al., 2013, Havel et al., 2014), it was necessary to establish that endogenous PRAS40 and SRSF1 could be detected in U2OS cells. Therefore, SRSF1 and PRAS40 protein levels were investigated in a panel of human cell lines; HEK293, HeLa, MCF-7 and U2OS.

The cells were grown at 37^{o}C in growth medium until 80% confluent before harvesting, separating whole cell extracts via SDS-PAGE and western blotting. Anti-PRAS40 and anti-SRSF1 antibodies were used with an α-Tubulin antibody as a loading control (Figure 4.4A-B). In Figure 4.3A, it was noted that the PRAS40 antibody gave an additional signal of a higher molecular weight which was presumably cross reactivity or an isoform of PRAS40 with a higher molecular weight. Depletion of PRAS40 (shown later in Section 4.2.6) resulted in a decrease in the lower molecular weight signal, identifying this as PRAS40. SRSF1 and PRAS40 could be detected in all 4 cell lines (Figure 4.4A-B). As the cell lines expressed endogenous PRAS40 and SRSF1, it was therefore possible to study the effect of depletion of the proteins in these cell lines.

In order to create cells expressing FLAG-tagged SRSF1 and PRAS40, U2OS Flp-In cells were co-transfected with pcDNA5 plasmids containing either the PRAS40 or SRSF1 coding sequences. The pcDNA5 plasmid contains a tetracycline inducible promoter and thus the expression of the protein can be controlled. The cells containing the insert were selected using hygromycin B and selection was maintained throughout culture.

The selected cells were incubated at 37^{o}C with media supplemented with 0-1 μg/ml tetracycline for 48 hours before harvesting and separating whole cell lysates via SDS-PAGE. Western blot analysis (Figure 4.4C-D) showed that both proteins were induced, as indicated by an additional signal from the anti-SRSF1 and anti-PRAS40 antibodies and a signal from the anti-FLAG which is not present in the un-induced cells. A higher molecular weight signal was seen using the PRAS40 antibody without the addition of

tetracycline. As this was not detected with the anti-FLAG antibody, it is possible that this a cross-reactive band as seen in Figure 4.4A.

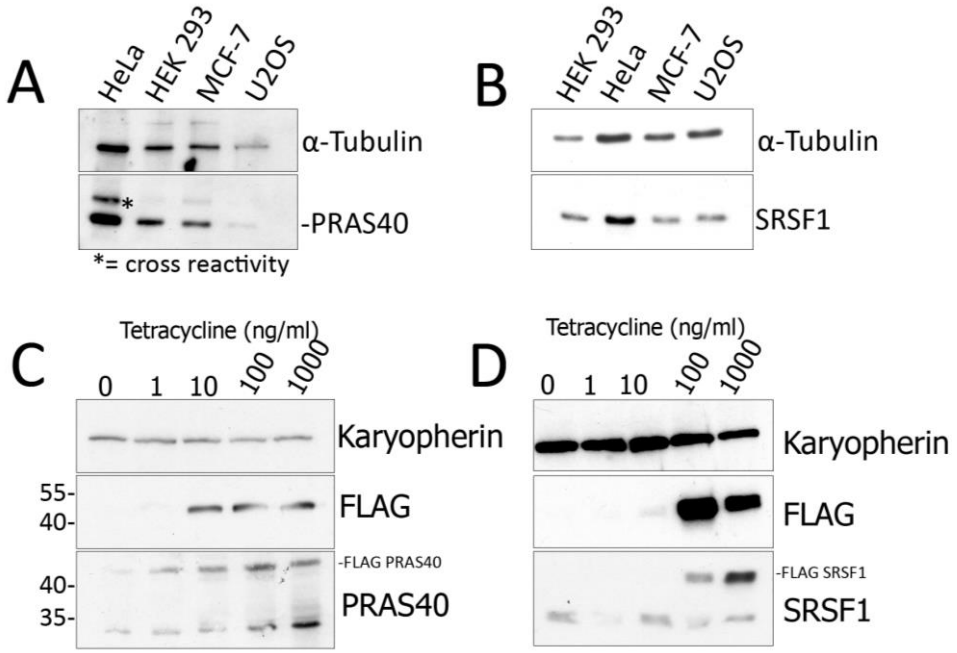

Figure 4.4. SRSF1 and PRAS40 expression in cell lines. **(A)** and **(B)** Endogenous PRAS40 and SRSF1 expression across different cell lines. Cells harvested from HeLa, HEK 293, MCF-7 and U2OS cells were grown until 80% confluent before whole cell extracts were separated by SDS-PAGE. Western blot analysis was performed using anti-PRAS40 and anti-SRSF1 antibodies to show endogenous expression. * indicates cross reactivity. **(C)** and **(D)** Western blots to show expression of the FLAG-tagged proteins. U2OS cells containing FLAG-tagged PRAS40 or SRSF1 were induced with 0-1 µg/ml tetracycline, to induce expression, for 48 hours before harvesting. Whole cell extracts were separated by SDS-PAGE before western blotting using antibodies raised against FLAG, PRAS40, SRSF1, or Karyopherin.

This data as a whole suggested that the proteins were being expressed in U2OS cells and that 1 µg/ml was an appropriate concentration of tetracycline to use for future experiments using the FLAG-SRSF1 and FLAG-PRAS40 stable cell lines as this showed a high level of expression.

4.2.2 Localisation of FLAG-SRSF1 and PRAS40

In order to ensure that FLAG-tagged SRSF1 and PRAS40 were being localised as expected, immunofluorescence was utilised. SRSF1 is normally localised in the nucleus in nuclear speckles (Koizumi et al., 1999, Caceres et al., 1997) whilst PRAS40 shuttles between the nucleus and the cytoplasm and has been shown to co-localise with RPL11 in the nucleoli in U2OS cells (Havel et al., 2014).

U2OS SRSF1 or PRAS40 expressing cells were grown on cover slips and incubated for 48 hours at 37°C in media supplemented with 1 µg/ml tetracycline. The cells were then used in immunofluorescence with anti-FLAG (to target the FLAG-tagged SRSF1 or PRAS40), anti-fibrillarin (to show nucleolar localisation) and DAPI (to stain the nucleus) (figure 4.5). The anti-FLAG antibody gave signal mostly in the nucleus with the expression of FLAG-SRSF1, as shown by co-localisation with DAPI. There was no evidence of any enrichment in the nucleoli as the signal was evenly distributed throughout the nuclei and did not have any stronger signal coinciding with the fibrillarin signal. There was very little SRSF1 detected in the cytoplasm.

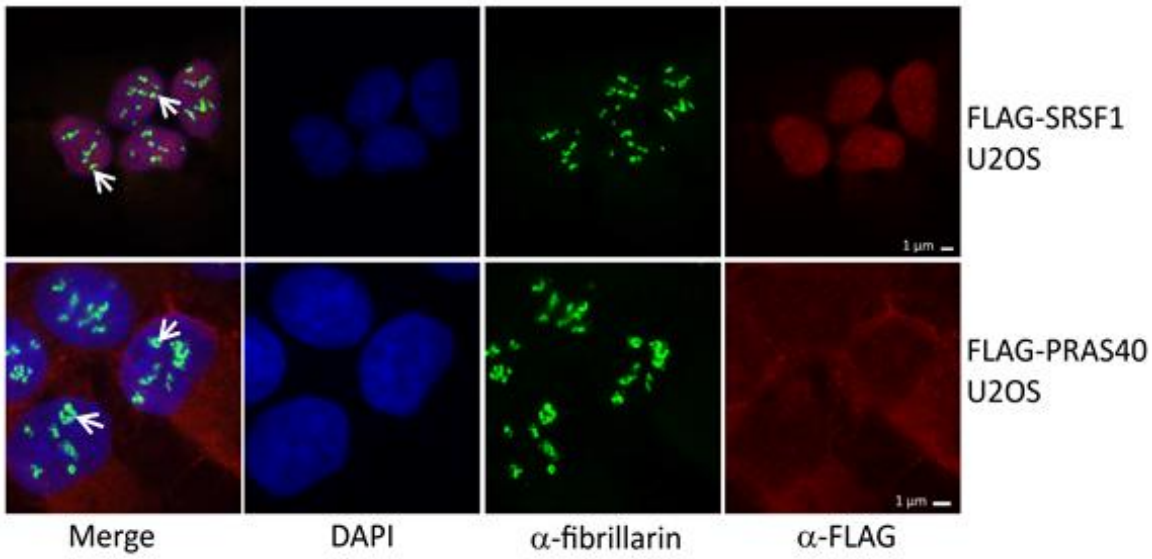

Figure 4.5. Localisation of FLAG-tagged SRSF1 and PRAS40. U2OS cells stably expressing tetracycline-inducible FLAG-SRSF1 or FLAG-PRAS40 were grown at 37°C on cover slips for 48 hours in medium containing 1 µg/ml tetracycline to induce protein expression. Cells were fixed in 4% paraformaldehyde and incubated with anti-FLAG (red) anti-fibrilliarin to indicate the nucleoli (green) and DAPI to stain the nuclei (blue). Examples of nucleoli are shown with white arrows. Scale bar represents 1 µm.

In the cells expressing FLAG-PRAS40, the anti-FLAG antibody was detected most strongly in the cytoplasm. There was little co-localisation of the FLAG antibody with the DAPI staining, suggesting that FLAG-PRAS40 is not found in the nucleus. Similarly to the FLAG-SRSF1 cells, there was no enrichment of FLAG-PRAS40 in the nucleoli as demonstrated by no co-localisation of the FLAG antibody signal with that of the fibrillarin antibody. This suggests the possbility that the FLAG-PRAS40 used in this project is not normally localised in the nucleoli of U2OS cells, in contrast to what was previously found using a different FLAG-PRAS40 expression system (Havel et al., 2014).

4.2.3 Expression of FLAG-tagged SRSF1 and PRAS40 increase p53 levels in U2OS cells

SRSF1 over-expression was shown to induce p53 in BJ-TT cells, however, the effect of PRAS40 over-expression on p53 had not been reported, therefore SRSF1 and PRAS40 over-expression studies were carried out to see whether this could be replicated in U2OS cells. U2OS Flp-In cells expressing either PRAS40 or SRSF1 were incubated in growth medium supplemented with 0-1µg/ml tetracycline for 48 hours. Whole cell lysates were separated by SDS-PAGE before western blotting (Figure 4.6). Karyopherin was used as a loading control.

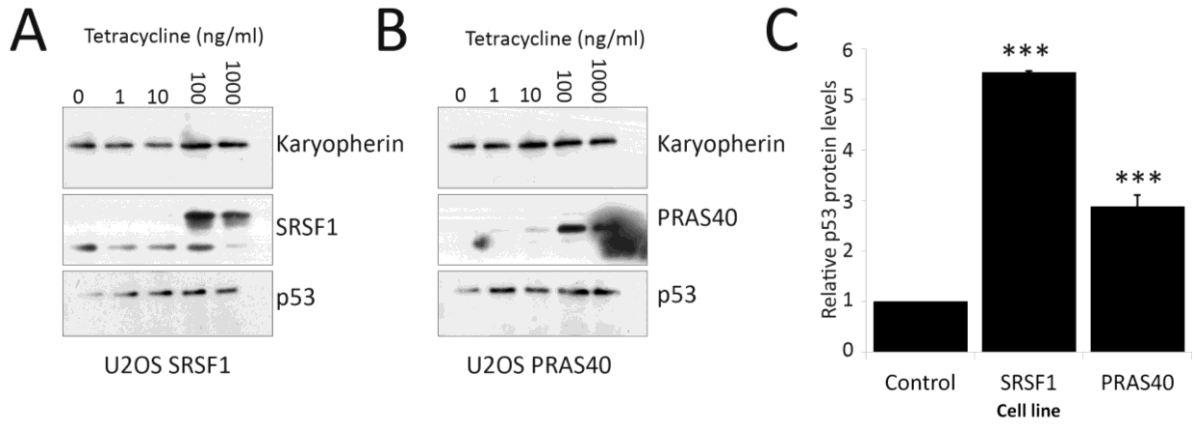

Figure 4.6. The effect of SRSF1 and PRAS40 over-expression on p53. (A+B) Representative western blots to show the effect of over-expression of SRSF1 and PRAS40 on p53 levels. U2OS cells expressing FLAG-SRSF1 or FLAG-PRAS40 were induced with 1-1000 ng/ml tetracycline as indicated for 48 hours prior to harvesting. Whole cell extracts were separated by SDS-PAGE and immunoblotted using antibodies against the endogenous proteins, p53, and karyopherin as indicated on the right. Karyopherin was used as a loading control. (C) Densitometry analysis of A+B. Each bar in C represent the mean ± the standard deviations of the means, n=3. Statistical analysis was determined by paired t test. NS= non-significant, *= P ≤ 0.05, **= p≤ 0.01, ***= p≤ 0.001.

In agreement with previous findings (Fregoso et al., 2013), expression of FLAG-tagged SRSF1 (Figure 4.6A) resulted in an increase in p53 protein levels, suggesting that expression of SRSF1 induces p53. When analysed by densitometry analysis (Figure 4.6C), this expression gave, on average, a 5.5 fold increase in the levels of p53. Interestingly, expression of the FLAG-tagged PRAS40 (Figure 4.6B) also resulted in an increase in p53 protein levels. When analysed by densitometry analysis (Figure 5.6C), the expression of PRAS40 resulted in, on average, a 3 fold increase in the levels of p53. This suggests that, in U2OS cells, expression of FLAG-tagged PRAS40 or SRSF1 results in an increase in p53 levels.

Previously it was shown that over-expression of PRAS40 combined with over-expression of RPL11 resulted in reduced p53 protein levels compared to expression of

RPL11 alone (Havel et al., 2014). However, the effect of PRAS40 over-expression alone on p53 levels was not reported and thus cannot be compared to the results shown here.

4.2.4 SRSF1 and PRAS40 over-expression does not affect cell cycle progression

As the data suggested that FLAG-PRAS40 and FLAG-SRSF1 expression in U2OS cells appeared to result in increases in p53 protein levels, it was important to investigate the effects on cell cycle progression as p53 is a regulator of the cell cycle through its downstream effectors such as p21, GADD45 and 14-3-3 (Waldman et al., 1995, Carrier et al., 1994, Hermeking et al., 1997).

U2OS cells were induced for 48 hours with 1 μg/ml tetracycline and fixed in ice cold ethanol before propidium iodide staining and flow cytometry analysis. Figure 4.7A depicts the representative dot plots and histograms produced whilst Figure 4.7B is a graphical representation of the percentage of cells at each stage in the cell cycle.

The control cells, on average, had 54% of cells in G1/G0, 25% of cells in S phase and 21% of cells in G2/M phase. There was no significant change in the cells expressing FLAG-tagged SRSF1 or PRAS40. When analysed using a paired t-test, the p value for the percentage of cells in G2/M for SRSF1 compared to the control was 0.89 and thus not significant. The p value for the G2/M for PRAS40 compared to the control was 0.2031, also considered to be not significant. This suggests that PRAS40 and SRSF1 over-expression had no effect on cell cycle progression in U2OS cells, despite increases in p53 protein levels, possibly suggesting that they do not increase p53 activity.

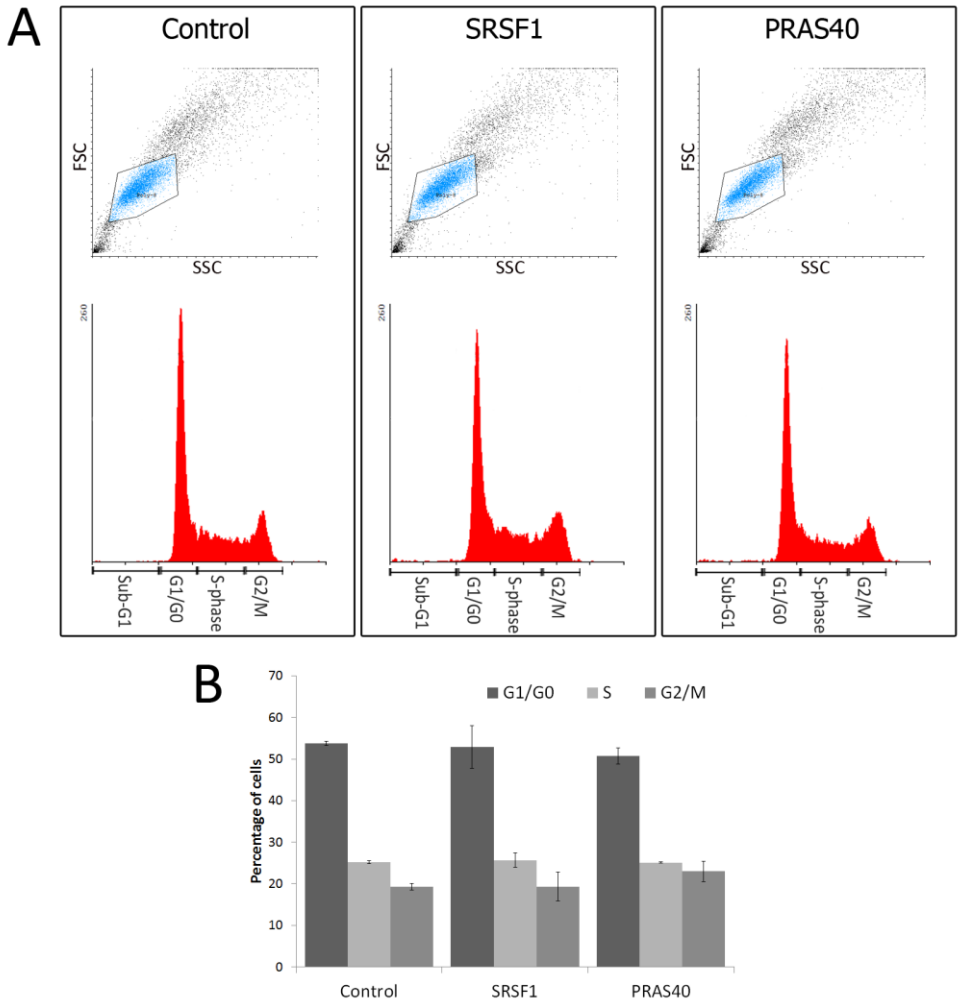

Figure 4.7. Cell cycle analysis of SRSF1 and PRAS40 over-expression. (A) Control, SRSF1 or PRAS40 expressing U2OS cells were induced with 1 µg/ml tetracycline for 48 hours before harvesting, fixing in ethanol and propidium iodide staining before flow cytometric analysis. The forward scatter (FSC) was plotted against the side scatter (SSC) and the live population of cells was gated on as shown in blue. The different stages of the cell cycle were then selected from the histograms. (B) Graphical representation of the percentage of cells at each stage in the cell cycle. Each bar in B represents the means ± the standard deviations of the means, n=3.

4.2.5 SRSF1, but not PRAS40, co-purifies with the 5S rRNA.

In order to investigate how SRSF1 and PRAS40 are involved in the 5S RNP pathway, immunoprecipitations were utilised to study the association of SRSF1 and PRAS40 with the 5S rRNA. The experiment was carried out in the presence or absence of ActD as the ability of these proteins to bind the 5S RNP could be dependent upon ribosomal stress. Cells expressing either control, SRSF1 or PRAS40 flag-tagged proteins were induced for 48 hours with 1 µg/ml tetracycline followed by 5 ng/ml ActD treatment for 16 hours. The cells were incubated with anti-FLAG agarose for 2 hours before RNA extraction and northern blotting using ^{32}P labelled probes that anneal to the 5S and 5.8S rRNAs

(Figure 4.8A). The northern blot was exposed to a phosphorimager screen and scanned to visualise the rRNAs. The FLAG-tag alone from the control cells expressing an empty pcDNA5 vector, did not show any interaction with the 5S or 5.8S rRNAs, suggesting that any binding seen was specific.

The 5S rRNA or the 5.8S rRNA did not co-purify with FLAG-PRAS40 in control or ActD treated cells, suggesting that it PRAS40 not found in a complex with the 5S RNP in normal cells or those experiencing ribosomal stress. FLAG-SRSF1 co-purified with both the 5.8S and the 5S rRNA. SRSF1 interacts with translating ribosomes (Sanford et al., 2005, Sanford et al., 2004) and thus interaction with the ribosomal proteins would be expected. However, when densitometry analysis was utilised (Figure 4.8B), it revealed that SRSF1 interacted with a higher percentage of the 5S rRNA than the 5.8S rRNA. As there 5.8S rRNA is mostly found in ribosomes whilst a high proportion of the 5S rRNA is non-ribosomal (Sloan et al., 2013a), this implies that SRSF1 may be interacting with free 5S rRNA as well as ribosome bound. Upon ActD treatment, the percentage of the total 5S rRNA and 5.8S rRNA bound by SRSF1 decreased suggesting that when the cells are undergoing ribosomal stress, SRSF1 interaction with ribosomes and/or the 5S RNP is decreased.

As PRAS40 did not co-immunoprecipitate with the 5S rRNA, it was decided to look for interactions between PRAS40 and RPL5 and RPL11 as it had been previously reported that RPL11 and PRAS40 interacted (Havel et al., 2014). Control, RPL5 and RPL11 Flp-In cells were induced for 48 hours with media containing 1 µg/ml tetracycline. The cells were lysed and incubated with anti-FLAG agarose for 2 hours prior to washing, elution, separation of the proteins by SDS-PAGE and western blotting (Figure 4.8C). MDM2 and PRAS40 antibodies were utilised as MDM2 is known to interact with RPL5 and RPL11 (Marechal et al., 1994, Lohrum et al., 2003) and thus acts as a control. The FLAG-only control did not show any interaction with MDM2 whilst RPL5 and RPL11 showed interactions with MDM2.

As with MDM2, PRAS40 did not co-purify with the FLAG-only control (Figure 4.8C). PRAS40 also did not co-purify with RPL5, suggesting that PRAS40 does not interact with RPL5. However, PRAS40 co-purified with the FLAG-RPL11, suggesting that PRAS40 is found in complexes with RPL11 exclusively and not RPL5 or the 5S rRNA. This further supports the evidence that PRAS40 may not interact with the 5S RNP.

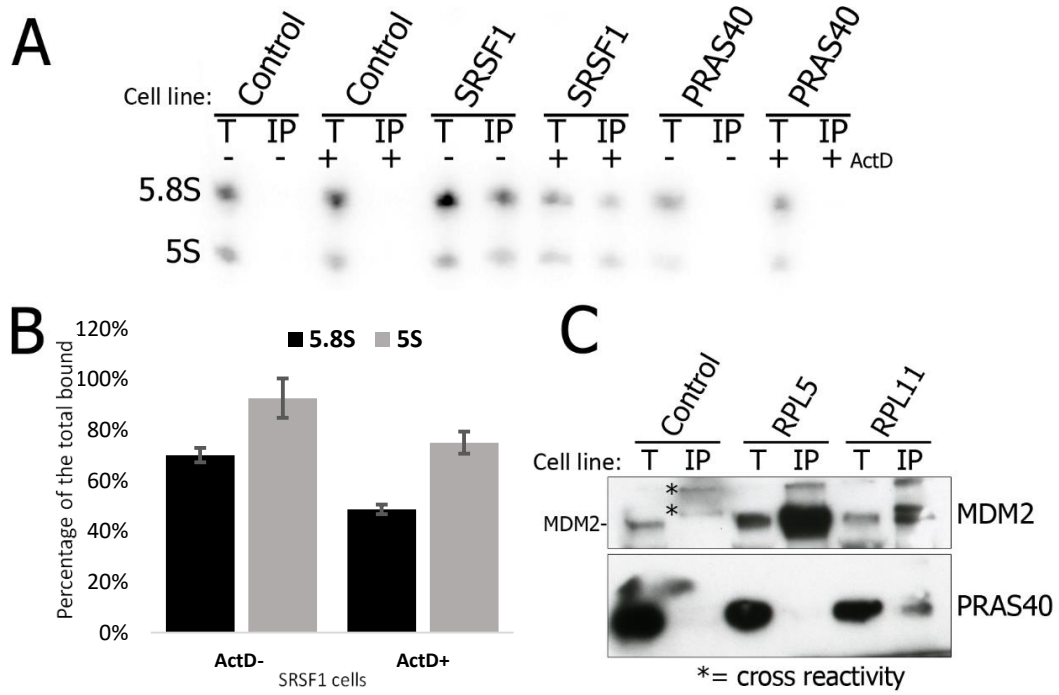

Figure 4.8. SRSF1 interacts with the 5S RNP whilst PRAS40 only interacts with RPL11. (A) Representative northern blot of three repeats to show co-purification of PRAS40 and SRSF1 with the 5S and 5.8S rRNAs. U2OS cells expressing empty vector, FLAG-SRSF1 or FLAG-PRAS40 were induced for 48 hours with 1 µg/ml tetracycline before treatment with 5ng/ml ActD in half of the samples for the final 16 hours. Cells were harvested and lysed before being incubated with anti-FLAG agarose for 2 hours before washing. The bound complexes were eluted and RNA extracted before separating with acrylamide gel electrophoresis and northern blotting using ^{32}P ATP labelled probes that hybridise to the 5S and 5.8S rRNAs. T denotes the 5% total loaded. **(B)** Densitometry analysis of A. The percentage of 5S and 5.8S bound from the total is shown. **(C)** Western blot to show immunoprecipitation using control, FLAG-RPL5 or FLAG-RPL11 U2OS cells. The cells were grown for 48 hours in media supplemented with 1 µg/ml tetracycline to induce the expression of the FLAG-tagged proteins. Cells were harvested and the lysates incubated with anti-FLAG agarose for 2 hours. The bound complexes were eluted and separated by SDS-PAGE before western blotting using antibodies raised against MDM2 and PRAS40. Each bar in B represents the means ± the standard deviations of the means, n=2.

4.2.6 Depletion of PRAS40 increases p53 activity whilst depletion of SRSF1 reduces p53 activity.

In order to investigate the role of SRSF1 and PRAS40 in p53 signalling, PRAS40 or SRSF1 were depleted in U2OS and MCF-7 cells. Both U2OS cells and MCF-7 cells have wild type p53 and so act as a good comparison. U2OS or MCF-7 cells were incubated in growth medium post-transfection with 20 µM siRNA duplexes targeting control, SRSF1 or PRAS40 for 48 hours prior to harvesting. Whole cell lysates were separated by SDS PAGE and analysed by western blot using antibodies raised against SRSF1 and PRAS40 (Figure 4.9A-B) and p53 and p21 (Figure 4.9C). Karyopherin was used as a loading control. Treatment of U2OS or MCF-7 cells with siRNAs targeting PRAS40 and SRSF1 achieved a good level of knockdown of PRAS40 (Figure 4.9A) and SRSF1 (Figure 4.9B).

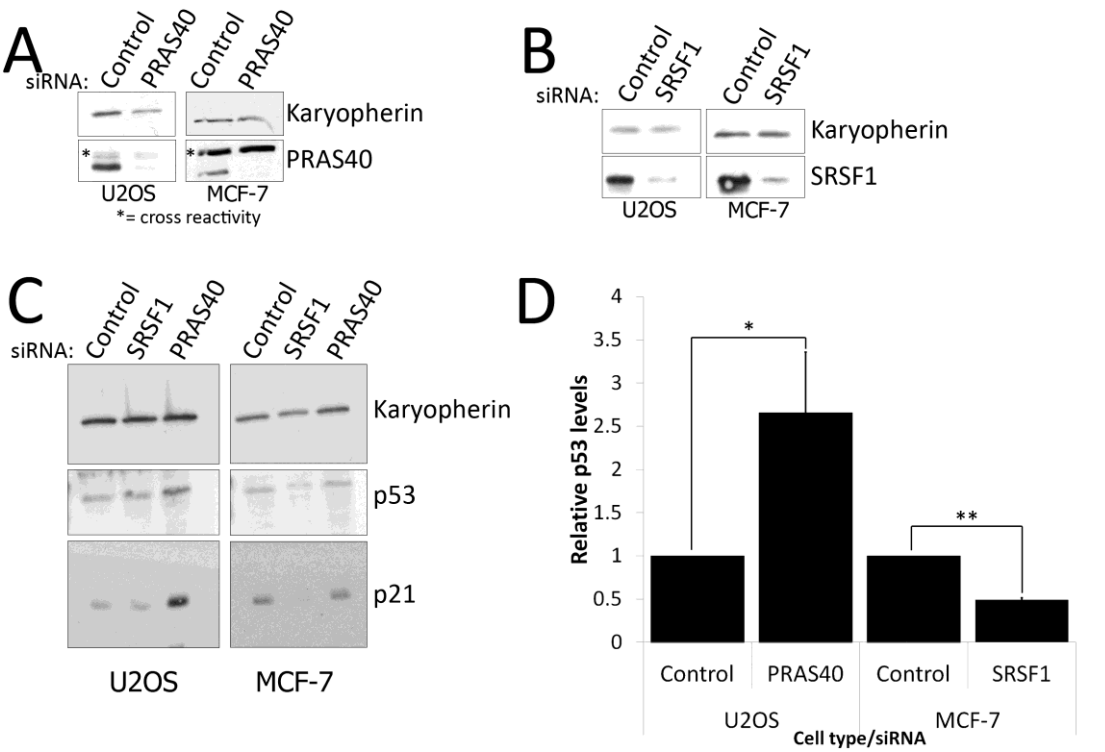

Figure 4.9. PRAS40 and SRSF1 depletion affects p53 and p21 levels. (A+B) Representative western blots to show the depletion of PRAS40 and SRSF1 in U2OS and MCF-7 cells. U2OS cells or MCF-7 cells were incubated with control, PRAS40 or SRSF1 siRNAs for 48 hours. Whole cell lysates were separated by SDS PAGE and western blotted for SRSF1 or PRAS40. **(C)** Representative western blot to show the effect of PRAS40 and SRSF1 depletion on p53 and p21 protein levels. U2OS or MCF-7 cells were treated with control, SRSF1 or PRAS40 siRNAs for 48 hours prior to harvesting. Whole cell extracts were separated by SDS-PAGE and western blotted using antibodies against p53 and p21. Karyopherin was used as a loading control. **(D)** Densitometry analysis of C. Each bar in D represents the means ± the standard deviation of the means, n=3. Statistical analysis was determined by paired t test. NS= non-significant, *= P ≤ 0.05, **= p≤ 0.01, ***= p≤ 0.001.

In U2OS cells SRSF1 depletion did not affect p53 levels or p21 levels (Figure 4.9C). This suggested that SRSF1 depletion in U2OS cells does not induce p53. PRAS40 depletion caused, on average, a 2.5 fold increase in p53 levels (Figure 4.9D). There was also an increase in the p53 downstream effector, p21, suggesting that p53 levels and the activity of p53 were both increased.

In MCF-7 cells, SRSF1 depletion resulted in the p53 protein levels reducing by half (Figure 4.9D). There was also a decrease in p21 levels. In contrast to the situation in U2OS cells, depletion of PRAS40 in MCF-7 cells had no noticeable impact on p53 or p21 protein levels. This data shows that SRSF1 depletion results in a decrease in p53 activity in MCF-7 cells and whereas PRAS40 depletion results in an increase in p53 levels and activity in U2OS cells. This could be due to differences between the cell lines as later discussed.

4.2.7 SRSF1 and PRAS40 have opposing effects on ActD treatment

It was previously demonstrated that SRSF1 depletion could counteract the p53 response following ActD treatment whilst PRAS40 depletion had the opposite effect, resulting in increased p53 levels (Fregoso et al., 2013, Havel et al., 2015) however this had not been shown in the same cell lines and so it was decided to repeat these experiments in U2OS cells. U2OS cells were incubated in growth medium for 48 hours post-transfection with 20 µM SRSF1 or PRAS40 siRNA duplexes, followed by 5 ng/ml ActD treatment for the final 18 hours. Western blot analysis was carried out using antibodies raised against p53 along with a karyopherin antibody for a loading control (Figure 4.10A).

In cells treated with the control siRNA, ActD treatment resulted in increased p53 levels compared to the untreated. However, in the SRSF1 depleted cells, the drug had a much milder effect with p53 protein levels reduced by half (Figure 4.10A+B). Interestingly, PRAS40 depletion had the opposite effect and resulted in a 2 fold increase in p53 in ActD treated cells (Figure 4.10A+B). In both cases, these results were found to be not quite significant by statistical analysis, although a general trend can be seen to support the previously published data and suggests that SRSF1 and PRAS40 may be acting antagonistically in the 5S-RNP pathway.

In order to determine whether the activity of p53 was affected, U2OS cells stably expressing a p53-regulated luciferase reporter under the control of p53 were incubated post-transfection with 20 µM siRNA duplexes targeting SRSF1 or PRAS40 for 48 hours. In the final 18 hours, 5 ng/ml ActD was added to simulate ribosomal stress. As seen in Figure 4.10C, ActD treatment resulted in, on average, a 7 fold increase in luciferase levels above the control, suggesting p53 activation.

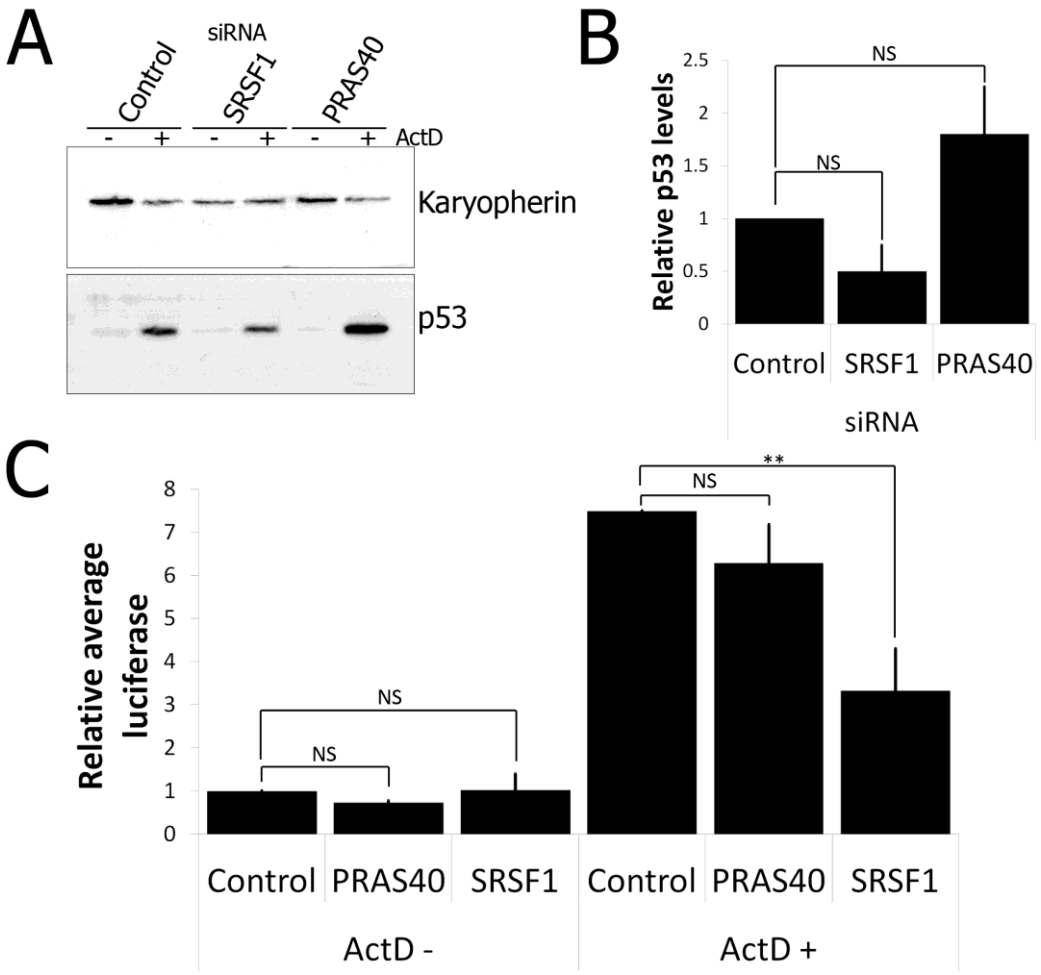

Figure 4.10. SRSF1 and PRAS40 have opposite effects upon ActD treatment. (A) Western blot to show p53 signalling after ActD treatment. U2OS cells were incubated at 37°C post-transfection with 20 µM control, SRSF1 or PRAS40 siRNA duplexes for 48 hours. In the final 18 hours, 5 ng/ml ActD or an ethanol control was added. Cells were harvested and whole cell extracts separated by SDS-PAGE. Levels of p53 were analysed by western blot with karyopherin as a loading control. **(B)** Densitometry analysis was carried out on A using Image J software and the levels of induction normalised to the loading control. **(C)** U2OS cells stably expressing a p53-regulated luciferase reporter were depleted of SRSF1 or PRAS40 for 48 hours, followed by 5 ng/ml ActD treatment for the final 18 hours, as in A. Luciferase assays were used to determine firefly luciferase levels. A Bradford assay was used to normalise Luciferase levels to protein levels. Each bar in B+C represents the mean ± the standard deviation of the mean, n=3. Statistical analysis was determined by paired t test. NS= non-significant, *= P ≤ 0.05, **= p≤ 0.01, ***= p≤ 0.001.

Depletion of PRAS40, however, showed a slight, but non-significant, reduction in this level whilst SRSF1 depletion shows a significant reduction in luciferase levels. This suggests that SRSF1 is required for both the increase in p53 levels and activity whilst PRAS40 depletion increases p53 levels, although not significantly, but does not significantly impact upon p53 activity. Interestingly, in the untreated cells, depletion of PRAS40 did not illicit increased p53 activity. As it has been previously seen that PRAS40 depletion results in an increase in p53 protein levels, this suggests that PRAS40 depletion is able to increase p53 protein levels but does not affect p53 activity above

that of ActD treatment alone. This could be the result of saturation whereby an increase in p53 levels can no longer result in an increase in p53 activity.

4.2.8 Depletion of SRSF1 and PRAS40 counteract the p53 response from RPL5 and RPL11 over-expression.

It was shown previously that RPL5 knockdown was able to counteract the p53 response from SRSF1 over-expression (Fregoso et al., 2013). In order to determine whether SRSF1 had an effect on RPL11-mediated p53 signalling as well as RPL5-mediated p53 signalling, the effect of depletion of SRSF1 on the p53 response from RPL5 or RPL11 expression was investigated.

U2OS cells expressing FLAG-tagged RPL11 and RPL5 U2OS cells (Figure 4.11A) were incubated in growth medium for 48 hours post-transfection with 20 μM SRSF1 siRNA duplexes at 37^{o}C. For the final 36 hours, 1 μg/ml tetracycline was added to the medium to induce the expression of the FLAG-tagged proteins. It was important to ensure that the knockdown had occurred before the expression of the FLAG-tagged proteins. After harvesting the cells and separating whole cell lysates by SDS-PAGE, a western blot was carried out with anti-p53, using anti-karyopherin as a loading control (Figure 4.11B).

When densitometry analysis was applied to the blots (Figure 4.11C), it was observed that when SRSF1 was depleted, the p53 response was decreased in the RPL5 cells. A greater reduction in p53 protein levels was seen in the RPL11 cells, which is interesting because RPL11 was suggested to not be involved in the SRSF1-RPL5 mediated p53 response.

Furthermore, to investigate the role of PRAS40 on p53 signalling via the 5S RNP the effect of PRAS40 depletion on p53 protein levels as a result of SRSF1, RPL5, or RPL11 expression was studied. U2OS cells over-expressing RPL5, RPL11, or SRSF1 were transfected with 20 μM SRSF1 (RPL5 and RPL11 only) or PRAS40 siRNA duplexes. In the final 36 hours, 1 μg/ml tetracycline was added to the medium to induce the expression of RPL5, RPL11, (Figure 4.11D) or SRSF1 (Figure 4.11E). Western blots (Figure 4.11F) were carried out as previously described.

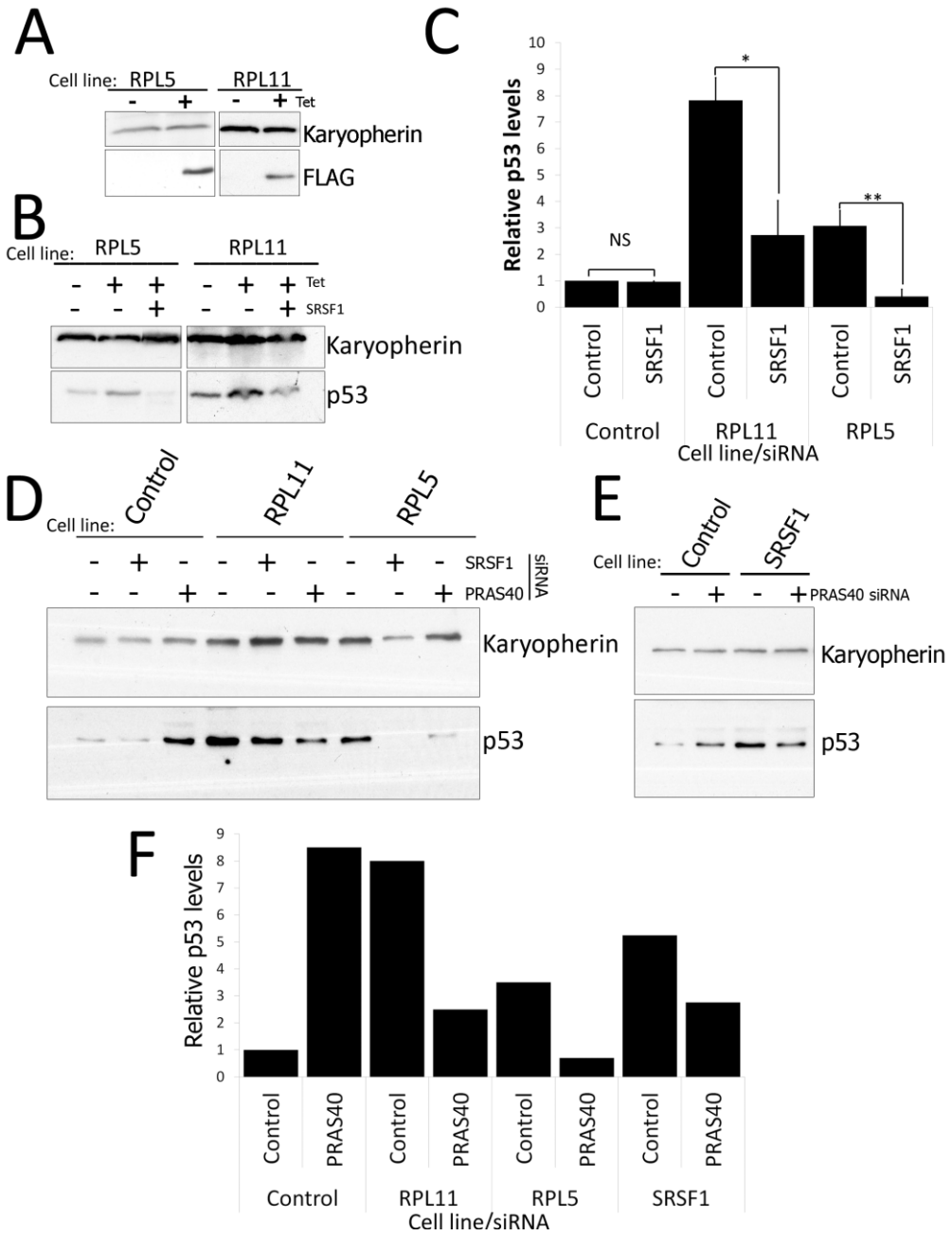

Figure 4.11 Depletion of SRSF1 or PRAS40 counteracts the p53 response from RPL5 and RPL11 over-expression. (A) Western blot analysis of RPL5 and RPL11 expression in U2OS cells. Cells expressing FLAG-tagged RPL5 or RPL11 were incubated in growth medium supplemented with 1 µg/ml tetracycline for 48 hours to induce the expression of the proteins. Whole cell extracts were separated by SDS-PAGE and western blotted using an anti-FLAG antibody. Karyopherin was used as a loading control. **(B)** Western blot analysis of the effect of SRSF1 depletion in U2OS cells expressing RPL5 and RPL11. U2OS cells expressing FLAG-tagged RPL5 or RPL11 were incubated with growth medium post-transfection with 20 µM SRSF1 siRNA for 48 hours. In the final 36 hours, 1 µg/ml tetracycline was added to the medium to induce expression of the FLAG-tagged proteins. Whole cell extracts were separated by SDS-PAGE and western blotted using an anti-p53 antibody with anti-karyopherin as a loading control. **(C)** Densitometry analysis of B. Bars represent the mean ± the standard deviation of the mean, n=3. NS= non-significant, *= P ≤ 0.05, **= p≤ 0.01, ***= p≤ 0.001. **(D)** Western blot to show the effect of SRSF1 and PRAS40 depletion on the p53 induction after RPL5 and RPL11 expression. Inducible U2OS cells expressing empty vector, FLAG-RPL11 or FLAG-RPL5 were treated with siRNA against SRSF1 for 48 hours followed by induction of RPL5 or RPL11 with 1µg/ml tetracycline for the final 36 hours. Cells were harvested and whole cell extracts separated by SDS-PAGE before western blotting for p53. Karyopherin was used as a loading control. **(E)** As in A, using FLAG-SRSF1 cells with a depletion of PRAS40. **(F)** Densitometry analysis of A+B showing the effect of PRAS40 depletion on p53 levels after expression of RPL11, RPL5, or RPL11, n=1.

As previously demonstrated in Figure 4.11B, RPL5 and RPL11 expression resulted in increased p53 in an SRSF1-dependent manner. In Figure 4.11D and 4.11F, PRAS40 depletion resulted in a similar decrease in p53. This suggests that PRAS40 and SRSF1 may both required for p53 induction upon the expression of RPL5 and RPL11. Furthermore, the effect of SRSF1 over-expression combined with depletion of PRAS40 was studied. If SRSF1 and PRAS40 were acting antagonistically, an additive effect would be expected. However, these results show that SRSF1 over-expression induces p53 in a PRAS40-dependent manner (Figure 4.11E-F). All of this data taken together suggest that SRSF1 and PRAS40 are required for p53 activation via the 5S RNP. This is in contrast to the previous findings that depletion of SRSF1, RPL5 or RPL11 counteracts the p53 response after ActD treatment but PRAS40 depletion does not.

4.2.9 Knockdown of SRSF1 and PRAS40 have effects the localisation of 5S RNP components.

Havel et al. (2014) hypothesised that PRAS40 sequesters RPL11 to the nucleolus to prevent interactions between RPL11 and MDM2, however the effect of SRSF1 on RPL5 or RPL11 localisation had not been studied. In order to investigate this, PRAS40 or SRSF1 were depleted in cells expressing FLAG-tagged RPL5 and RPL11 and, using immunofluorescence, localisation of the FLAG-tagged proteins was studied. U2OS expressing FLAG-RPL5 or FLAG-RPL11 were transfected with siRNAs targeting SRSF1 or PRAS40 and grown on coverslips in growth medium 48 hours with the addition of 1 µg/ml tetracycline, to induce the RPL5 or RPL11 expression, for the final 24 hours. Cells were then fixed in 4% paraformaldehyde and used in immunofluorescence with anti-FLAG antibody and DAPI staining to show the location of the nucleus (figure 4.12).

In control cells, RPL11 was strongly localised in the nucleoli with the FLAG-antibody showing even distribution of RPL11 throughout the nucleus and cytoplasm. However, SRSF1 depletion resulted in a reduction of cytoplasmic staining in the cells. PRAS40 depletion resulted in an enrichment of cytoplasmic RPL11 and some loss of nucleolar staining suggesting that PRAS40 may play a role in the cytoplasmic and nucleolar localisation of RPL11.

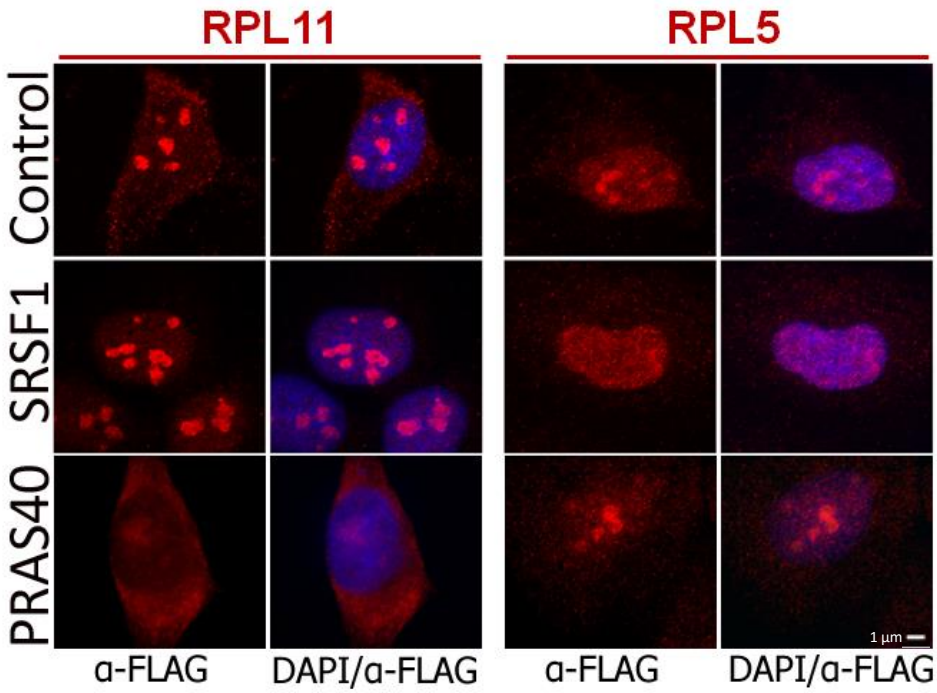

Figure 4.12. SRSF1 and PRAS40 depletion affects localisation of RPL5 and RPL11. U2OS cells containing a tetracycline-inducible FLAG-tagged RPL5 or RPL11 were grown for 48 hours in the presence of SRSF1, PRAS40 or control siRNAs. In the final 24 hours, 1µg/ml tetracycline was added to induce the expression of RPL5 or RPL11. The cells were fixed in paraformaldehyde and used in immunofluorescence with anti-FLAG antibody (red). DAPI (blue) was used to stain the nucleus. Scale bar represents 1 µm.

The control RPL5 cells showed the strongest anti-FLAG signals in the nucleoli and the nucleus and with almost no cytoplasmic signals. In the SRSF1 depleted cells, the enrichment of nucleolar FLAG signal was reduced, suggesting that SRSF1 may be required for nucleolar localisation of RPL5. In the PRAS40 depleted cells, RPL5 showed a very similar localisation to the control cells, with strong nucleolar and nuclear signals. This suggests that whilst PRAS40 had an effect on RPL11 localisation, it is not involved in RPL5 localisation. SRSF1 depletion affected the localisation of both RPL5 and RPL11 thus supporting the hypothesis that SRSF1 interacts with the 5S RNP but, as PRAS40 only affected RPL11 localisation, this may support previous findings that PRAS40 only interacts with RPL11.

4.2.10 Depletion of SRSF1 affects integration of the 5S RNP into the ribosome

As SRSF1 depletion had an impact on the localisation of both RPL5 and RPL11 it was possible that this may impact upon the ability of RPL5 and RPL11 to be incorporated into ribosomes. Therefore, cell lysates from cells expressing FLAG-tagged RPL5 and RPL11 with control or SRSF1 siRNA treatment were applied to a glycerol gradient to

identify the proportion of the protein in small, free complexes as opposed to large, ribosome complexes.

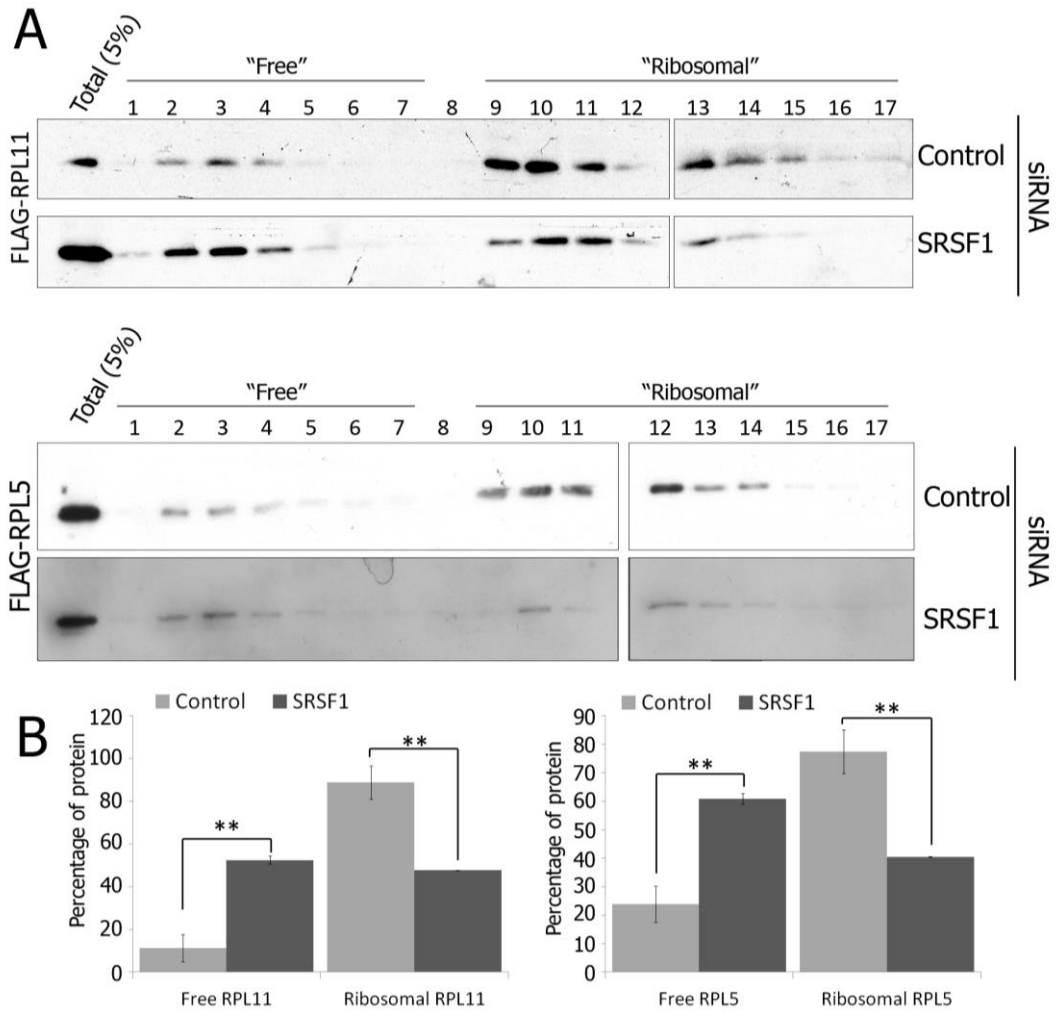

Figure 4.13. Depletion of SRSF1 affects integration of RPL5 and RPL11 into the ribosome. (A) Western blot to show the presence of RPL11 and RPL5 in large and small complexes. Cells expressing FLAG-tagged RPL11 or RPL5 were induced for 48 hours with 1µg/ml tetracycline before harvesting. Lysates were applied to a glycerol gradient to separate the larger and smaller complexes. Fractions through the gradient were collected and separated by SDS-PAGE before western blotting with anti-FLAG antibody. (B) Densitometry analysis of A using Image J software. Fractions 1-8 were denoted as 'free' whilst fractions 9-17 were denoted as 'ribosomal'. Bars in B represent the mean ± the standard deviations of the means, n=3. Statistical analysis determined by paired t test. NS= non-significant, *= P ≤ 0.05, **= p≤ 0.01, ***= p≤ 0.001.

To do this, U2OS cells expressing FLAG-tagged RPL5 or RPL11 were depleted of SRSF1 and induced by incubating in medium containing 1 µg/ml tetracycline for 48 hours at 37^{o}C. The cells were harvested and lysates applied to a 10-40% glycerol gradient and subjected to ultra-centrifugation. The gradient was fractionated and used separated by SDS-PAGE before western blotting with an anti-FLAG antibody to detect the FLAG-tagged proteins in different gradient fractions (Figure 4.13A). Densitometry analysis

was utilised to quantify the percentage of the protein (Figure 4.13B) in either the 'free' (Figure 4.13A fractions 1-8) or 'ribosomal' fractions (Figure 4.13A fractions 9-17).

In the control cells, 90% of the FLAG-tagged RPL11 protein was detected in the ribosome fractions, however only 60% of the FLAG-tagged RPL5 was detected in the ribosome fractions. This is in agreement with previous findings that there is more non-ribosomal RPL5 in the cells than RPL11 (Sloan et al., 2013a). When SRSF1 was depleted, the percentage of FLAG-RPL11 and FLAG-RPL5 incorporated into ribosomes significantly reduced to 48% and 45% respectively. This indicated that SRSF1 may be required for efficient integration of the 5S RNP into the ribosome or that SRSF1 is required for ribosome production.

4.2.11 Knockdowns of SRSF1 and PRAS40 have no significant effect on rRNA processing in pulse-labelling experiments

As SRSF1 had an impact on both localisation of RPL5 and RPL11 and their integration into the ribosome and PRAS40 depletion affected RPL11 localisation, it was important to study the impact of SRSF1 depletion on ribosome biogenesis as many ribosome biogenesis factors affect localisation and ribosomal integration of the 5S RNP (Sloan et al., 2013a). In order to do this, cells were phosphate starved with phosphate free medium before the addition of ^{32}P orthophosphate medium and a chase period with standard medium. This method allows for the specific evaluation of newly synthesised rRNAs that have incorporated labelled phosphate.

U2OS cells were depleted of SRSF1 or PRAS40 along with RPL5, as a control for the disruption of ribosome production, for 48 hours prior to the pulse-labelling experiment. After the three hour chase with normal medium, RNA was extracted from the cells and separated using a glyoxal-agarose gel (Figure 4.14A- top panel) to separate the rRNA species. The U.V scanned 18S rRNA was used as a loading control. RPL5 depletion resulted in an almost complete loss of the labelled 28S rRNA with an accumulation of the 47/45S and 32S rRNA precursors, in agreement with previously published data (Sloan et al., 2013a). In the cells depleted of SRSF1, there was a slight 32S pre-rRNA increase. In the cells depleted of PRAS40 there was a slight increase in the 47/45S pre-rRNAs and a slight decrease in 32S pre-rRNA. However, when analysed with a paired t-test all p-values were greater than 0.5, thus suggesting that depletion of SRSF1 or PRAS40 has no significant effect on ribosome biogenesis. This suggests that

the effects seen on localisation and integration are more likely to be direct rather than due to defects in ribosome biogenesis.

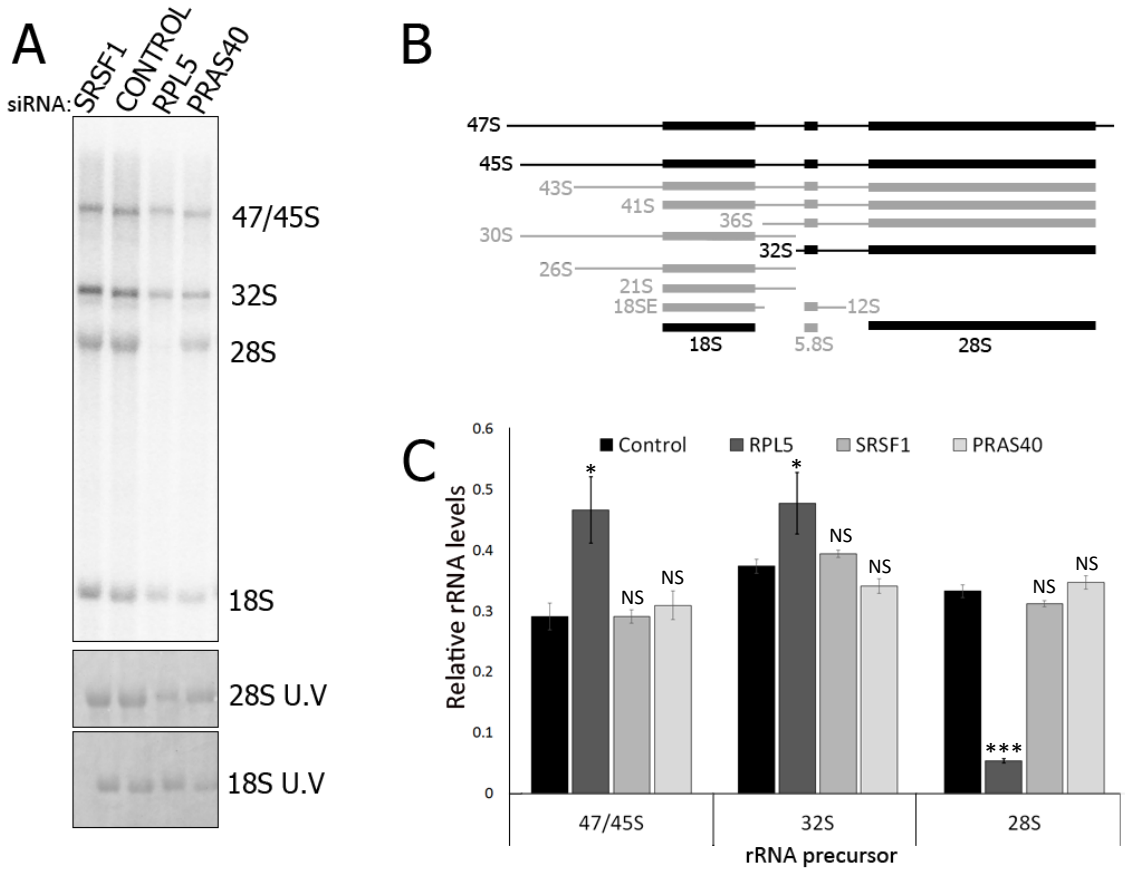

Figure 4.14. Analysis of the effect of depletion of SRSF1 or PRAS40 on rRNA processing. (A) Pulse-labelling experiment with ^{32}P orthophosphate to show the newly synthesised rRNA precursors. U2OS cells were transfected with siRNAs targeting control, RPL5, SRSF1 or PRAS40 siRNAs for 48 hours prior to 1 hour labelling with a 3 hour chase period with standard media. RNA was extracted and separated by acrylamide gel electrophoresis, transferred to a nylon membrane and exposed to a phosphorimager screen. **(B)** Schematic representation of the rRNA processing events in humans. The precursors visible in A are shown in black. **(C)** Densitometry analysis of A analysed using ImageQuant™. Each bar represents the means ± the standard deviation of the means, n=3. Statistical analysis determined by paired t test. NS= non-significant, *= P ≤ 0.05, **= p≤ 0.01, ***= p≤ 0.001.

4.2.12 p53 induction from Deguelin treatment is RPL5-dependent.

Finally, the effect of drug treatments on p53 that were linked to SRSF1 and PRAS40 were investigated. Deguelin is an AKT inhibitor (Chun et al., 2003, Baba et al., 2015) which results in mTORC1 inhibition (Jin et al., 2007) and thus would be expected to trigger 5S RNP mediated p53 signalling via PRAS40. AKT is an activator of mTORC1 (Sengupta et al., 2010, Rosen and She, 2006, Manning and Cantley, 2007) which, in turn, activates PRAS40 and results in p53 inhibition via RPL11 (Havel et al., 2014). However, previous reports have shown that deguelin treatment was able to counteract

the p53 response after ActD treatment (Chen et al., 2014) and, therefore, the role of the 5S RNP-signalling after deguelin treatment was investigated.

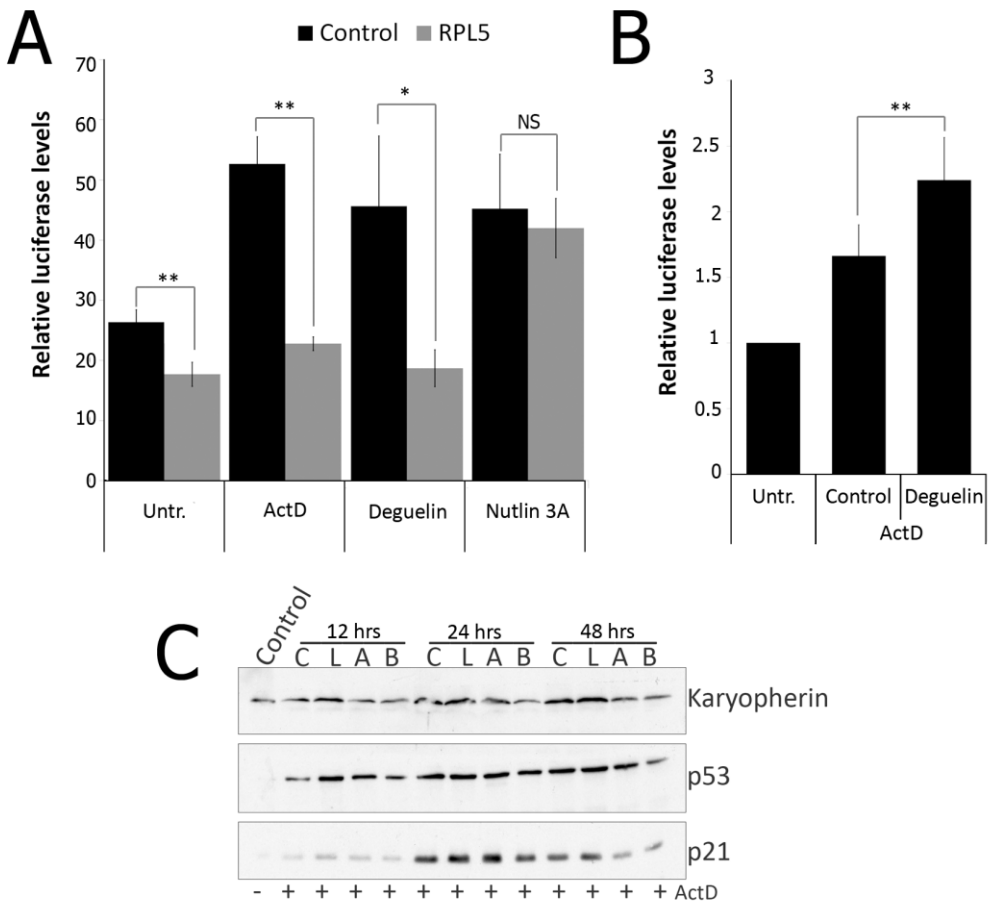

Figure 4.15 The effect of deguelin, L-leucine and arginine treatment in U2OS cells. (A) U2OS cells containing a p53 regulated luciferase reporter were incubated at 37°C post-transfection with 20 µM control or RPL5 siRNA duplexes in growth medium for 48 hours. For the final 24 hours, 100nM deguelin was added, or for the final 16 hours 5 nM ActD, or 5 µM Nutlin 3A. Cells were harvested and assayed for luciferase compared to untreated cells (Untr.). **(B)** U2OS cells containing a p53 regulated luciferase reporter were incubated in growth medium at 37°C for 48 hours. After 24 hours, 100 nM deguelin was added to the medium followed by 5 nM ActD for the final 16 hours. Cells were harvested and assayed for luciferase compared to untreated cells (Untr.). **(C)** U2OS cells were incubated at 37°C in growth medium with the addition of 10 mM L-leucine (L), arginine (A) or both (B) for 12, 24 or 48 hours as indicated. C denotes the control. For the final 18 hours, 5 nM ActD was added. Whole cell extracts were separated by SDS-PAGE and western blotted using antibodies raised against karyopherin, p53 or p21. Each bar in A and B represents the means ± the standard deviation of the means, n=3. Statistical analysis was carried out using a paired t-test. NS= non-significant, *= P ≤ 0.05, **= p≤ 0.01, ***= p≤ 0.001.

U2OS cells stably expressing a p53 luciferase reporter were incubated with 20µM control or RPL5 siRNA in growth medium for 48 hours at 37°C. For the final 24 hours, 100 nM deguelin was added. As a control, cells were treated with 5 nM ActD or 5 µM Nutlin 3A for 16 hours. ActD and Nutlin 3A were chosen as controls as ActD activates p53 through the 5S RNP whilst Nutlin 3A activates p53 independent of the 5S RNP

(Lindstrom and Nister, 2010, Bursac et al., 2012). After harvesting, the cells were lysed and assayed for luciferase activity (Figure 4.15A).

All three drugs resulted in an increase in luciferase levels, suggesting an increase in p53 activity. In agreement with previous findings, RPL5 depletion resulted in a significant decrease in luciferase levels after ActD treatment, but not after Nutlin 3A treatment (Lindstrom and Nister, 2010, Sloan et al., 2013a, Bursac et al., 2012). RPL5 depletion also resulted in a significant decrease in luciferase levels after deguelin treatment. This suggests that the p53 response from deguelin treatment is dependent upon RPL5.

Deguelin treatment was previously shown to counteract the p53 response after ActD treatment (Chen et al., 2014). However, if deguelin activates p53 via the 5S RNP as shown in Figure 4.14A, then this would not be expected.

Therefore, U2OS cells stably expressing a p53 reporter were incubated in growth medium at 37^{o}C for 48 hours to achieve the desired cell density. After 24 hours, 100 nM deguelin was added to the medium followed by 5 nM ActD for the final 16 hours. After harvesting, the cells were lysed and assayed for luciferase activity (Figure 4.15B). The data shows that ActD treatment increased luciferase levels above the untreated cells. Furthermore, deguelin and ActD treatment have a significant additive effect over treatment with ActD alone. This supports the previous finding (Figure 4.15A) that deguelin activates p53 via the 5S RNP.

Furthermore, the ability of L-leucine or arginine treatment to counteract the p53 response after treatment of cells with ActD was investigated. Both arginine and L-leucine trigger the mTOR signalling pathway (Kong et al., 2012, Lynch, 2001) and L-leucine treatment has been shown to be a successful treatment in DBA patients (Pospisilova et al., 2007). Therefore, U2OS cells were grown in medium at 37^{o}C with the addition of 10 mM L-leucine, arginine or both. For the final 18 hours, 5 nM ActD was added. Treatment of ActD resulted in an increase in p53 and p21 levels, as expected (Figure 4.15C). However, treatment with leucine, arginine, or a combination of both, did not decrease the p53 levels as expected suggesting that, under the conditions used, treatment of cells with leucine or arginine was not sufficient to reduce p53 levels after treatment with ActD.

4.3 Discussion

This chapter aimed to gain a greater understanding of the role of SRSF1 and PRAS40 as regulators of the 5S RNP-p53 pathway through studying the effect over-expression and depletion of the two proteins.

The first factor studied was SRSF1, an alternative splicing factor, commonly upregulated in cancers (Anczukow et al., 2012), that has many roles within the cell such as mRNA nuclear export, mRNA translation and nonsense mediated decay (Long and Caceres, 2009). SRSF1 regulates translation through its ability to positively regulate mTOR signalling pathways (Michlewski et al., 2008). However, SRSF1 had also been linked to RPL5-mediated p53 signalling to induce senescence (Fregoso et al., 2013).

The presented in this thesis showed that FLAG-SRSF1 was localised in the nucleus similar to what had been seen by previous groups (Koizumi et al., 1999, Caceres et al., 1997). However, it also suggested that SRSF1 may affect localisation of RPL5 and RPL11. Depletion of SRSF1 resulted in a loss of cytoplasmic signal from FLAG-RPL11 and nucleolar localisation of RPL5. Therefore, it is possible that SRSF1 is required for the localisation of RPL11 in the cytoplasm and RPL5 into the nucleolus. However, it is not clear whether this is direct or indirect as SRSF1 has many roles in the cell such as splicing and translation and thus could be affecting a number of other factors with these effects. However, as SRSF1, whether directly or indirectly, affected localisation of the 5S RNP components, this led to the question as to whether SRSF1 played a role in 5S RNP recruitment into the ribosome.

In yeast, Rrs1 and Rpf2 form a complex with the 5S RNP and are responsible for the integration of the 5S RNP into 90S pre-ribosomes containing the 35S pre-rRNA (Zhang et al., 2007, Morita et al., 2002, Kharde et al., 2015, Madru et al., 2015). However, in human ribosome biogenesis, the 5S RNP recruitment relies more upon the nucleolar protein, PICT-1 than the Rpf2/Rrs1 homologues (Sloan et al., 2013a). This suggesting that human 5S RNP incorporation may vary to yeast. Indeed, depletion of SRSF1 resulted in an accumulation of free, non-ribosome bound, FLAG-RPL5 and FLAG-RPL11 in U2OS cells compared to the control (Figure 4.13). This suggested that SRSF1 was required for the integration of RPL5 and RPL11 into ribosomes. One caveat with this

experiment is that the RPL5 and RPL11 proteins investigated are not the endogenous proteins. However, studying the endogenous proteins would be more problematic as the majority of the protein would already be incorporated into ribosomes and thus the ability to see changes in integration would be determined by ribosome turnover rates. A way to counteract this would be to investigate the integration of the 5S rRNA using pulse-labelled cells. This would allow for visualisation of the newly-synthesised 5S rRNA and would help to determine the integration of the endogenous 5S RNP.

As the data presented in this chapter implies that SRSF1 depletion results in the 5S RNP not being incorporated into ribosomes, this would imply that SRSF1 depletion should activate p53 by an accumulation of non-ribosomal 5S RNP, unless it was part of the complex involved in activating p53. It was demonstrated that depletion of SRSF1 in MCF-7 cells resulted in a decrease in p53 and p21 protein levels (Figure 4.9). This suggested that SRSF1 may form part of the 5S RNP complex that activates p53 and, along with the 5S RNP (Sloan et al., 2013a), is involved in maintaining p53 homeostasis. Interestingly, Fregoso et al. (2013) demonstrated that SRSF1 inductions in BJ-TT cells caused an increase in p53 levels. In this study, expression of FLAG-SRSF1 for 48 hours in U2OS cells showed a slight increase in p53 and p21 protein levels when analysed by western blot (Figure 4.6) however the previous observations in BJ-TT cells showed a much greater induction of p53. Whilst Fregoso et al. (2013) showed that SRSF1 expression led to senescence, they did not study the cell cycle progression. p53 induction can result in an accumulation of cells in G1 or G2 (Agarwal et al., 1995, Waldman et al., 1995), however the cells expressing SRSF1 showed no cell cycle defects (Figure 4.7), further supporting the fact that SRSF1 expression has only a small effect on p53 in U2OS cells. BJ-TT cells are primary cells and so the signalling pathways will be more akin to normal cell signalling. Therefore, in the future, it would be beneficial to attempt to express SRSF1 in more clinically relevant cells in order to see how these cells are affected. As ribosome biogenesis defects often lead to anaemia and blood related disorders, cells from a red-blood cell lineage may be the most appropriate choice to carry the study forward.

Furthermore, the data presented in this chapter demonstrates that, in agreement with Fregoso et al. (2013), SRSF1 interacts with RPL5, but it was also determined that SRSF1 associates with the 5S rRNA, possibly outside of ribosomes (Figure 4.8). As SRSF1

associates with translating ribosomes (Sanford et al., 2005, Sanford et al., 2004), interaction with ribosome components would be expected. However the proportion of 5S rRNA bound was greater than 5.8S, suggesting that whilst SRSF1 is interacting with ribosomes, it is also interacting with free 5S rRNA, and thus, possibly, the 5S RNP. It was not possible to detect RPL11 interaction with SRSF1 due to poor quality antibodies. However, RPL11 requires binding of RPL5 to the 5S rRNA before it is recruited (Scripture and Huber, 2011) and thus it is possible that SRSF1 interacts with the complex before RPL11 binding. Further investigations of the interaction with SRSF1 and RPL11 are, therefore, required.

Previous studies concluded that depletion of PICT-1, a factor shown to affect 5S RNP incorporation into the ribosome, resulted in a severe reduction in the 28S and the 5.8S rRNAs (Sloan et al., 2013a) and thus it is possible that SRSF1 depletion would have a similar effect. However, no significant difference in the ratios of the mature rRNAs or the rRNA precursors was seen in the control cells compared to those depleted of SRSF1, suggesting that SRSF1 is not required for rRNA processing. This was surprising as depletion of SRSF1 had such an impact on 5S RNP recruitment into the ribosome and suggests that SRSF1 may be directly involved in 5S RNP recruitment instead of simply affecting ribosome biogenesis as a whole. However, it is possible that SRSF1 depletion causes subtle defects that were not detectable by pulse-labelling and thus more detailed analysis of the pre-rRNA precursors is required. Unfortunately it was not possible, due to time constraints, to investigate this any further. In order to further this investigation, the pulse-labelling could be attempted with different time points as there may be very subtle defects. It could also be possible to utilise northern blots using probes specific to different pre-rRNA precursors to look at the rRNA processing in more detail.

One important tool for studying 5S RNP-mediated p53 signalling is through the use of ActD. ActD is a drug that selectively inhibits RNA polymerase I (Sobell, 1985) and activates p53 through the 5S RNP pathway exclusively (Bursac et al., 2012, Donati et al., 2013, Sloan et al., 2013a). SRSF1 depletion had been shown to counteract the effect of p53 induction in BJ-TT cells (Fregoso et al., 2013), and this was able to be repeated in U2OS cells (Figure 4.10). Furthermore, Fregoso et al. (2013) had demonstrated that RPL5 depletion could counteract the p53 response from SRSF1

over-expression in BJ-TT cells and, therefore, it was important to determine whether this could be repeated in U2OS cells to further address the role of SRSF1 in 5S RNP-mediated p53 signalling. As the p53 inductions were not very strong with SRSF1 expression, RPL5 and RPL11 cells were utilised instead and it was found that SRSF1 could counteract the p53 induction from both RPL5 and RPL11 expression (Figure 4.11). These data further supported the evidence that there is a possibility that SRSF1 forms a complex with the 5S RNP to activate p53.

The 5S RNP-p53 signalling pathway appears extremely complicated but the regulation of the pathway may be a step closer to being understood. This chapter has shown that SRSF1 interacts with the 5S rRNA, possibly outside of the ribosome, and is involved in integration of the 5S RNP into the ribosome and thus may be an important assembly factor. It has given evidence towards a model whereby SRSF1 is involved in integration of the 5S RNP into the ribosome and aids in the activation of p53 as summarised in Figure 4.16. Whilst interaction with RPL11 has not yet been confirmed, it is probable that SRSF1 is able to interact with the entire 5S RNP in order to activate p53 as all three components of the RNP are required for p53 activation.

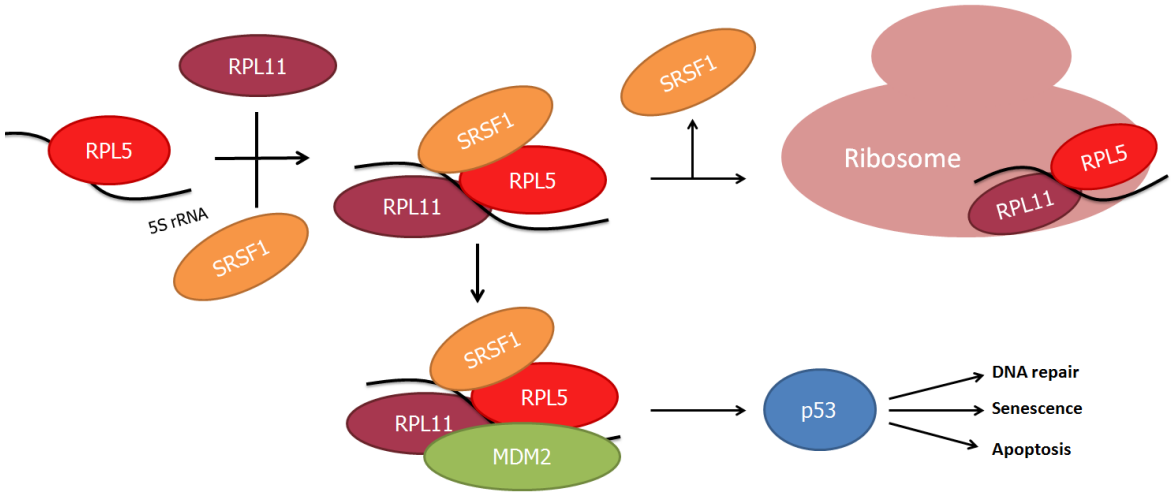

Figure 4.16 A proposed model for SRSF1 in the 5S-RNP mediated activation of p53. RPL5 and the 5S rRNA recruit RPL11 and SRSF1. In normal cells, SRSF1 is lost and the 5S RNP is incorporated into the ribosome. However, in ribosomal stress, the 5S RNP with SRSF1 binds to MDM2 and blocks its ubiquitin ligase activity, leading to p53 activation. p53 affects DNA repair, senescence and apoptosis through its role as a transcription factor.

The second factor investigated in this chapter was PRAS40. PRAS40 is a substrate of AKT (Kovacina et al., 2003) that is involved in negatively regulating mTOR signalling in cells (Thedieck et al., 2007, Fonseca et al., 2007). PRAS40 was shown to sequester

RPL11 to the nucleolus and act as a negative regulator of RPL11-mediated p53 signalling (Havel et al., 2014).

This study has shown FLAG-PRAS40 was localised in the cytoplasm and did not co-localise with the nucleolar marker, fibrillarin (Figure 4.5). This is contradictory to the findings by Havel et al. (2014) who demonstrated, in U2OS cells, that FLAG-PRAS40 co-localised with RPL11 in the nucleoli. Furthermore, investigations revealed that PRAS40 depletion reduced the nucleolar localisation of FLAG-RPL11 but not RPL5 (Figure 4.12). Havel et al. (2015) suggested that PRAS40 blocked translocation of RPL11 from the nucleoli to the nucleoplasm and so this data may support that theory, despite the fact that FLAG-PRAS40 could not be detected in the nucleoli. Therefore, it would be beneficial to investigate the localisation of the endogenous PRAS40, especially with leptomycin B treatment to affect nuclear shuttling. Further investigations could also be carried out into the effect of over-expression of PRAS40 on the localisation of RPL11 to see whether this results in an increase in nucleolar RPL11. Furthermore, the PRAS40-RPL11 complex was suggested to contain other components (Havel et al., 2014). This raised the question about the identity of the other components- the obvious candidates being RPL5 and the 5S rRNA. However, PRAS40 did not associate with the 5S rRNA, in the presence or absence of ribosomal stress (Figure 4.8). Using FLAG-RPL5 or FLAG-RPL11 expressing cells, there was also no co-purification of PRAS40 and RPL5. In agreement with Havel et al. (2014), however, PRAS40 did co-purify with RPL11 (Figure 4.8). As the data presented in this chapter in conjunction with previous findings suggests that PRAS40 is involved in nucleolar localisation of RPL11 and that PRAS40 only interacts with RPL11, this implies that the role of PRAS40 is specific to RPL11 and may sequester RPL11 away from the other components of the 5S RNP, preventing 5S RNP formation and p53 activation.

PRAS40 was shown to increase p53 protein levels further than ActD treatment alone in U2OS cells (Havel et al., 2014) and the data in this chapter was in agreement with this. However, when the activity of p53 was studied using a p53-regulated Luciferase reporter, it was found that PRAS40 had little impact on luciferase levels after ActD treatment. This suggests that PRAS40 increased p53 levels but not activity after ActD treatment. It is possible that this was the result of a plateau of p53 activity that cannot be increased any further, regardless of p53 levels in the cell. However, further

investigations into the role of PRAS40 on p53 activity would be beneficial to further elucidate the role of PRAS40 in p53 signalling. Expression of FLAG-PRAS40 in U2OS cells resulted in elevated p53 and p21 protein levels, although to a lesser extent than the expression of SRSF1 (Figure 4.6). It was previously demonstrated that over-expression of PRAS40 resulted in inhibition of mTORC1 (Sancak et al., 2007) and thus it is possible to hypothesise, based on the observation that over-expression of PRAS40 activates p53, that this feedback mechanism would result in decreased phosphorylation of PRAS40, release of RPL11 from PRAS40 and activation of p53. Despite increases in p53 and p21 (Figure 4.6), over-expression of PRAS40 did not result in p53-mediated cell cycle arrest (Figure 4.7). It would, therefore, be beneficial to investigate apoptosis with Annexin V and 7-AAD or β-galactosidase with flow cytometry as these would give an indication as to whether apoptosis or senescence was occurring.

Furthermore, PRAS40 depletion in U2OS cells resulted in increased p53 and p21 levels (Figure 4.9) which supports evidence from the previous study (Havel et al., 2014). As SRSF1 was shown to decrease p53 levels, these opposing effects may suggest that they may have antagonising roles in p53 signalling. However, the effect of SRSF1 depletion was more pronounced in the MCF-7 cells than in the U2OS where SRSF1 depletion had very little impact on p53 levels. Similarly, the depletion of PRAS40 had very little effect on p53 expression in MCF-7 cells but had a greater impact in U2OS cells. Both MCF-7 and U2OS cells have wild type p53 but are lacking ARF. U2OS cells have MDM2 over-expression (Florenes et al., 1994) and thus the p53 response may be greater in the MCF-7 cells due to less inhibition. Furthermore, MCF-7 cells express constitutively active PRAS40, phosphorylated at T246 (Huang and Porter, 2005). T246 phosphorylation was shown to be crucial for interactions between RPL11 and PRAS40 (Havel et al., 2014) and thus these cells would have a higher level of suppression of p53. It is possible that depletion of PRAS40 in these cells is not sufficient to induce p53. It would be important in the future to perform the same experiment in more clinically relevant cell lines as these would give a clearer indication of how this is affecting diseases such as a Diamond Blackfan Anaemia. Interestingly, the data indicated that PRAS40 may also be required for p53 activation via RPL5, RPL11 and SRSF1. This suggests that whilst phosphorylated PRAS40 inhibits interactions between RPL11 and MDM2, PRAS40 may also be required for activation of p53 via the 5S RNP and p53. This

leads to contradictory roles for PRAS40 in 5S RNP-mediated p53 signalling. However, it is possible that phosphorylated PRAS40 may retain RPL11 in the nucleolus whilst PRAS40 is also required in a different capacity to activate p53 signalling via the 5S RNP complex, without direct interaction with the complex. Therefore, it is currently unclear as to the role in PRAS40 in 5S RNP-mediated p53 signalling and further investigation is required to elucidate the role of PRAS40. For example, PICT-1 was initially also believed to sequester RPL11 to the nucleolus (Sasaki et al., 2011), however it has since been demonstrated to be crucial for 5S RNP recruitment into the ribosome. Due to time constraints, experiments investigating the role of PRAS40 in the recruitment of the 5S RNP could not be optimised. However, this could still be studied in the future using gradients to investigate the incorporation of the 5S rRNA, RPL5, or RPL11 into the ribosome.

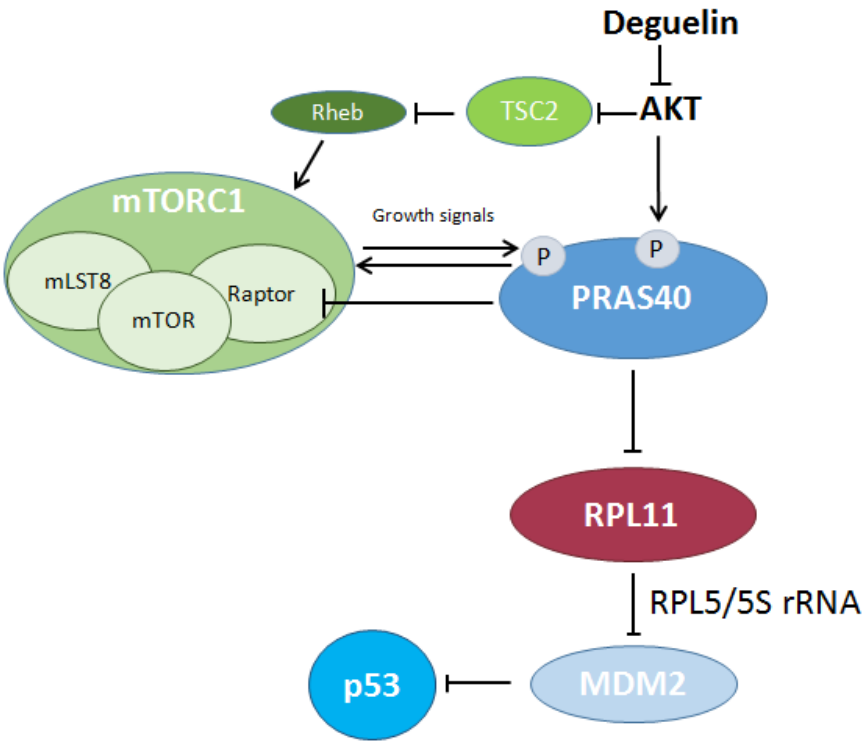

Figure 4. 17. The mechanism of Deguelin activating p53 via the 5S RNP. A Schematic representation of how AKT inhibitors such as deguelin activate p53. Deguelin inhibits AKT, preventing phosphorylation of PRAS40 via mTORC1 and AKT. Non-phosphorylated PRAS40 does not bind to RPL11 and thus RPL11 can bind to the 5S RNP to inactivate MDM2 and stabilise p53.

Finally, inhibitors of AKT were shown to activate p53 via the 5S RNP. AKT is an activator of mTORC1 which, in turn, activates PRAS40 by phosphorylation and results in p53 inhibition. It was, therefore, possible that AKT inhibition would induce p53. Inhibition of AKT with deguelin resulted in an RPL5 dependent p53 activation (Figure 4.15).

Furthermore, deguelin treatment in combination with ActD treatment resulted in an increase in p53 levels compared to ActD treatment alone, similar to what was seen with a depletion of PRAS40. This suggests that inhibition of AKT results in a 5S RNP-dependent activation of p53 as summarised in Figure 4.17 and, therefore, conversely that activation of AKT may counteract 5S RNP-dependent p53 activation and thus could be a potential therapeutic for the treatment of ribosomopathies.

In this chapter, L-leucine or arginine treatment was not able to reduce the p53 response after treatment of cells with ActD. Many similar experiments have been carried out with serum starvation first (Ou et al., 2014, Singh et al., 2011) however, serum starvation abrogates ribosome biogenesis and activates p53 via RPL11 (Strezoska et al., 2000, Ahamad et al., 2015, Bhat et al., 2004). As only one concentration of leucine or arginine was tested, it would be beneficial to attempt to treat cells with different concentrations in the future without having to serum starve cells first as greater concentrations have been suggested to have an effect without prior starvation (Kitsy et al., 2014). Furthermore, addition of L-glutamine to the medium may improve the uptake of leucine (Xu et al., 2013, Nyman et al., 1983).

The role of PRAS40 is not yet clear, but the data from this study suggests that the role of PRAS40 in regulation of RPL11 is more complex than originally demonstrated (Havel et al., 2014). The data is in agreement with previously published data and showed that PRAS40 is exclusively associated with RPL11 and may prevent RPL11 from interacting with MDM2 and, presumably, the other 5S RNP components (Havel et al., 2014). This would propose a mechanism for negatively regulating the 5S RNP and, as treatments for Diamond Blackfan Anaemia appear to target this pathway, could be further assessed. This chapter identified that PRAS40 over-expression as well as depletion of PRAS40 induces p53, possibly as a result of a feedback loop with mTORC1, suggesting there may be multiple mechanisms of regulating p53 via PRAS40. Furthermore, inhibition of AKT resulted in p53 activation, presumably through abrogating the RPL11-PRAS40 interactions. However, most surprisingly, the data indicated the possibility that PRAS40 may be required for p53 activation upon RPL5, RPL11, or SRSF1 expression. These findings have led to an increased knowledge about the role of PRAS40 in suppressing 5S RNP-regulated p53 signalling but also raised new questions about why PRAS40 may be required for p53 activation via the 5S RNP.

CHAPTER FIVE: THE ROLE OF PHOSPHORYLATION OF RPL5 IN CELL SIGNALLING VIA P53

5.1 Introduction

Ribosomes are important molecular machines required for the translation of mRNA into proteins. Production of ribosomes requires large quantities of cellular energy and, as a result, impacts upon the proliferation rate of cells (Warner, 1999a). Furthermore, ribosome biogenesis defects result in the activation of p53 (Holzel et al., 2010, Donati et al., 2012). Recently, a link between recurrent mutations in ribosomal proteins and ribosome biogenesis factors and diseases has been reported, and many of these diseases have been linked to the dysregulation of p53 (Fumagalli and Thomas, 2011).

TP53 is a tumour suppressor gene that is frequently deleted or inactivated in cancers. The TP53 gene encodes the p53 protein which, when activated, coordinates with a variety of signalling pathways that result in DNA repair, apoptosis, senescence or G1 cell cycle arrest (Ko and Prives, 1996, Timofeev et al., 2013, Sperka et al., 2012, Sugikawa et al., 1999). The major repressor of p53 is Mouse Double Minute 2 (MDM2-HDM2 in humans), an ubiquitin ligase which promotes the ubiquitination and degradation of p53 by targeting it to the 26S proteasome (Honda and Yasuda, 1999). In stressed conditions, MDM2 activity is suppressed and this leads to the stabilisation and activation of p53 (Michael and Oren, 2003). One other suppressor of p53 is MDMX, an MDM2 homolog that can bind to p53 to inhibit p53 activity (Danovi et al., 2004) but lacks E3 ubiquitin ligase activity and thus cannot target p53 for degradation (Jackson and Berberich, 2000). MDMX is involved in stabilising MDM2 (Gu et al., 2002) and the interaction between MDM2 and MDMX is required for the E3 ubiquitin ligase activity of MDM2 (Kawai et al., 2007). Furthermore, MDM2 is able to polyubiquitinate MDMX and target it for degradation (Pan and Chen, 2003), resulting in a feedback loop between these two proteins.

One prominent factor contributing to diseases as a result of ribosome biogenesis defects is the 5S RNP. The 5S RNP is a processing intermediate of the ribosomal large subunit, consisting of RPL5, RPL11, and the 5S rRNA. There are multiple ways that the 5S RNP is able to activate p53- through interactions with ARF and MDM2. Interestingly,

the 5S RNP is not only responsible for the regulation of p53 under ribosomal stress situations, it is also important for normal p53 homeostasis (Sloan et al., 2013a). All three components of the 5S RNP are involved with mutually dependent interactions with MDM2, thus stabilising p53 (Sloan et al., 2013a, Donati et al., 2013), as shown in Figure 5.1. Furthermore, the 5S RNP was shown to be essential for the cellular p53 response to ARF (Sloan et al., 2013a). ARF binds to and inhibits MDM2, promoting p53 stabilisation (Honda and Yasuda, 1999).

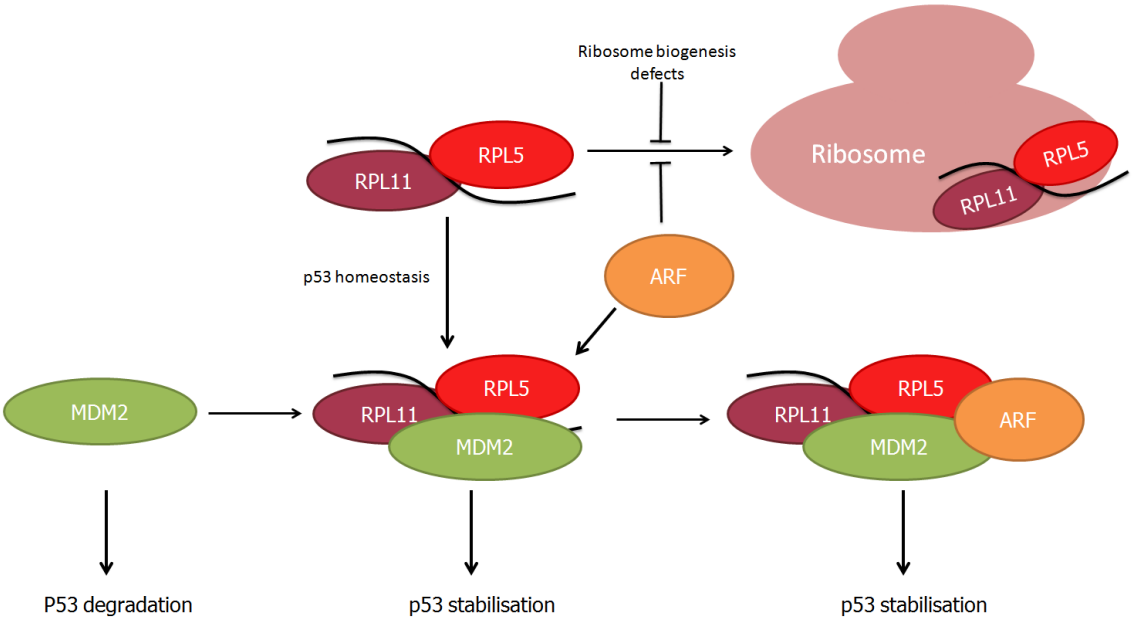

Figure 5.1 The 5S RNP activates p53 via interactions with MDM2. A schematic representation of the 5S RNP-p53 signalling pathway in response to ribosome biogenesis defects or over-expression of the oncogene, ARF. In unstressed cells, MDM2 polyubiquitinates p53 and targets it for proteasomal degradation via the 26S proteasome and the 5S RNP binds to MDM2 and maintains normal p53 homeostasis. When ribosome biogenesis defects occur, the integration of the 5S RNP into the ribosome is blocked and more of the free 5S RNP interacts with MDM2, blocking its ubiquitin ligase activity. When ARF is over-expressed, ribosome biogenesis is disrupted and ARF interacts with the free 5S RNP to promote p53 stabilisation.

Furthermore, it has also been demonstrated that the 5S rRNA, RPL5 and RPL11 play a vital role in regulation of MDMX. The interaction between RPL11 and MDM2 was shown to be induced upon nucleolar stress, resulting in MDMX degradation and p53 activation (Gilkes et al., 2006). In contrast, the 5S rRNA was shown to associate with MDMX and protect it from degradation by MDM2 (Li and Gu, 2011). These data suggest that the 5S RNP may have different roles in the regulation of MDMX under different cellular conditions.

In addition to their interactions with p53, RPL5 and RPL11 have also been implicated in the activation of p73 (Zhou et al., 2015), a p53 homolog (Jost et al., 1997, Kaghad et al., 1997) which can transactivate many of the same gene targets as p53 (Zhu et al., 1998). These data taken together suggest a diverse role for the 5S RNP in the regulation of p53.

The mechanisms of regulating the 5S RNP-p53 pathway are not yet fully understood. Sloan et al. (2013a) proposed that any proteins involved in ribosomal integration, localisation or formation of the 5S RNP were crucial in regulating p53. One way that the 5S RNP-p53 pathway could be regulated is through post-translational modifications such as phosphorylation and NEDDylation.

MDM2 promotes NEDDylation of RPL11 and protects RPL11 from destabilisation (Xirodimas et al., 2008, Sundqvist et al., 2009). ActD treatment was found to decrease NEDDylation of RPL11 and cause re-localisation of RPL11 to the nucleoplasm, where it can activate p53 (Sundqvist et al., 2009). This suggests that NEDDylation of RPL11 is a mechanism by which to prevent activation of p53 via RPL11. However, Mahata et al. (2012) found that, in cells undergoing ribosomal stress, RPL11 and NEDD8 were both crucial for the acetylation, and thus activation, of p53. The authors demonstrated that NEDDylation of RPL11 was required for the recruitment of RPL5 and RPL11 to promoter sites of p53 regulated genes in an MDM2-dependent manner. These data, when assessed in combination with the later findings that the 5S RNP is crucial for p53 activation (Donati et al., 2013, Sloan et al., 2013a), indicate that NEDDylation is an important regulator of 5S RNP-mediated p53 activation.

Whilst only NEDDylation has been shown to regulate the 5S RNP signalling to date, phosphorylation may also be important for protein regulation. Phosphorylation is a key regulator in p53 signalling. p53 is phosphorylated at a number of different sites to regulate function, for example, p53 can be activated by phosphorylation of serine 37 by ATR and CK2 in response to chemotherapeutic drugs or U.V light (Meek, 1999). In addition, phosphorylation of p53 serine 15 has been demonstrated to be critical for stimulating transactivation at p53-responsive promoters (Loughery et al., 2014). Phosphorylation of MDM2 by the kinases ATM and C-Abl inhibits p53 degradation upon DNA damage (Goldberg et al., 2002, Maya et al., 2001). Furthermore, DNA damage results in phosphorylation of MDMX via Chk1, Chk2 and ATM, activating p53

(Jin et al., 2006, Chen et al., 2005). These data taken together show an important role for phosphorylation in the regulation of p53.

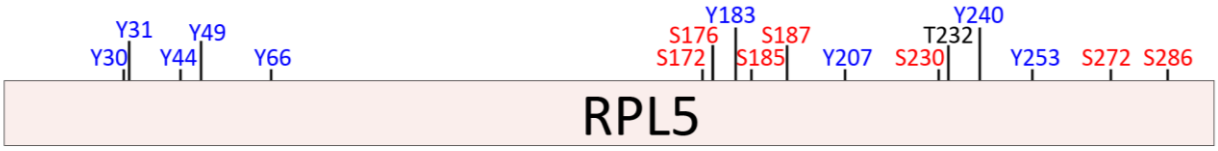

Figure 5.2 Reported phosphorylation sites on RPL5. Schematic representation of the RPL5 protein with the phosphorylation sites as reported on Phosphosite (http://www.phosphosite.org). RPL5 contains 17 reported phosphorylation sites, 9 tyrosine phosphorylation sites (blue), 7 serine phosphorylation sites (red) and 1 threonine phosphorylation site (black).

Many ribosomal proteins such as RPS3 (Kim et al., 2009, Lee et al., 2010), RPS6 (Ruvinsky and Meyuhas, 2006, Khalaileh et al., 2013), and RPS14 (Ventimiglia and Wool, 1974) have been shown to be phosphorylated. Furthermore, it has been shown *in vitro* that RPL5 has the potential to be phosphorylated (Park and Bae, 1999). As RPL5 and RPL11 are crucial to 5S RNP-mediated p53 signalling, phosphorylation of these proteins may play a role in the regulation of this pathway. According to Phosphosite (http://www.phosphosite.org), RPL5 has 17 reported phosphorylation sites (Figure 5.2). Out of the 17 phosphorylations, 9 of these are tyrosine. Tyrosine phosphorylations are uncommon in cells (Cooper, 1989) and, therefore it is possible that these may play an important role in the regulation of RPL5. It is important to note that these are reported phosphorylation sites and thus may not necessarily be representative of the actual phosphorylation sites in RPL5. Interestingly, some of the mutations have been reported in multiple data sets, for example phosphorylation of Y30 was demonstrated in 14 different data sets, whilst Y31 has been reported in 3.

Currently, very little is known about RPL5 phosphorylation or the kinases and phosphatases that may be involved. RPL5 phosphorylation by CK2 at serine residues was shown to prevent binding of RPL5 to the 5S rRNA *in vitro* (Park and Bae, 1999). Thus, phosphorylation of RPL5 by CK2 could affect 5S RNP formation and, subsequently, ribosome biogenesis. RPL5 has also been shown to interact with, and potentially be phosphorylated by, one other kinase, the Death-associated protein kinase (DAPk) (Bialik et al., 2008).

As 5S RNP-p53 signalling has been implicated in diseases, it is crucial that the regulation of this pathway is fully elucidated. Ribosomopathies are diseases of ribosome dysfunction and many have reported changes in p53. One such ribosomopathy is Diamond Blackfan Anaemia (DBA), which is a rare autosomal recessive condition characterised by a reduction in erythroid production and thus anaemia. 50-70% of all DBA patients present with a mutation in ribosomal proteins, including RPS7, RPS19, RPS27, RPS26, RPL27, RPL5, RPL11 (Smetanina et al., 2015, Wang et al., 2015, Mirabello et al., 2014, Konno et al., 2010). These mutations are mostly deleterious resulting in haploinsufficiency. The pathogenesis of DBA is thought to be attributed to defects in ribosome biogenesis resulting in p53 activation and apoptosis of the red blood cell precursors, which rely upon a high rate of ribosome biogenesis (Dai and Lu, 2004, Zhang et al., 2011).

Little is currently known about how phosphorylation regulates the 5S RNP. Therefore, the aim of this chapter was to characterise the role of RPL5 phosphorylation in regulating the 5S RNP-p53 pathway and ribosome biogenesis. To do this, several mutants of RPL5 were expressed in human U2OS cells and studied for their ability to activate p53, their effect on cell cycle, and their ability to integrate into the ribosome.

5.2 Results

5.2.1 Tyrosines 30, 31 and 44 of RPL5 are located on the interaction surface with the ribosome and, thus, if phosphorylated, may block ribosomal integration.

Several tyrosine residues in RPL5 have been reported to be phosphorylated. The phosphorylation sites were mapped onto the structure (Figure 5.3A) of the human ribosome (Khatter et al., 2015) and assessed for their proximity to the 5S rRNA or the large subunit of the ribosome.

The three most interesting phosphorylation sites seen in this study were tyrosines 30, 31 and 44 (Y30, Y31 and Y44) as shown in yellow, green and orange respectively in Figure 5.3A-B. Y30, Y31 and Y44 were shown to be located on the interaction surface between the 28S rRNA and RPL5 (Figure 3.5B). This was interesting as phosphorylation at these sites could affect RPL5 being integrated into the ribosome. None of the three

phosphorylation sites appeared to be in a position to disrupt the RPL5 and 5S rRNA interaction.

Furthermore, when aligning RPL5 protein sequences across a spectrum of eukaryotic organisms, it was clear that the Y30, Y31 and Y44 sites were generally highly conserved (Figure 5.3C). However, Y31 was not conserved in two lower eukaryotes (*D. discoidium* and *T. gondii*). Furthermore, in zebrafish (*D. rerio*), Y31 is mutated to phenylalanine (F); this is interesting as phenylalanine cannot be phosphorylated and thus suggests phosphorylation of this site is species specific. These data taken together imply that it is possible that phosphorylation of Y30, Y31 and Y44 may disrupt integration of the 5S RNP into the ribosome.

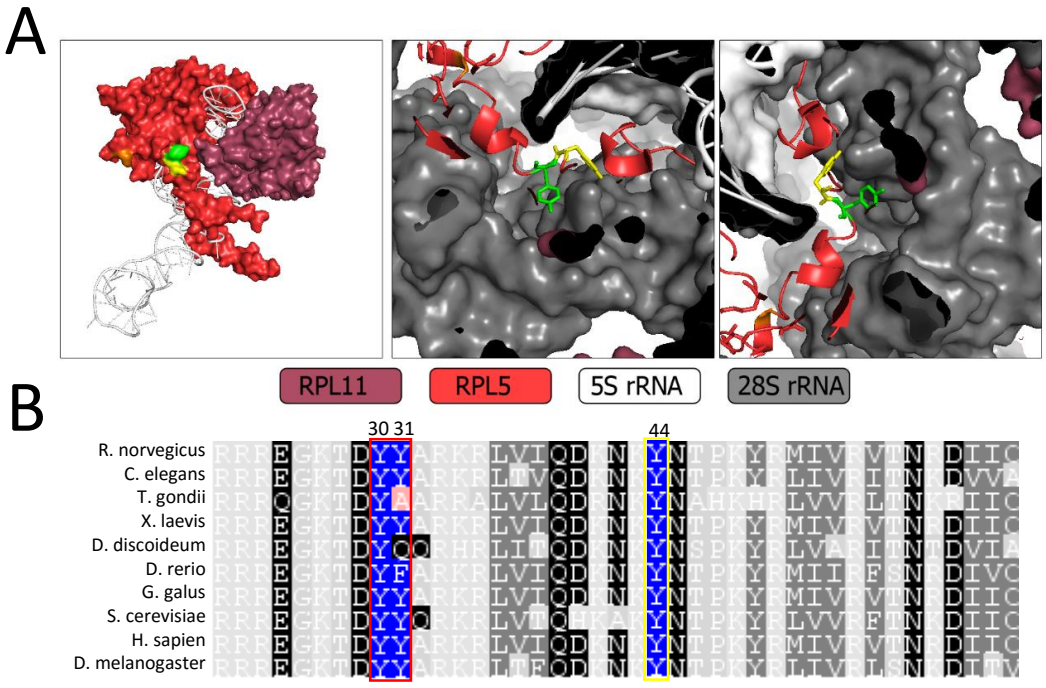

Figure 5.3. RPL5 Y30, Y31 and Y44 are located within the interaction surface with the ribosome and Y30 and Y44 are highly conserved in eukaryotes. (A) The cryo-EM structure of the 5S RNP showing the position of Y30, Y31 and Y44 of RPL5. The first image depicts the human ribosome structure (Khatter et al., 2015) of RPL5 (red), RPL11 (purple) and the 5S rRNA (white). The three potentially phosphorylated tyrosines are shown: Y30 in yellow, Y31 in green and Y44 in orange. The second and third images depicts Y30 and Y31 (yellow and green), showing their close interaction the 28S rRNA in grey **(C)** Sequence alignment showing the sequence similarity between the RPL5 in various eukaryotic organisms. Y30 and 31 are indicated with a red border and Y44 with yellow.

Therefore, it was important to validate these predicted phosphorylation sites and test whether phosphorylation of RPL5 in U2OS cells could be detected. In order to do this, Phos-Tag acrylamide gels were utilised. In Phos-Tag acrylamide gels, the

phosphorylated proteins are retarded in their migration through the gel and thus phosphorylated proteins become separated from non-phosphorylated.
U2OS cells expressing either an empty pcDNA5 vector or the RPL5-FLAG pcDNA5 vector were incubated in growth medium with the addition of 1 µg/ml tetracycline for 48 hours. Whole cell lysates were then separated by SDS-PAGE using 13% acrylamide gels with or without the addition of the Phos-Tag. Anti-FLAG antibody was utilised and showed FLAG-RPL5 expression in the induced cells.

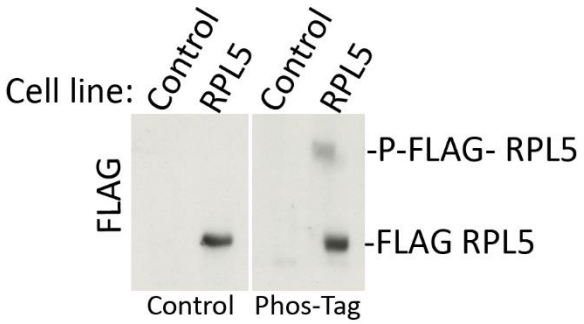

Figure 5.4. Phosphorylation of RPL5 in cells. U2OS Flp-In cells stably transfected with an RPL5-FLAG pcDNA5 construct were incubated at 37°C in growth medium containing 1 µg/ml tetracycline for 48 hours to induce the FLAG tagged protein. Whole cell lysates were separated via SDS-PAGE using either 13% acrylamide or 13% acylamide with PhosTag acrylamide prior to transferring to a nitrocellulose membrane via western blotting. Anti-FLAG antibody was used to probe the membranes

The PhosTag gel showed an additional signal that was not present on the control gel, suggesting the presence of phosphorylated RPL5 in U2OS cells (Figure 5.4).

5.2.2 Creation of inducible cell lines expressing FLAG-tagged RPL5 phosphorylation mutants

In order to study the effect of phosphorylation of RPL5, RPL5 mutants were generated that attempted to mimic phosphorylation (Y to E) or prevented phosphorylation (Y to F) of sites Y30, Y31 and Y44. U2OS cell lines were generated that would express FLAG-tagged wild type or mutant RPL5 upon induction with tetracycline. To do this, pcDNA5-FRT plasmids containing wild type RPL5 were subjected to site-directed mutagenesis using primers specific to each mutation. The resultant plasmids were then co-transfected into Flp-In U2OS cells with a vector expressing Flp recombinase (pOG44). Six of the intended cell lines were successfully created- Y30E, Y30F, Y44F, Y44E, and a double mutant of Y30F Y31F.

In order to establish their ability to express the proteins, the cells expressing the wild type or mutant RPL5 were incubated at 37°C with medium containing 1 µg/ml tetracycline for 48 hours before harvesting, separating whole cell extracts by SDS-PAGE and western blotting for RPL5 (Figure 5.5A). Upon expression of the wild type or RPL5 mutants, a larger molecular weight protein of RPL5 origin was detected using an anti-RPL5 antibody, indicating that the FLAG-RPL5 mutants were expressed as the FLAG-tag increases the size of the protein, thus affecting the migration through the gel. However, there were clear differences between the levels of induction of the proteins with the Y30F mutant exhibiting the highest induction of RPL5.

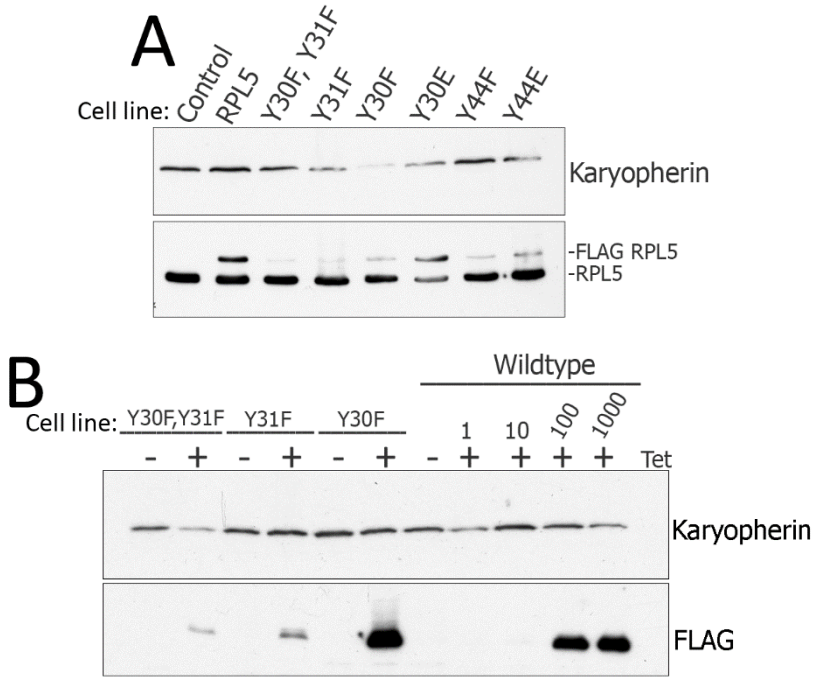

Figure 5.5. Inductions of RPL5 and the mutants in U2OS cell lines. (A) U2OS cell stably expressing RPL5 wild type and mutant proteins, as shown, were grown for 48 hours in the presence of 1µg/ml tetracycline before harvesting the cells. Whole cell lysates were separated by SDS-PAGE and western blotted for RPL5 using Karyopherin as a loading control. The endogenous (End) and FLAG-tagged (FLAG) RPL5 are denoted on the left. **(B)** U2OS cells expressing RPL5 Y30F Y31F, Y31F or Y30F were induced for 48 hours with 1000 ng/ml tetracycline as indicated. The wild type RPL5 U2OS cells were similarly induced for 48 hours with a range of tetracycline concentrations from 1ng/ml-1000ng/ml tetracycline. Whole cell extracts were separated by SDS-PAGE and analysed by western blotting.

As the level of protein expression for some mutants did not reach levels of wild type protein, comparable conclusions from these mutants would be inaccurate. Therefore, in order to rectify this issue, the Y30F Y31F, Y31F and Y30F mutants were incubated, as before, in normal medium containing 1000ng/ml tetracycline for 48 hours and compared to a 48 hour induction of the Wild type at varying tetracycline concentrations (Figure 5.5B). By analysing at the signal from the anti-FLAG antibody, it

was clear that the wild type protein expression did not increase proportionally with increased tetracycline levels and that it was not possible to reduce the expression of the wild type protein to the levels seen for the Y31F and Y30F/Y31F mutants. As the expression of some of the mutants was poor this meant that downstream experiments were often difficult as there was not enough expression to detect signals via western blot. Therefore, only the two highest expressing mutants, Y30E and Y30F, were carried forward for further studies.

5.2.3 Mutation of RPL5 tyrosine 30 does not affect the localisation of RPL5.

In addition to affecting protein-protein interactions, phosphorylation can also affect localisation of proteins (Stulke, 2010). In order to ascertain whether the mutations in RPL5 affected localisation of the protein, U2OS cells expressing either the mutant or wild type FLAG-tagged RPL5 were cultured with 1 µg/ml tetracycline-containing medium, at 37°C, for 48 hours before fixing in 4% paraformaldehyde and analysing by immunofluorescence using antibodies raised against the FLAG tag and fibrillarin (to highlight the nucleoli). DAPI staining was used to indicate the nucleus (Figure 5.6).

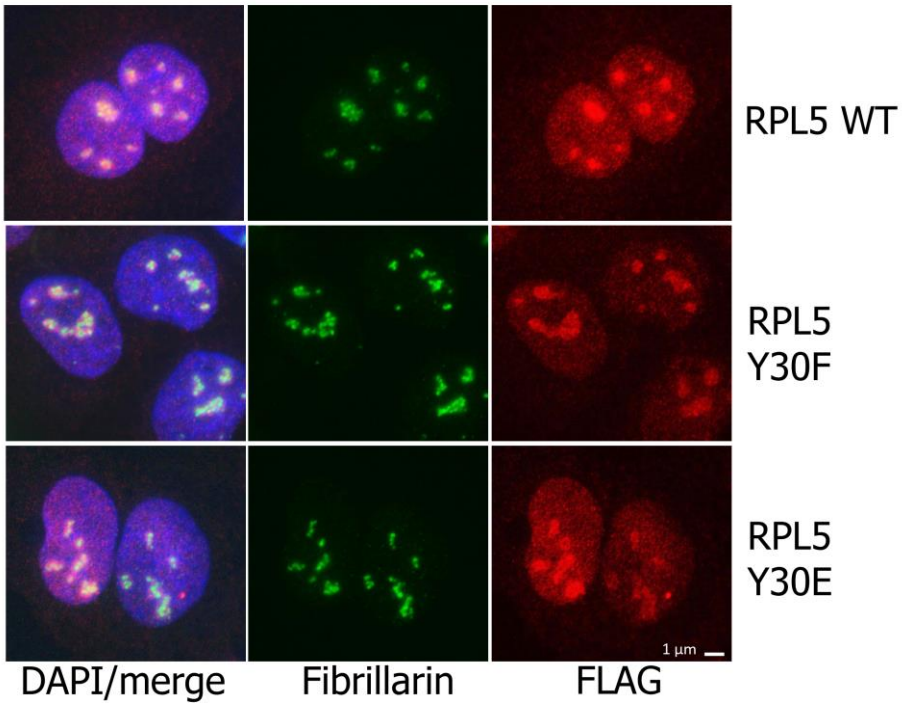

Figure 5.6. Mutation of Y30 does not affect localisation of RPL5. U2OS cells expressing wild type, Y30E or Y30F FLAG-tagged RPL5 were grown on coverslips and induced with 1 µg/ml tetracycline for 48 hours. The cells were fixed in 4% paraformaldehyde and prepared for immunofluorescence using anti-Fibrillarin (green), anti-FLAG (red) and DAPI (blue). Scale bar represents 1 µm.

In agreement with previous studies, FLAG-RPL5 signal was enriched in the nucleolus and nucleoplasm (Sloan et al., 2013a). Very little cytoplasmic FLAG-signal was detected, due to the short induction times as 5S RNP incorporation into the ribosome is slow (Sloan et al., 2013a). As both the wild type and mutant RPL5 showed the same localisation pattern, this suggests that mimicking phosphorylation, or lack thereof, tyrosine 30 does not affect the regions in which RPL5 is localised.

5.2.4 Mutating Y30 to phenylalanine or glutamic acid does not affect RPL5-5S rRNA interactions.

RPL5 has been previously demonstrated to be required for the maturation of the 5S rRNA and formation of the 5S RNP is essential for ribosome production and, as all three factors of the 5S RNP are required to bind MDM2, p53 activation (Donati et al., 2013, Sloan et al., 2013a). RPL5 association with the 5S RNA is crucial for the production of the 5S RNP, therefore it was important to establish whether RPL5 mutants could associate with the 5S rRNA.

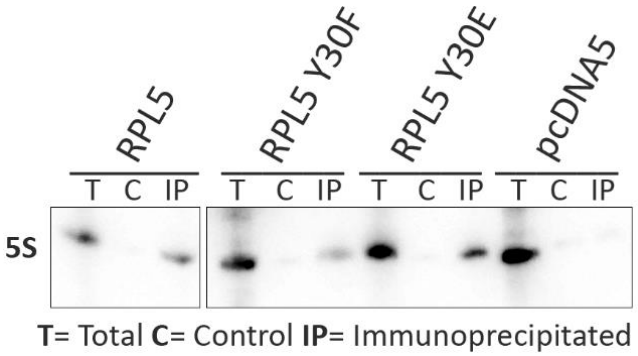

Figure 5.7. Interactions between RPL5 wild type and mutants with the 5S rRNA. Representative northern blot from three independent experiments to show association of wild type or mutant RPL5 with the 5S rRNA. U2OS cells expressing the wild type or mutant RPL5 were induced as in A for 48 hours before harvesting. Cell lysates were incubated with IgG sepharose with anti-FLAG antibody for 2 hours prior to washing, elution and RNA extraction. RNAs were separated by acrylamide gel electrophoresis and northern blotted and probed using ^{32}P labelled probes against 5S rRNA. T denotes 10% RNA totals.

U2OS cells expressing either an empty pcDNA5 vector (control), wild type, Y30E RPL5, or Y30F RPL5 were grown in medium containing 1 µg/ml tetracycline at 37^{o}C for 48 hours before harvesting and lysing the cells. For the RNA immunoprecipitations, IgG sepharose alone (control) or in conjunction with anti-FLAG antibody (IP) were incubated with the lysates followed by RNA extractions, acrylamide gel electrophoresis, and northern blotting. The blots were incubated with ^{32}P-labelled probes specific for the 5S and 5.8S rRNAs (Figure 5.7).

The empty pcDNA5 vector control and no antibody control (denoted c) showed little–to-no signal in the IP lane, showing that the FLAG-tag or IgG sepharose alone do not associate with the 5S rRNA. Wild type RPL5 and the mutants all co-purified with 5S (Figure 5.7). This suggests that mutations of Y30 to phenylalanine or glutamic acid does not affect RPL5-5S RNA interactions and means that these mutants still have the potential to form the 5S RNP.

5.2.5 Expression of RPL5 Y30E results in increased levels of non-ribosomal RPL5 compared the RPL5 Y30F mutant and wild type RPL5.

As the mutants interacted with the 5S rRNA, the next step was to investigate whether they were still incorporated into ribosomes. To do this, a 10-40% glycerol gradient was utilised to separate the free and ribosomal bound RPL5.

U2OS cells expressing either the wild type or Y30 mutant FLAG-tagged RPL5 were incubated for 48 hours with 1 µg/ml tetracycline-containing media before harvesting. The cells were lysed and applied to a 10-40% glycerol gradient and separated by ultracentrifugation, before collecting 200 µl fractions from the gradient. The fractions were separated by SDS-PAGE and used in western blot analysis using an anti-RPL5 antibody (figure 5.8A) in order to see the endogenous RPL5. The ‘free’ and ‘ribosomal’ fractions were defined as fractions 1-7 and 9-20 respectively based on previous experiments (Sloan et al., 2013a). Magnesium was omitted from the lysate during sonication in order to prevent the nucleoli staying intact as this is where most of the RPL5 is present (Figure 5.6).

The endogenous RPL5 showed the same distribution through the fractions (Figure 5.6A labelled end) upon expression of the wild type or mutant FLAG-RPL5. The wild type RPL5 gave a strong RPL5-FLAG signal in the ribosomal fractions (Figure 5.8A fractions 9-20). When densitometry analysis (Figure 5.8B) was carried out, it revealed that the wild type had, on average, 20% of the protein in the ‘free’ fractions (Figure 5.8A fractions 1-7). Similar results were seen with the Y30F mutant, with no significant difference between the percentages of free RPL5 present when using a paired t-test.

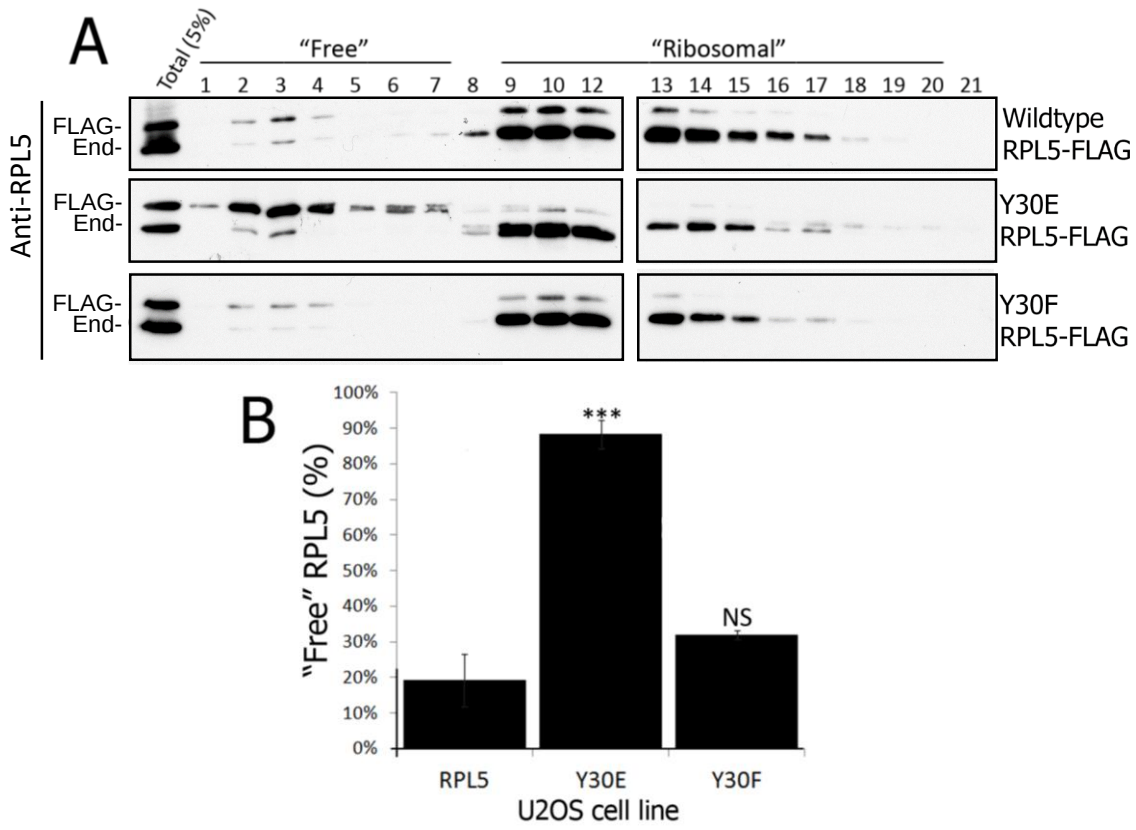

Figure 5.8. Mimicking phosphorylation of RPL5 Y30 leads to decreased ribosomal integration of RPL5. (A) Representative western blots from three independent experiments to show incorporation of wild type or mutant RPL5 into the ribosome. U2OS cell lines expressing FLAG-tagged wild type, Y30F, or Y30E RPL5 were grown in the presence of 1 µg/ml tetracycline for 48 hours to induce protein expression. The cells were harvested and lysates prepared and applied to a 10-40% glycerol gradient to separate the large and small complexes. The resultant gradient was fractionated, separated by SDS-PAGE and analysed by western blot using an anti-RPL5 antibody. **(B)** The western blot in A was subjected to densitometry analysis and the density of the FLAG-tagged RPL5 signal was quantified using ImageJ. Each bar in B represents the means ± the standard deviation of the means, n=3. Statistical significance was determined using a paired t-test. NS= non-significant, *= P ≤ 0.05, **= p≤ 0.01, ***= p≤ 0.001.

The Y30E mutant, however, showed a very different profile from the wild type and Y30F mutant. Most of the FLAG-RPL5 Y30E was in the 'free' fractions (Figure 5.8A fractions 1-7). The Y30E mutant had around 85% of protein in the 'free' fractions (Figure 5.8B) which was highly significantly different from the wild type when analysed using a paired t-test. This suggested that mimicking phosphorylation of Y30 may prevent integration of RPL5 into the ribosome, whilst preventing phosphorylation has no effect.

5.2.6 RPL5 shows evidence of differential phosphorylation between non-ribosomal and ribosomal RPL5

As the data suggested that phosphorylation of tyrosine 30 prevents ribosomal integration of RPL5, it was important to establish whether there was preferential

phosphorylation of free RPL5 over ribosomal RPL5. In order to investigate this, Phos-Tag acrylamide was utilised as it enables separation of phosphorylated and non-phosphorylated proteins as described in section 5.2.1. As the previous experiment (Figure 5.4) had shown a 'smear' of RPL5 phosphorylation, the gels were separated for longer using a lower percentage of acrylamide to allow for better resolution.

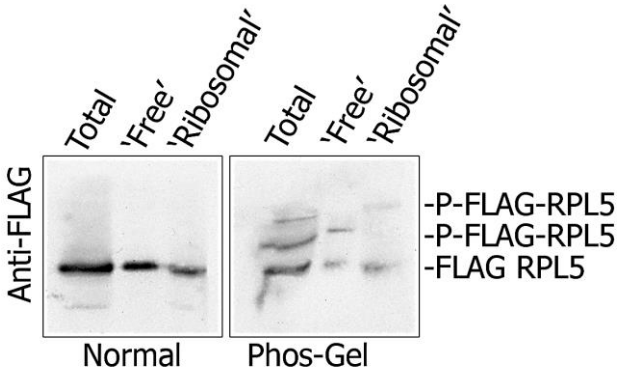

Figure 5.9. RPL5 is differentially phosphorylated when incorporated into ribosomes. U2OS Flp-In cells stably transfected with an RPL5-FLAG pcDNA5 construct were incubated with 1 µg/ml tetracycline for 48 hours to induce the FLAG tagged protein. The cells were harvested, lysed and separated by 10-40% glycerol gradient centrifugation. Fractions were taken from the resultant gradient and fractions 1-8 were combined and denoted 'free' 5S RNP whilst fractions 9-16 were combined and denoted 'ribosomal' 5S RNP. The pooled fractions were separated via SDS-PAGE using either 7% acrylamide or 7% acylamide with PhosTag acrylamide prior to transferring to a nitrocellulose membrane via western blotting. Anti-FLAG antibody was used to probe the membranes. The signals indicating FLAG-RPL5 and the two phosphorylated RPL5 signals are indicated on the right.

U2OS cells expressing wild type FLAG-RPL5 were incubated with 1 µg/ml tetracycline-containing medium, at 37°C, for 48 hours prior to harvesting. The cells were lysed and applied to a 10-40% glycerol gradient before ultracentrifugation to separate the large and small complexes. The resultant gradient was sequentially aliquoted into 200µl fractions. Based on previous gradient experiments (Figure 5.8), fractions 1-7 and fractions 9-20 were pooled as the 'free' and 'ribosomal' pools of RPL5 respectively. The pooled protein fractions were separated using a 7% acrylamide gel or a 7% acrylamide gel containing 50µmol/L Phos-tag and 100µmol/L $MnCl_2$ (Figure 5.9). The proteins were transferred via western blotting to a nitrocellulose membrane and probed using an anti-FLAG antibody.

With the standard 7% acrylamide SDS-PAGE gel, there was a single signal from the anti-FLAG antibody that denoted the FLAG-RPL5 protein. However, the Phos-tag gel had multiple signals of a higher molecular weight than the FLAG-RPL5 signal (denoted P-FLAG-RPL5), suggesting the presence of phosphorylated proteins. A higher molecular

weight signal was detected specifically in the total protein and the 'free' fraction that did not appear to be detected in the 'ribosomal' fraction. Furthermore, an additional higher molecular weight signal was detected in the 'ribosomal' pool but appeared not to be in the 'free' pool. This suggests that different phosphorylations may be present in the 'free' and 'ribosomal' pools of RPL5 and may support the theory that phosphorylation may regulate 5S RNP integration into the ribosome.

5.2.7 FLAG-RPL5 Y30 mutants associate with MDM2 and p53

Looking at the cryo-EM structure of the human ribosome, the RPL5 Y30 residue appeared to be in close proximity to helices 83-85 of the 28S rRNA. This region has been shown to bind to RPL11 in the same region that MDM2 binds (Zheng et al., 2015), thus suggesting that phosphorylation of this site may disrupt 5S-RNP MDM2 interactions. Therefore, immunoprecipitations were utilised to look for interactions between wild type or mutant RPL5 and MDM2.

U2OS cells expressing either an empty pcDNA5 vector (control), wild type, Y30E RPL5, or Y30F RPL5 were grown in medium containing 1 µg/ml tetracycline at 37°C for 48 hours before harvesting and collection of lysates. The lysates were incubated with anti-FLAG conjugated agarose beads for 2 hours before elution of the protein, separation by SDS-PAGE and western blot analysis (Figure 5.10). The control FLAG-tag did not co-purify MDM2 or p53, suggesting any interactions were specific to RPL5.

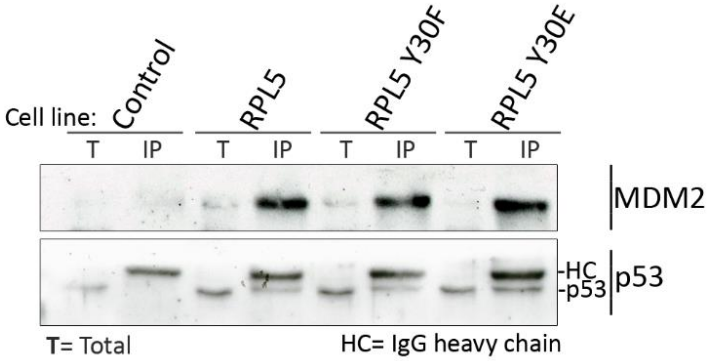

Figure 5.10. The RPL5 mutants interact MDM2 and p53. Representative western blot from two independent experiments to show RPL5 association with MDM2 and p53. U2OS cells expressing the wild type or mutant RPL5 were incubated in growth medium containing 1 µg/ml tetracycline for 48 hours before harvesting. Cell lysates were collected and incubated with FLAG-conjugated agarose for 2 hours followed by elution, separation by SDS-PAGE and western blotting. T denotes 5% total protein. HC indicates cross-reactivity with the IgG heavy chain.

MDM2 co-purified with RPL5 and both of the mutants strongly, suggesting that phosphorylation of Y30, or lack thereof, has no effect on RPL5 association with MDM2. Furthermore, the 5% total protein (denoted T) for both the MDM2 and p53 was extremely weak, suggesting that the percentage being bound by RPL5 was much higher than the 5% total loaded. This suggests that RPL5 associates with a large percentage of the MDM2 in the cell.

The p53 binding site (Chi et al., 2005) and binding sites for the 5S RNP (Zhang et al., 2011, Zheng et al., 2015) do not overlap on MDM2, suggesting that the 5S RNP and p53 may simultaneously interact with MDM2. Marechal et al. (1994) detected the presence of an RPL5-MDM2-p53 complex in cells. Therefore, it was possible that the MDM2-5S RNP complex also contained p53. Furthermore, (Bursac et al., 2012) showed that p53 co-purified with MDM2, RPL5 and RPL11 and it has also been shown that RPL11 is recruited to promoter sites of p53 regulated genes (Sundqvist et al., 2009). However, it was recently shown that p53 does not associate with RPL5 or RPL11 (Zhou et al., 2015) and thus the presence of p53 in the RP-MDM2 complex remained uncertain. Therefore, a p53 antibody was utilised in the western blots to investigate p53 association with RPL5. Interestingly, the data shows that p53 co-purified with both the wild type RPL5 and mutants (Figure 5.10). This suggests that it is possible that p53 interacts with the 5S RNP/MDM2 complex.

5.2.8 The wild type and mutant RPL5 all increase p53 levels but the Y30F mutation does not increase p53 activity

It has been shown that expression of RPL5 in U2OS cells causes an increase in p53 (Horn and Vousden, 2008). It has also been hypothesised that anything regulating 5S RNP integration into the ribosome would be central in regulating p53 stabilisation via MDM2 (Sloan et al., 2013a). As mimicking phosphorylation of Y30 appeared to block integration of RPL5 into the ribosome, expression of the RPL5 Y30E or the Y30F mutant was investigated to see whether it would result in a changes in p53 activation compared to wild type RPL5.

U2OS cells expressing FLAG-tagged wild type, Y30E or Y30F RPL5 were grown at 37^{o}C in media containing 1 μg/ml tetracycline for 48 hours to induce the expression of the proteins. The cells were harvested and whole cell extracts separated by SDS-PAGE and

analysed by western blot for p53 and its downstream effector, p21 (Figure 5.8A). Karyopherin was used as a loading control.

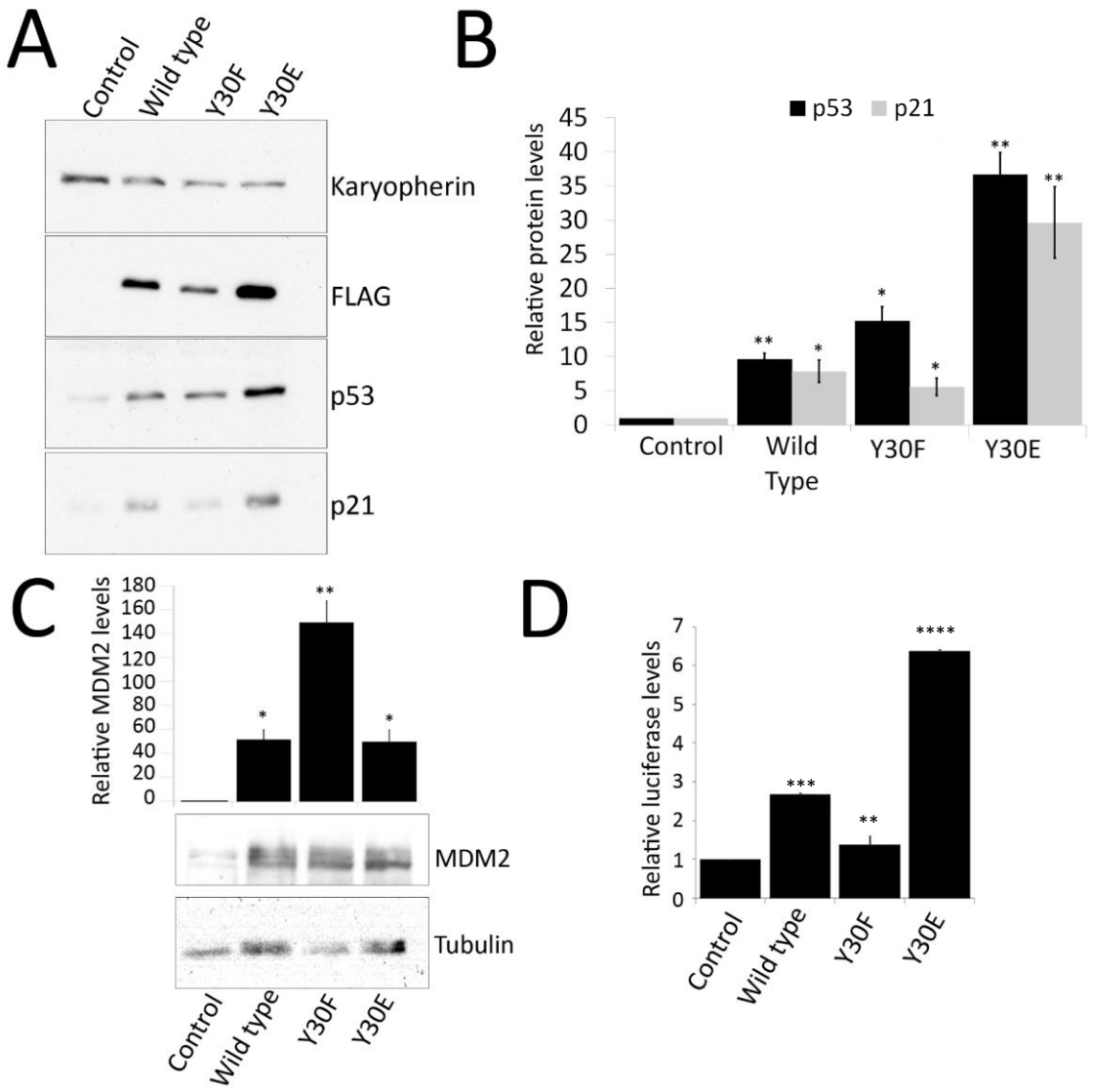

Figure 5.11. Expression of the RPL5 mutants affects p53 protein levels and activity. (A) Representative western blot from three independent experiments to show how expression of the proteins affects p53 and p21 levels. U2OS cells expressing either wild type or mutant FLAG-tagged RPL5 were induced with 1 µg/ml tetracycline for 48 hours prior to harvesting and separating the whole cell lysates by SDS-PAGE before western blotting. Karyopherin was used as a loading control. **(B)** Densitometry analysis of p53 and p21 levels in A relative to the control. Each bar in B represents the means ± the standard error of the means from three experiments. **(C)** Representative western blot from two independent experiments to show the effect of RPL5 wild type or Y30 mutant expression on MDM2 protein levels. U2OS cells expressing wild type or mutant FLAG-tagged RPL5 were expressed as in A, separated by SDS-PAGE and analysed by western blotting using anti-MDM2 and p53 antibodies with an anti-α-tubulin loading control. Bars represent Densitometry analysis of C. **(D)** Luciferase assay to determine p53 activity upon expression of the wild type or mutant FLAG-RPL5. U2OS cells expressing the proteins as in A+B were transfected with a plasmid containing Renilla luciferase under a constitutive promoter and a plasmid containing firefly luciferase under the control of p53 regulated promoters. Luciferase was measured after 48 hours and normalised against relative protein concentrations. Each bar in B, C and D represents the means ± the standard deviation of the means, n=3. Statistical analysis determined by a paired t test. NS= non-significant, *= $P \leq 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$.

Both the mutants and the wild type cell lines expressed significantly more p53 protein than the control, with the Y30E mutant showing the greatest increase in p53 (Figure 5.11 A-B). Whilst the difference between the p53 levels upon expression of the Y30E mutant were significant when compared to wild type (P=0.007), the difference between p53 levels upon expression of Y30F mutant compared to wild type were not. This suggests that RPL5 Y30F expression results in a similar p53 induction to wild type but expression of RPL5 Y30E results in a greater p53 induction. Interestingly, when studying the effect of the p53 downstream effector, p21, it was noted that expression of the mutant or wild type RPL5 resulted in increased p21 levels compared to control. However, when analysed by densitometry (Figure 5.11B), it revealed that expression of the Y30F mutant resulted in a lesser p21 response compared to the wild type or Y30F mutant RPL5. This suggested that whilst p53 protein levels had increased with RPL5 Y30F expression compared to the control cells, the amount of p53 activity had not increased proportionally.

Increased p53 expression results in increased MDM2 levels due a feedback loop between p53 and MDM2 (Barak et al., 1993). However, as the main regulator of p53 activity, increased levels of MDM2 can result in suppression of p53 (Bond et al., 2004, Jones et al., 1998). Therefore, the effect of wild type or mutant RPL5 expression on MDM2 protein levels were also studied. U2OS cells expressing wild type or mutant RPL5 were expressed as previously described and whole cell lysates separated by SDS-PAGE before western blotting. The resultant western blot revealed that expression of wild type RPL5 resulted in an increase in MDM2 protein levels (Figure 5.11C). Expression of the Y30E mutant resulted in a similar increase in MDM2 levels to the wild type. However, when analysed by densitometry analysis (bars- Figure 5.11C), it appeared that the MDM2 increase upon induction of the Y30F mutant was much greater than that upon induction of the wild type or Y30E mutant.

To further explore the effect of the RPL5 mutants on p53 activity rather than protein levels, plasmids containing p53-regulated Luciferase were utilised (Figure 5.11D). Cells containing wild type or mutant RPL5-FLAG were co-transfected with the pG13-LUC plasmid, which contains 13 copies of the p53 binding consensus linked to a luciferase reporter gene (el-Deiry et al., 1993), and the control pRL-TK renilla luciferase plasmid (Promega). Twelve hours after transfection, the cells were incubated in medium

supplemented with 1 µg/ml tetracycline for 48 hours to induce the FLAG-tagged proteins. The cells were then lysed and assayed for luciferase activity using the Promega Dual Luciferase kit. The results were normalised against Renilla luciferase activity to account for any differences in transfection efficiency.

The results (Figure 5.11D) show that expression of wild type RPL5 results in just under a 3-fold increase in p53 transcriptional activity whereas expression of the RPL5 Y30E mutant brings about a 6-fold increase in p53 activity. Interestingly, induction of the Y30F mutant showed only a 50% increase in p53-regulated luciferase, supporting the hypothesis that the Y30F mutant increases p53 protein levels but does not lead to as successful of an activation of p53.

These data suggest that expression of RPL5 harbouring a mutation of Y30 to glutamic acid results in increased p53 protein levels and increased activation of p53. However, expression of RPL5 harbouring a mutation of Y30 to phenylalanine results in an increase in p53 protein levels but the p53 is less active.

5.2.9 Expression of the wild type RPL5 and the mutants all lead to decreased cellular growth rate.

As the induction of RPL5 and the mutants increased p53 levels, it was possible that this would impact upon growth of the cells. It had been noted during the previous experiments that the mutants tended to grow much slower than the wild type, which in turn, was slower than the control cells. Therefore, it was important to study this further with growth assays.

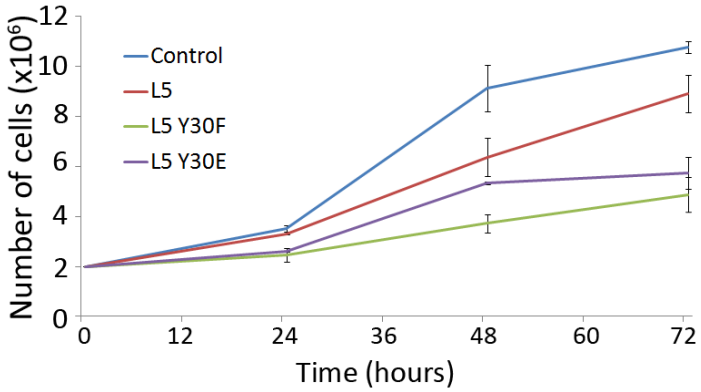

Figure 5.12. RPL5 expression slows the growth of U2OS cells. Cell counting experiment to show the effect of RPL5 expression on the cell growth over a 72 hour period. U2OS cells expressing FLAG-tagged wild type, Y30E, or Y30F mutant RPL5 cells were grown in the presence of 1 µg/ml tetracycline. Live cell numbers were counted at the time intervals as indicated. Each point on the graph represents the mean ± the standard deviations of the means, n=3.

U2OS cells expressing the FLAG-tagged wild type or mutant RPL5 constructs were incubated with 1 μg/ml tetracycline-containing medium, at 37^{o}C, to induce the expression of the proteins. Cells were counted at 24, 48 and 72 hours and total cell number was plotted (Figure 5.12). Expression of the wild type RPL5 reduced cell number; however the reduction in cell number of the two mutants was more marked. As p53 is involved in cell cycle regulation through p21, and apoptosis, this suggests that expression of the two mutant RPL5 proteins may be impeding cell cycle progression, despite the evidence for differences in their ability to activate p53.

5.2.10 Expression of the wild type, Y30E or Y30F mutants leads to an accumulation of cells in G1/G0 in a p53-dependent manner

Whilst the effect of altered RPL5 protein levels on p53 expression has been extensively studied, few experiments have studied the effect of altered RPL5 protein levels on cell cycle progression. Teng et al. (2013) reported that depletion of RPL5 had no effect on cell cycle progression, whilst Singh et al. (2014) demonstrated in mice that RPL5 haploinsufficiency resulted in delayed G2 progression. One study involving U2OS cells showed that RPL5 expression promoted p53-dependent cell cycle arrest (Dai and Lu, 2004). Furthermore, it has also recently been determined that RPL5 over-expression, in p53 null SAOS-2 cells, induces apoptosis (Zhou et al., 2015). Therefore, to further elucidate the role of RPL5 over-expression on cell cycle progression, flow cytometry using Propidium Iodide (PI) was utilised (Figure 5.13). U2OS cells expressing control or FLAG-tagged RPL5 wild type or the Y30 mutants were induced with 1 μg/ml tetracycline for 48 hours, harvested and fixed in cold 70% ethanol before staining with propidium iodide and analysing by flow cytometry (Figure 5.13A-B). The cell cycle analysis was gated as shown in blue (Figure 5.13A) and only includes cells with a uniform size and shape.

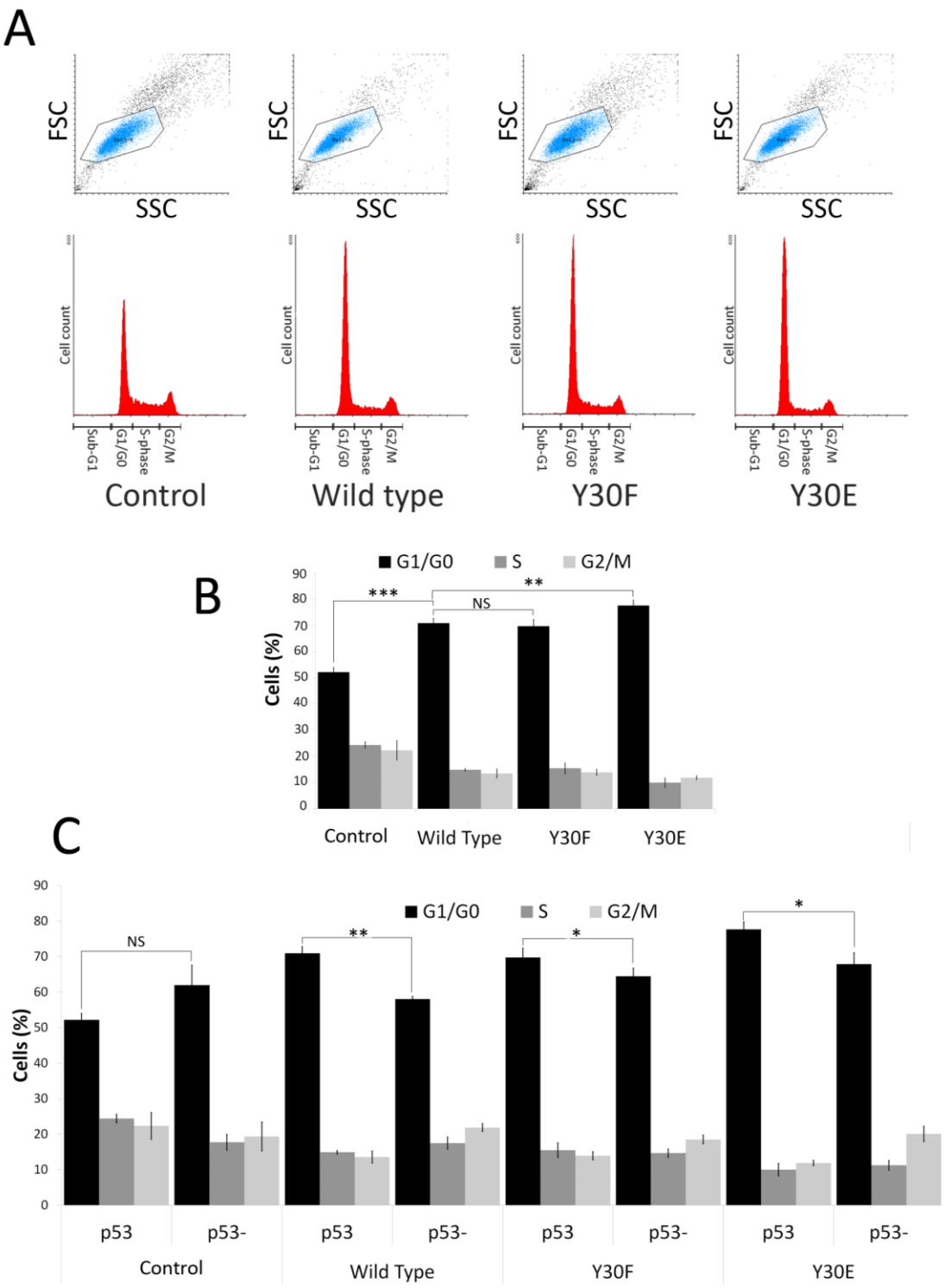

Figure 5.13. Expression of RPL5 induces an accumulation of cells in G1. (A) Representative distribution of cells from cell cycle analysis. U2OS cells expressing empty vector, wild type RPL5 or the Y30 mutants were induced with 1 µg/ml tetracycline for 48 hours prior to ethanol fixation and cell cycle analysis using propidium iodide. The forward scatter (FSC) was plotted against the side scatter (SSC) and the live population of cells was gated on as shown in blue. **(B)** Quantitation of the cell cycle distribution in A. **(C)** Quantification of the cell cycle distribution as in A+B, including a depletion of p53 for 48 hours using 20 µM p53 siRNA. Bars in B and C represent the means ± the standard deviation of the means, n=3. Statistical analysis determined by a paired t test. NS= non-significant, *= P ≤ 0.05, **= p≤ 0.01, ***= p≤ 0.001.

Expression of FLAG-RPL5, FLAG-RPL5 Y30E, or FLAG-RPL5 Y30F resulted in an accumulation of cells in G1/G0, which is consistent with p53 activation. Statistical analysis using a paired t-test showed a small, but significant, difference between the percentage of cells in G1 between the wild type and the Y30E mutant with a p-value of 0.0032. The difference between the control and the wild type RPL5 had a very significant p-value of <0.0001 whilst the wild type compared to the Y30F mutant had a non-significant p-value of 0.5155. As the Y30E mutant shows the greatest level of p53 induction, it suggests that this increased p53 induction leads to increased cell cycle arrest. In order to determine whether the G1/G0 accumulation was caused by an increase in p53 activity, the inductions were carried out 12 hours post-transfection with 20 µM p53 siRNA duplex (Figure 5.13C). In all cases, the depletion of p53 resulted in a reduction in the number of cells in G1/G0. Statistical analysis was carried out between the single and double knockdowns and, with p-values between 0.016 and 0.045, there was a significant decrease upon p53 knockdown in all cases, suggesting that the accumulation of cells in G1/G0 was p53 dependent.

This is surprising as the Y30F mutant showed similar cell cycle defects to the wild type, despite having less of an effect on p21 levels although this could be due to other factors as discussed later.

5.3 Discussion

Post-translational modifications such as ubiquitination, NEDDylation, phosphorylation and acetylation are key in the regulation of p53 signalling (Meek, 1999, Xirodimas et al., 2004, Brooks and Gu, 2006, Vogelstein et al., 2000, Kumazawa et al., 2015). As NEDDylation has been demonstrated to regulate p53 signalling via RPL11 (Mahata et al., 2012, Sundqvist et al., 2009, Xirodimas et al., 2008), in this study it was proposed that other types of post-translational modifications, such as phosphorylation, could be important in regulating p53 signalling via 5S RNP. p53 signalling is regulated by multiple phosphorylation events in p53, MDM2, and MDMX (Chen et al., 2005, Goldberg et al., 2002, Jin et al., 2006, Loughery et al., 2014, Maya et al., 2001, Meek, 1999) and thus it was possible that, as regulators of p53, RPL5 and RPL11 may also be phosphorylated to modulate p53 signalling. Previous *in vitro* studies identified two different kinases that interact with RPL5 (Bialik et al., 2008, Park and Bae, 1999). Furthermore, phosphorylation of RPL5 at serine residues has been shown to prevent

interaction with the 5S rRNA *in vitro* (Park and Bae, 1999), however there have been no *in vivo* experiments and no phosphorylation sites have yet been characterised that affect 5S RNP integration into the ribosome and result in the activation of p53. This chapter focused on the characterisation of reported phosphorylation sites that, based on the cryo-EM structure of the human ribosome, may affect integration of the 5S RNP into the ribosome.

Analysis of the cryo-EM structure indicated that Y30, Y31 and Y44 of RPL5 were in close proximity to the 28S rRNA in the human ribosome (Figure 5.3) and thus mutations were introduced that mimicked phosphorylation (Y to E) or could not be phosphorylated (Y to F). Upon expression of the mutant RPL5 proteins, it was decided that only the Y30 mutations could be reliably used for experimentation due to poor expression and survival of the cells expressing the other RPL5 mutants. The toxicity of these mutations suggested a vital role in cell survival and thus they could prove to be extremely interesting to study. It would be of interest in the future to try to improve their expression. It is possible that only a small proportion of the cells were expressing the proteins and therefore clonally selecting the cell colonies could help to rectify this problem.

Whilst mutations that attempted to mimic phosphorylation of, or lack thereof, Y30 did not appear to affect the areas in which RPL5 was localised (Figure 5.6), mimicking phosphorylation of Y30 did appear to abrogate RPL5 incorporation into the ribosome (Figure 5.8). As the Y30E mutant was expressed more strongly than the other two proteins, an excess of 'free' protein may be expected. If the rate of incorporation of the 5S RNP into ribosomes was steady between the wild type and mutants, there should have been equal amounts of each mutant incorporated into the ribosome regardless of how much additional 'free' RPL5 was present. These data taken together indicate that mimicking phosphorylation of RPL5 at Y30 by mutating it to glutamic acid may prevent integration of RPL5 into the ribosome. As discussed in previous chapters, this could be due to the mutations having a direct effect on ribosomal incorporation of RPL5 or it may abrogate interactions with proteins responsible for RPL5 recruitment. This could be further investigated by looking for interactions between these mutants and proteins known to be involved in 5S RNP recruitment- such as PICT-1.

Furthermore, glycerol gradients were utilised to determine whether it was possible detect phosphorylated RPL5 in ribosomes. The small 'free' 5S RNP fractions were directly compared to the large 'ribosomal' bound 5S RNP fractions. A higher molecular weight signal was present on a phos-tag western blot in the free pool that was not present in the 'ribosome' pool. Furthermore, there was a higher molecular weight signal present in the 'ribosome' pool that was not present in the 'free' pool, suggesting the evidence of differential phosphorylation (Figure 5.9). However, this experiment only demonstrates that phosphorylation is present in 'free' and 'ribosomal' pools, but does not reveal which residues are phosphorylated. The experiment was carried out with the expression of RPL5-FLAG cells using an anti-FLAG antibody and not the endogenous protein and, therefore, is not necessarily representative of the endogenous protein. However, the RPL5 antibodies were not specific enough to use; resulting in too many off-target signals. Another limitation of this study is that this is an artificial situation and has already induced ribosomal stress due to over-expression of ribosomal proteins resulting in p53 activation. Therefore, this is not a true representation of what would happen in a normal, unstressed cell. If a more specific RPL5 antibody were used, this may give a better result with the endogenous protein. It would also be possible to utilise an anti-phosphotyrosine antibody in an immunoprecipitation experiment followed by gradient centrifugation and western blotting for RPL5 as this would only show phosphorylated tyrosine.

As RPL5 over-expression has been shown to stabilise p53 (Horn and Vousden, 2008), it was unsurprising that expression of the wild type, Y30E, or Y30F RPL5 resulted in increased p53 levels (Figure 5.11A-B). In addition, RPL5 Y30E expression resulted in a greater increase in p53 levels compared to the wild type. This is presumably as a result of RPL5 Y30E not being incorporated into ribosomes or due to the higher levels of expression of the RPL5 Y30E mutant, although it is not yet clear which.

It has been demonstrated that MDM2 binds to the RPL11 in the same region that RPL11 binds helices 83-85 of the 28S rRNA (Zheng et al., 2015). As the Y30 residue was in very close proximity to the 28S rRNA binding site (Figure 5.3A), it was possible that mutating this site would affect RPL5-MDM2 interactions. However, the wild type, Y30E, and Y30F RPL5 mutations associated with MDM2 (Figure 5.10) thus suggesting

that mimicking phosphorylation of RPL5 Y30 does not abrogate MDM2-RPL5 association.

Furthermore, the levels of MDM2 protein were analysed upon induction of the wild type or mutant FLAG-RPL5 proteins (Figure 5.11C) and it was found that expression of the wild type or mutant RPL5 increased MDM2 expression, possibly as a result of the MDM2-p53 feedback loop whereby p53 increases can drive MDM2 expression (Barak et al., 1993). Furthermore, whilst the interaction between the 5S RNP and p53 had previously been debated (Zhou et al., 2015, Bursac et al., 2012, Marechal et al., 1994), in this study, association between RPL5 and p53 was detected (Figure 5.10). This suggested that RPL5 associates with p53 and MDM2 and that mimicking phosphorylation of Y30 does not affect these associations. As RPL11 had been previously shown to be recruited to promoter sites of p53 regulated genes (Sundqvist et al., 2009), the data in this study may support the theory that the 5S RNP complex with p53 may be recruited to p53 promoters.

When the downstream effector of p53, p21, was analysed, expression of the Y30F mutation appeared to have a lesser impact on p21 levels compared to the wild type or Y30E expression. This was supported by the fact that expression of the RPL5 wild type or Y30E mutant in cells transfected with a p53-driven luciferase reporter resulted in an increase in luciferase levels, but expression of the Y30F mutant had no significant effect (Figure 5.11D). This may suggest that expression of RPL5 Y30F is not sufficient to increase activity of p53. This could potentially mean that the Y30 site of RPL5 is required for adequate activation of p53 as summarised in figure 5.14.

One explanation for the reduced p53 activity could be due to interactions with p73 as RPL5 and RPL11 can activate the p53 homolog, p73. RPL5 and RPL11 bind to the N-terminal transactivation domain of p73, blocking MDM2 binding to this domain and increasing p73 activity (Zhou et al., 2015). As p73 has the ability to transactivate many of the same genes involved in cell cycle arrest and apoptosis as p53 (Jost et al., 1997, Zhu et al., 1998), this may provide insight into why the RPL5 Y30F mutant does not increase p53 activity but can still affect cell cycle progression.

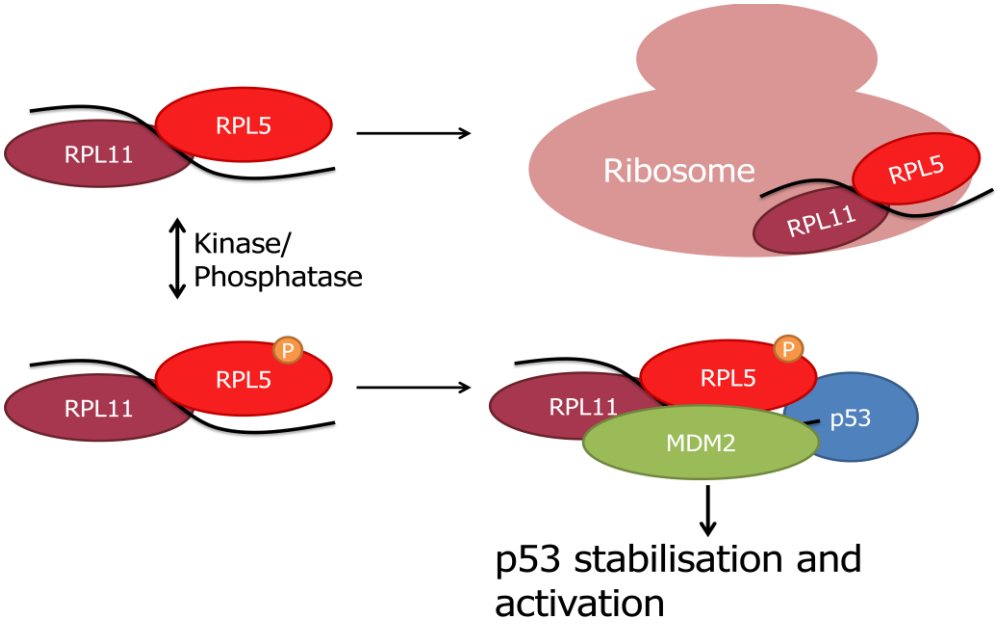

Figure 5.14 Schematic representation of the proposed role of phosphorylation in 5S RNP signalling. Non-phosphorylated RPL5 is incorporated as part of the 5S RNP complex into the ribosome. If RPL5 is phosphorylated, the 5S RNP is not incorporated into ribosomes and can interact with MDM2 and p53 to promote p53 stabilisation and activation.

It was previously demonstrated, using the pG13 luciferase reporter, that p73 induction results in a small increase in luciferase levels compared to p53. However, when p53 and p71 were both expressed, the luciferase levels remained similar to that of p73 induction alone (Vikhanskaya et al., 2000). Therefore, one possible explanation for the reduced luciferase levels upon induction of RPL5 Y30F compared to the control is that expression of RPL5 Y30F may lead to an increase in p73 levels and thus cause only a small luciferase increase, despite p53 increases.

Another explanation for the lack of p53 activity in the Y30F mutation could be explained by interactions with MDMX. Unlike MDM2, MDMX does not have E3 ligase activity and instead inhibits p53 by binding to its transactivation domain (Danovi et al., 2004, Jackson and Berberich, 2000). This means that MDMX inhibits p53 without affecting p53 levels. Recently, the 5S rRNA was shown to be involved in the stability of MDMX (Li and Gu, 2011) whilst RPL11-MDM2 interactions were shown to be involved in the degradation of MDMX under ribosomal stress conditions (Gilkes et al., 2006). Although, currently it is believed that RPL5 and RPL11 do not interact with MDMX (Gilkes et al., 2006), given that the 5S rRNA is often found in a complex with RPL5 and/or RPL11, it is possible that RPL5 Y30F, in conjunction with the 5S rRNA and/or RPL11 results in increased stability of MDMX and thus p53 activity but not levels are

affected as shown in Figure 5.15. Furthermore, as MDMX regulates the stability of MDM2 (Gu et al., 2002), this may explain the higher MDM2 protein levels seen upon expression of the RPL5 Y30F mutant. In order to further test these theories, it would be beneficial in the future to assess the mutants for their ability to increase p73 activity or their interactions with MDMX in order to understand the mechanisms behind the differences in p53 activity between the mutants.

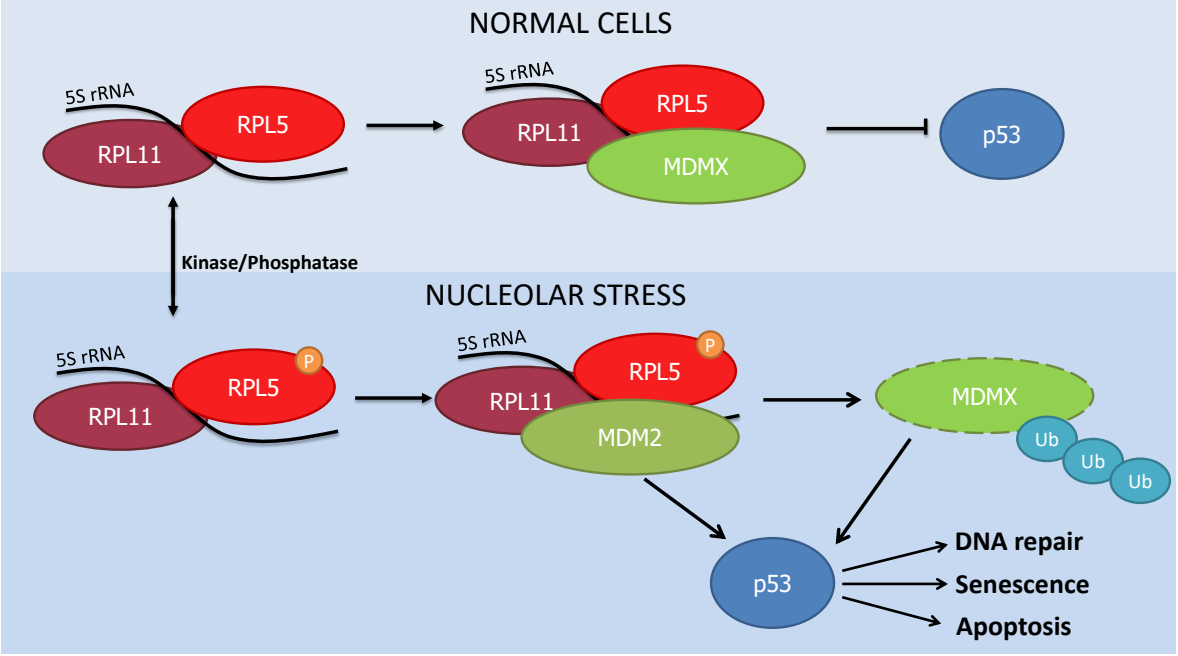

Figure 5.15. Proposed model for phosphorylation in the regulation of p53 via MDMX. In normal cells, RPL5 (unphosphorylated at Y30) with RPL11 and the 5S rRNA bind to MDMX and inactivate p53. Upon nucleolar stress, RPL5 (phosphorylated at Y30) with RPL11 and the 5S rRNA bind to MDM2 to promote ubiquitination of MDMX and activate p53.

In this chapter, it was shown that expression of RPL5, whether wild type or mutant, in U2OS cells resulted in a decreased total cell number compared to the control (Figure 5.12) and caused cells to accumulate in G1/G0 in a p53-dependent manner (Figure 5.13). This is in agreement with results from several groups (Hiebert et al., 1995, Agarwal et al., 1995, Kastan et al., 1991) who have shown that p53 activation causes G1 cell cycle arrest in a range of cell types. The Y30E mutant showed a small but significantly higher proportion of cells in G1/G0 than the wild type expression. This correlates with the higher p53 levels seen in these cells, whereas the Y30F mutation had a similar profile to the wild type. The gating used for the cell cycle analysis is only focused upon regularly shaped cells and specifically excludes dead cells and debris. To further explore the effect of these mutations on cell death, Annexin V and 7-AAD staining could be used to look for signs of apoptosis (Zembruski et al., 2012).

One limitation of this chapter as a whole is that phosphorylation mimics are not always an accurate representation of phosphorylation in the cell. Phosphorylated tyrosine is a difficult amino acid to mimic; glutamic acid makes a good mimic for phosphoserine (Maciejewski et al., 1995) as they have similar structures. However, there is no natural amino acid that is similar to phosphotyrosine. However, if the kinase or phosphatase could be identified, this would allow the phosphorylation to be up-regulated or down-regulated by manipulating the kinase or phosphatase levels in cells. Whilst no kinases have yet been identified, investigation into the possible kinases involved through the use of the Group based predicition system, GPS (Xue et al., 2008), identified several kinases with the potential to phosphorylate tyrosine 30 of RPL5- Anaplastic lymphoma kinase (Alk), the Proto-oncogene tyrosine-protein kinase MER, Fibroblast growth factor receptor 3 kinase, Janus kinase 3 (JAK3), the insulin receptor tyrosine kinase and the tyrosine-protein kinase HCK. Further investigation into these tyrosine kinases may reveal an insight into whether this phosphorylation of RPL5 does, indeed, exist in cells.

In this chapter, the possibility that phosphorylation may be an important regulator involved in 5S RNP-mediated p53 signalling was highlighted. Mimicking phosphorylation of tyrosine 30 of RPL5 by mutating it to glutamic acid appears to prevent the integration of RPL5 into the ribosome and enhances p53 signalling. Interestingly, mimicking phosphorylation of tyrosine 30 also appears to be important for activation of p53, suggesting that it is an important regulation step in the pathway. As the 5S RNP has been implicated in diseases such as Diamond Blackfan Anaemia, a greater understanding of the regulation of the pathway could be crucial to the discovery of novel treatments for DBA. This chapter has highlighted the possibility that phosphorylation of RPL5 may be important for the activation of p53 and thus the kinases involved, if specific to RPL5, could be manipulated to affect 5S RNP-mediated p53 activation. However, at present, the specific Y30 phosphorylation has not been confirmed in cells and no tyrosine kinases have been identified that interact with RPL5 and therefore it remains unclear which kinases could be responsible for the phosphorylation of tyrosine residues in RPL5.

CHAPTER SIX: CONCLUSIONS AND DISCUSSION

6.1 Introduction

The aim of this project was to investigate the factors contributing to the regulation of the 5S RNP-mediated p53 signalling pathway. Ribosomes are crucial cellular machines that catalyse the translation of mRNA into proteins. Ribosome biogenesis is an extremely complex process and thus has to be tightly regulated. When ribosome biogenesis is disrupted, the 5S RNP, a component of the large ribosomal subunit, is free to bind to MDM2, the major suppressor of p53, and activate p53 (Sloan et al., 2013a, Donati et al., 2013). However, the regulation of this pathway is not yet fully understood.

In this thesis, I investigated factors involved in 5S RNP integration into the ribosome and post-translational modifications of RPL5. One primary mechanism of 5S RNP-mediated regulation of p53 is through control of the integration of 5S RNP into the ribosome. This project has shown that the large subunit ribosomal proteins, RPL7, RPL18, and RPL21, are ribosome biogenesis factors that appear to be required for the 5S RNP integration into the ribosome. Furthermore, this project has demonstrated *in vitro* that RPL11 can bind to a small fragment of the 28S rRNA that could possibly be used prevent interactions between the 5S RNP and MDM2.

Phosphorylation of p53 and its regulators plays a vital role in the regulation of p53 signalling pathways (Goldberg et al., 2002, Maya et al., 2001, Jin et al., 2006, Chen et al., 2005, Meek, 1999). I wanted to investigate the role of phosphorylation in regulating the 5S RNP to elucidate if phosphorylation of RPL5 could activate p53 signalling. I found that expression of a mutated RPL5 mimicking RPL5 phosphorylation (RPL5 Y30E) resulted RPL5 ribosomal integration defects coupled with p53 activation.

In this project I have also expanded on previous data showing that SRSF1 and PRAS40 interact with components of the 5S RNP to regulate p53 signalling (Fregoso et al., 2013, Havel et al., 2014). SRSF1 was demonstrated to associate with RPL5 and MDM2 to activate p53 (Fregoso et al., 2013), whilst PRAS40 was shown to associate with RPL11 and sequester it to the nucleoli, preventing it from binding to MDM2. However, studies have since shown that 5S RNA, RPL5 and RPL11 are all required for p53 activation (Donati et al., 2013, Sloan et al., 2013a) and thus I questioned whether these

proteins interacted with the 5S RNP. I have shown that SRSF1 interacts with the 5S rRNA and is required for the integration of RPL5 and RPL11 into the ribosome. Furthermore, I have demonstrated that both SRSF1 and PRAS40 are required for activation of p53 via the 5S RNP.

6.2 rRNA processing in humans

In eukaryotic organisms, three of the four rRNAs are transcribed by RNA polymerase I as a single 47S (35S in yeast) polycistronic precursor in the nucleolus (Nazar, 2004). This precursor then undergoes a series of processing steps in order to produce the mature 28S (25S in yeast), 18S and 5.8S rRNAs. It is known that ribosomal proteins are required throughout the ribosome biogenesis pathway (O'Donohue et al., 2010) however, most of the current understanding of rRNA processing has been from studies in yeast, and human rRNA processing is less understood.

In both yeast and humans, processing of the internal spacer region, ITS1, is an important step as it separates the large and small subunit rRNAs. In humans, there are two pathways involved in ITS1 processing. The major pathway involves cleavage at site 2 followed by processing by exonucleases, whereas the minor pathway involves cleavage at site 2a and acts to compensate for a block in site 2 cleavage (Sloan et al., 2013b). I demonstrated that depletion of RPL7 or RPL18 resulted in accumulation of the 36SC processing intermediate, suggesting a defect in site 2 cleavage. Site 2 is located within the internal transcribed space region 1 (ITS1), and is thought to be equivalent to A_3 cleavage in yeast (Sloan et al., 2013a) and thus responsible for the separation of the small and large subunit precursor rRNAs. It was previously demonstrated that the 36SC precursor accumulated upon depletion of RBM28 or BOP1 and could be rescued by depletion of the exonuclease responsible for processing the 36S precursor to the 32S precursor, XRN2 (Sloan et al., 2013a). Therefore, this suggests that, like RBM28 and BOP1, RPL7, RPL18, and RPL21 are also required for site 2 cleavage, furthering the current understanding of site 2 cleavage in humans. Interestingly, it was recently demonstrated that RPL7, along with RPL8, might be necessary for the association and function of the A_3 assembly factors in yeast (Jakovljevic et al., 2012), suggesting that RPL7 is involved in A_3/site 2 cleavage in both humans and yeast.

Further processing of the pre-rRNAs occurs within a second internal transcribed spacer region, ITS2. ITS2 contains two cleavage sites known as 3' and 4a and separates the 5.8S rRNA from the 28S rRNA. Cleavage at the 3'-region of ITS2 results in the generation of the 12S pre-rRNA (Hadjiolova et al., 1993) and further cleavage at site 4a results in a 7S pre-rRNA (Farrar et al., 2008). In eukaryotes, ITS2 excision is required for the formation of an evolutionarily conserved helix joining of the 3' end of the 5'8S rRNA and the 5' end of the 28S rRNA in mature ribosomes (Peculis and Greer, 1998) and thus is a crucial step in ribosome biogenesis. My data suggested that RPL21 depletion resulted in inefficient cleavage of the 32S rRNA, therefore presenting a role for RPL21 in ITS2 processing in humans.

Furthermore, I investigated the role of SRSF1 and PRAS40 in rRNA processing with pulse-labelling experiments. I found that neither depletion of SRSF1 nor PRAS40 showed any significant differences in the production of the 28S or 18S rRNAs over 3 experiments. However, SRSF1 depletion resulted in a slight increase in 32S with a slight decrease in 28S, suggesting that there could be very subtle large subunit processing defects, possibly in ITS2. Furthermore, PRAS40 depletion resulted in a slight increase in 47S with a slight decrease in 32S suggesting that PRAS40 may have subtle effects on ITS1 processing. However, as there was no significant differences, it would be of interest to investigate the effect of SRSF1 or PRAS40 using specific probes to ITS1 and ITS2 to reveal more subtle defects.

Therefore, these data taken together suggest a role for the ribosomal proteins, RPL7, RPL18, and RPL21, and possibly SRSF1 and PRAS40 in large subunit biogenesis, helping to unravel the complexity of human ITS1 and ITS2 processing.

6.3 The recruitment of the 5S RNP into the ribosome

There are several factors involved in regulating p53 via the 5S RNP. It has been suggested that any factors that affect the formation, localisation or ribosomal integration of the 5S RNP will have an impact on p53 signalling (Sloan et al., 2013a). Therefore, understanding factors involved in the recruitment of the 5S RNP into the ribosome is crucial to understanding p53 activation via the 5S RNP. However, this process is not yet fully elucidated in humans. In yeast, the recruitment of the 5S RNP into the ribosome is known to require Rrs1 and Rpf2. Rrs1 and Rpf2 form a complex

with the 5S RNP and are responsible for the integration of the 5S RNP into 90S pre-ribosomes containing the 35S pre-rRNA (Zhang et al., 2007, Morita et al., 2002). Recently, the crystal structure of Rrs1 and Rpf2 complex has been solved in *Aspergillus nidulans* (Asano et al., 2014, Kharde et al., 2015) and yeast (Madru et al., 2015) leading to a greater understanding of the role of the Rpf2/Rrs1 complex in 5S RNP recruitment in these organisms. RPL5 and RPL11 are delivered to the nucleus and probably assembled onto the 5S rRNA by the symportin, Syo1 (Calvino et al., 2015) to form the 5S RNP. When the 5S RNP is recruited into the ribosome, it is locked into conformation by the C-terminal tail of Rpf2, Rsa4 and Nsa2 to prevent any rotation (Kharde et al., 2015, Bassler et al., 2015) and docks onto helix 84 of the 25S rRNA (Rhodin et al., 2011). Rea1, a dynein-related nuclear AAA-ATPase, releases Rsa4 from the 5S RNP and rotates the structure by 180^{o} to form the correct conformation for the formation of the central protuberance (Bassler et al., 2015, Leidig et al., 2014).

Based on the *T. thermophila* crystal structure, I originally hypothesised that four ribosomal proteins, RPL7, RPL10, RPL18, and RPL21, formed a binding pocket for the 5S RNP in the ribosome. In light of the recent findings about the rotation of the 5S RNP, this may suggest that these proteins may not directly contact the 5S RNP in pre-ribosomes. However, I demonstrated that depletion of the ribosomal proteins RPL7, RPL18, and RPL21 affected ribosomal integration of the 5S RNP. However, it still remains unclear as to whether the defects in 5S RNP ribosomal integration are caused purely by ribosome biogenesis defects or whether these proteins play a direct role in 5S RNP recruitment, although the latter seems more likely as it was previously demonstrated that depletion of ribosome biogenesis factors reduced 5S rRNA recruitment into the ribosome, but that depletion of the factors involved in direct recruitment of the 5S RNP (RPL11, PICT-1, BOP1, and NOP2) had a stronger effect (Sloan et al., 2013a). At present, it appears that the RPL7, RPL18 and RPL21 depletions had a similar effect on 5S rRNA recruitment to RPL11, suggesting that they have a role in 5S RNP recruitment and are not just ribosome biogenesis factors. This suggests that the 5S RNP may not only contact the 28S rRNA upon binding, but may also require interaction with the surrounding ribosomal proteins. Interestingly, RPL10 depletion had no effect on the ribosomal integration of the 5S RNP. However, in yeast, RPL10

integration is affected by Rrs1 and Rpf2 depletion (Zhang et al., 2007), thus suggesting that, like in yeast, RPL10 is recruited after 5S RNP integration.

In human ribosomes, 5S RNP integration appears to be somewhat different to yeast. The human homologues of Rrs1 and Rpf2, RRS1 and BXDC1 have been shown to be involved in localisation of the 5S RNP but do not play a crucial role in ribosomal recruitment (Sloan et al., 2013a). Furthermore the nucleolar protein, PICT-1 (also known as Glioma tumour suppressor candidate region gene- GLTSRC2), directly contacts the 5S rRNA and has a more profound effect on 5S RNP recruitment into the ribosome than BXDC1 or RRS1. The data presented within this project may suggest that SRSF1 is also required for ribosomal recruitment of the 5S RNP. However, it is not clear whether SRSF1 is directly involved in the integration of the 5S RNP or whether it is involved in earlier steps such as formation of the 5S RNP particle. Previous reports demonstrated that depletion of the 5S RNP components, or PICT-1, resulted in rRNA processing defects (Sloan et al., 2013a), however the data in this study suggested that SRSF1 depletion did not. This may suggest that whilst SRSF1 and PICT-1 are both required for the ribosomal recruitment of the 5S RNP, they differ in the fact that PICT-1 is required for rRNA processing, but SRSF1 is not. This may show that SRSF1 has a more direct role in 5S RNP recruitment rather than affecting ribosome biogenesis as a whole, however, to fully explore this possibility, northern blot analysis of the pre-rRNA precursors upon depletion of SRSF1 is required.

Furthermore, I investigated the possibility that phosphorylation of RPL5 may affect integration of the 5S RNP into the ribosome. My data suggests that mimicking phosphorylation of RPL5 at Y30 prevents integration of RPL5 into the ribosome. The phosphorylation of serine residues on RPL5 was previously demonstrated to prevent interaction between RPL5 and the 5S rRNA *in vitro* (Park and Bae, 1999). This, together with the data presented in this thesis, would suggest that phosphorylation of RPL5 may play roles in both 5S RNP formation and 5S RNP integration into the ribosome. Interestingly, my data also showed evidence of phosphorylation present in ribosomal RPL5 that was not present in 'free' RPL5, suggesting that some phosphorylation sites may in fact be required for 5S RNP recruitment. In addition to Y30 there are many other phosphorylation sites in RPL5 and therefore investigating these other sites may provide greater insight into the role of phosphorylation in RPL5 regulation.

Furthermore, Phosphosite (http://www.phosphosite.org) also lists multiple phosphorylation sites for RPL11. Initial work in the Watkins lab by Zhao Zhao has revealed that mimicking phosphorylation of RPL11 may also have similar effects to that seen in this project (Wakins lab- unpublished data) and thus may be a mechanism for regulation of 5S RNP integration.

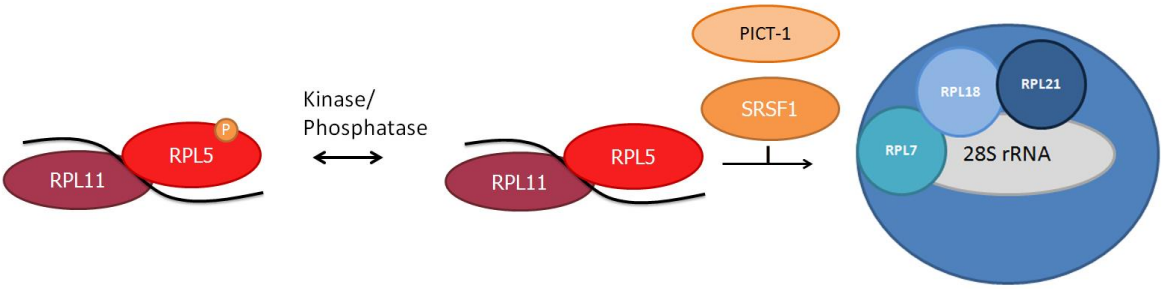

Figure 6.1 Proposed 5S RNP ribosomal integration pathway. Schematic representation of our proposed model of 5S RNP recruitment into the ribosome. Free pools of the 5S RNP contain phosphorylated RPL5. We propose that this phosphate group is removed by an unknown phosphatase before the 5S RNP is incorporated into the ribosome, although different sites may be phosphorylated at this stage. Incorporation of the 5S RNP into the ribosome requires PICT-1 and SRSF1 and also requires interactions with the 28S rRNA, RPL7, RPL18 and RPL21 in the pre-ribosome.

As I have also shown that SRSF1 is important for integration of the 5S RNP into the ribosome, it would be interesting to evaluate whether phosphorylation of RPL5 affects interactions between SRSF1 and RPL5. Similarly, SRSF1 phosphorylation is important for regulation of the localisation and function of SRSF1 (Hagopian et al., 2008, Lai et al., 2001, Ngo et al., 2005) and thus it is possible that phosphorylation of SRSF1 may affect its ability to interact with the 5S RNP. Therefore, studies into the effect of phosphorylation on SRSF1 and RPL5 interactions could further elucidate the role of SRSF1 in 5S RNP-mediated p53 signalling.

The data presented in this project has helped to further understand the factors involved in recruitment of the 5S RNP into the ribosome as summarised in Figure 6.1.

6.4 Regulation of the 5S RNP pathway

In this project, we also wanted to investigate factors that directly regulate p53 signalling via the 5S RNP. SRSF1 and PRAS40 had been previously demonstrated to be involved in p53 signalling via the 5S RNP and, therefore, we wanted to further elucidate these roles. Furthermore, as phosphorylation is a key regulator in p53

signalling pathways, we wanted to investigate whether phosphorylation of RPL5 affected the 5S RNPs ability to bind to MDM2 and activate p53.

6.4.1 PRAS40 and SRSF1

As previously described, one of the potential candidates for regulating the p53 response to ribosomal stress is PICT-1. PICT-1 interacts with RPL5 and RPL11 and it was suggested that PICT-1 sequesters RPL11 to the nucleolus (Sasaki et al., 2011) to prevent interactions between the 5S RNP and MDM2. The proline-rich Akt Substrate of 40kDa (PRAS40, also known as AKT1S1), was suggested to have a similar role to PICT-1 in sequestering RPL11 to the nucleolus to suppress p53 activation (Havel et al., 2014). Havel et al. (2014) demonstrated that nuclear PRAS40 was able to bind RPL11 and prevent activation of p53 via ActD treatment. Furthermore, this binding was dependent upon the phosphorylation of PRAS40 via mTORC1 and AKT at residues S221 and T246. Interestingly, the complex containing RPL11 and PRAS40 was found to have a high molecular weight and it was hypothesised that this could contain other unidentified proteins (Havel et al., 2014). However, my data suggests that these proteins are not the other 5S RNP components as PRAS40 did not co-immunoprecipitate with the 5S rRNA or RPL5. Therefore, we hypothesised that PRAS40 was involved in negative regulation of the 5S RNP pathway by sequestering RPL11 to the nucleolus. However, my data also presented some new observations regarding PRAS40 activity. PRAS40 appeared to be required for activation of p53 via SRSF1, RPL5, and RPL11, contradicting the previous findings that PRAS40 prevents p53 activation via RPL11 (Havel et al., 2014). However, unlike RPL5, RPL11 or SRSF1 depletion, PRAS40 depletion did not abrogate the p53 response after ActD treatment, suggesting that it is not essential for p53 activation upon ribosomal stress. This may present differing roles for PRAS40 in the regulation of the 5S RNP whereby it is both able to negatively and positively regulate p53 signalling. Phosphorylation of PRAS40 is essential for its interaction with RPL11, resulting in MDM2-mediated degradation of p53 (Havel et al., 2014). Therefore, it may be possible that altering the phosphorylation state of PRAS40 affects its role in 5S RNP signalling. Therefore, further investigation into PRAS40 signalling could focus on using PRAS40 phosphorylation mutants to evaluate the impact on p53 signalling.

One other factor shown to regulate p53 signalling upon ribosomal stress was Serine-Arginine Rich Splicing Factor 1 (SRSF1- also Alternative Splicing Factor- ASF). SRSF1 is a 28kDa member of the SR protein family of splicing factors which are involved in constitutive and alternative splicing as well as being involved in mRNA nuclear export, mRNA translation and nonsense mediated decay (Long and Caceres, 2009). Fregoso et al. (2013) demonstrated a role for SRSF1 in p53 signalling via RPL5. It was suggested that RPL5, MDM2 and SRSF1 form a complex to stabilise p53 and induce senescence. However, as Donati et al. (2013) and Sloan et al. (2013a) both demonstrated that RPL5, RPL11 and the 5S RNP were all required for p53 activation in response to ribosomal stress I questioned whether SRSF1 interacted with the 5S RNP as a complex. Indeed, my data also shows that SRSF1 interacts with the 5S rRNA, although it was not possible to show interactions with RPL11 due to problems with antibodies. Recent studies in the Watkins lab have, however, demonstrated that SRSF1 is able to interact with RPL11 *in vitro* and thus may strengthen the hypothesis that SRSF1 interacts with the entire 5S RNP (Watkins lab-unpublished data). Furthermore, my data suggests that SRSF1 may form part of the 5S RNP complex involved in activating p53 upon ribosomal stress and maintaining normal p53 homeostasis.

I have also shown that SRSF1 depletion resulted in a reduction in nucleolar RPL5 as well as a reduction in cytoplasmic RPL11. Indeed, it was previously demonstrated that depletion of any factors crucial for large subunit ribosome production resulted in an increased re-localisation of cytoplasmic RPL11 to the nucleoplasm (Sloan et al., 2013a). As RPL5, RPL11 and the 5S rRNA are integrated into the ribosome as a complex (Calvino et al., 2015, Kharde et al., 2015, Leidig et al., 2014, Madru et al., 2015), it follows that depletion SRSF1 will also affect 5S rRNA integration, however this should be followed up in future experiments by investigating 5S rRNA integration into the ribosome.

The data presented in this thesis provides a foundation for future studies into the potential therapeutic targets for ribosomopathies such as DBA and 5q-syndrome. As localisation and ribosomal integration of the 5S RNP are crucial factors in the regulation of the 5S RNPs ability to activate p53, it follows that targeting factors that affect localisation or integration of the 5S RNP may be a viable treatment option for patients with ribosomopathies. Furthermore, the data presented in this project reveals

that the 5S RNP complex may be more complex than previously suggested, as my data suggests that the 5S RNP complex also contains SRSF1.

6.4.2 Phosphorylation of RPL5

One further way that proteins are regulated is via post-translational modifications. Indeed, RPL11 has been demonstrated to be NEDDylated to regulate its stability and ability to activate p53 (Bursac et al., 2012, Mahata et al., 2012, Sundqvist et al., 2009, Xirodimas et al., 2008). However, the role of phosphorylation in 5S RNP regulation has yet to be reported in the literature. Phosphorylation of p53 is a key post-translational modification that regulates p53 signalling. p53 is phosphorylated at many different sites to regulate its function (Meek, 1999). In addition, phosphorylation of the p53 regulators, MDM2 and MDMX also regulates their ability to suppress p53 (Goldberg et al., 2002, Maya et al., 2001, Jin et al., 2006, Chen et al., 2005).

Therefore, I hypothesised that, as regulators of p53, the 5S RNP components may also be differentially phosphorylated to regulate p53 signalling. As a previous study demonstrated that RPL5 can undergo phosphorylation *in vitro* (Park and Bae, 1999), I focused my studies on RPL5.

At present, very little is known about phosphorylation of RPL5. Several phosphorylation sites have been reported on Phosphosite (http://www.phosphosite.org), but the relevance of these has not been investigated.

I have shown that the RPL5 Y30F mutation, to prevent Y30 phosphorylation, reduces the p53 response from RPL5 expression and that mutation to glutamic acid in an attempt to mimick phosphorylation affects RPL5 recruitment into the ribosome. This suggests that phosphorylation could be a key mechanism for controlling whether the 5S RNP is incorporated into the ribosome, and if it is not, how it affects p53 signalling. Unfortunately, there was not time within this project to identify the kinase responsible for the phosphorylation of RPL5 and to further investigate its existence in cells. RPL5 has been shown to interact with two different kinases (Park and Bae, 1999, Bialik et al., 2008), but neither are able to phosphorylate tyrosine residues. This should be investigated further, using a kinome screening technique, to reveal kinases and phosphatases involved in regulating the 5S RNP pathway.

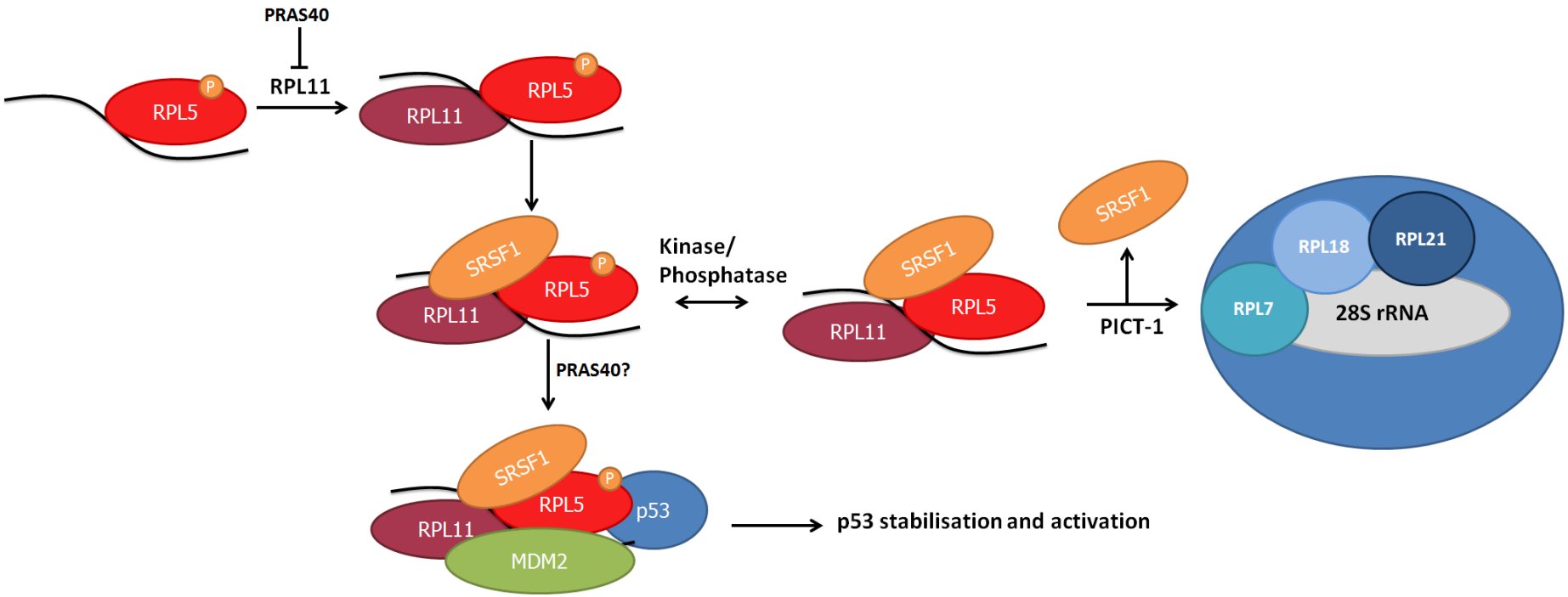

Figure 6.2 The proposed model for 5S RNP-p53 signalling via SRSF1 and PRAS40. The 5S RNP is formed by the binding of RPL5 to the 5S rRNA and subsequent RPL11 binding which can be blocked by the activation of PRAS40. After 5S RNP formation, we propose that SRSF1 is recruited. If RPL5 is de-phosphorylated, SRSF1 is required to integratte the 5S RNP into the ribosome, this process also requires PICT-1. If RPL5 remains phosphorylated, the 5S RNP is not incorporated into ribosomes, it can bind to MDM2 and p53 to mediate p53 stabilisattion and activation. PRAS40 is required for p53 activation although its role is not yet clear.

Interestingly, according to Phosphosite, T73 and Y119 are potential phosphorylation sites of RPL11 and these residues fall within the binding site of MDM2 (Zhang et al., 2011, Zheng et al., 2015). This could be a further mechanism for blocking RPL11-MDM2 interactions. Therefore, it would be beneficial to further investigate how these phosphorylations are regulated.

6.4.3 Summary

In summary (Figure 6.2), my data in combination with previous observations in the lab suggests that SRSF1 interacts with the 5S RNP to facilitate in the activation of p53. Furthermore, PRAS40 associates with RPL11 and possibly sequesters it to the nucleolus but, in direct contrast to the literature, also may be involved in the activation of p53.

Furthermore, my data suggests that phosphorylation of RPL5 at Y30 may be a requirement for p53 activation and thus it is possible that the phosphorylation of RPL5 may also regulate interactions between SRSF1 and the 5S RNP. Therefore, it would be beneficial to test association between SRSF1 and the RPL5 phosphorylation mutants to further understand this regulation pathway.

6.5 Future treatments for disease

This project has revealed many avenues that could be explored to aid in finding new therapeutics for ribosomopathies, such as DBA and 5q-syndrome. I have mainly focused my investigation on DBA as the role of the 5S RNP signalling in this disease has been well studied.

The interaction with SRSF1 and PRAS40 with the 5S RNP is extremely interesting as one of the most promising treatments for DBA currently available is leucine treatment (Jaako et al., 2012, Payne et al., 2012, Pospisilova et al., 2007). However, despite its efficiency at treating DBA, it has little effect in 5q-syndrome patients (Yip et al., 2012, Steensma and Ebert, 2013). Leucine is an activator of the mTOR pathway (Lynch, 2001). Interestingly, mTORC1 is able to phosphorylate PRAS40 at S221 and this phosphorylation was thought to be essential for RPL11-PRAS40 interactions (Havel et al., 2014). Therefore, it follows that leucine treatment is an effective therapy as it activates mTORC1 leading to phosphorylation of PRAS40 and thus RPL11 is sequestered to the nucleolus and can no longer interact with MDM2 to activate p53. Furthermore, SRSF1 is able to activate mTORC1 (Michlewski et al., 2008, Ben-Hur et al.,

2013, Karni et al., 2007), suggesting that SRSF1 and PRAS40 may work in a conjunction to maintain p53 levels within the cell via the 5S RNP. In this project, I was not able to demonstrate leucine treatment reducing p53 levels upon ribosomal stress. However, future experiments altering the dosage of leucine or the effectiveness of leucine uptake would be required to fully understand this pathway. Furthermore, leucine treatment in conjunction with PRAS40 activation could be a more effective treatment than either treatment alone and thus future experiments could be carried out to this effect.

In addition to leucine treatment, drugs that activate mTORC1 may be useful in treating ribosomopathy patients of whom have increased 5S RNP-mediated p53 levels. One of the main activators of mTORC1 in the cell is AKT (Huang and Manning, 2009) and my data shows that inhibition of AKT activates p53 via RPL5. Therefore, I hypothesise that activation of AKT could potentially block p53 accumulation through the 5S RNP. One activator that would be worth testing as a potential therapeutic for DBA and 5q- syndrome would therefore be SC-79 which activates AKT (Jo et al., 2012).

During the course of this project it was demonstrated that mimicking phosphorylation of RPL5 at Y30 prevented integration of the 5S RNP into the ribosome. Based on the data in Chapter 5 that mimicking phosphorylation of Y30 results in inefficient RPL5 incorporation into the ribosome, it is possible that blocking the kinase would potentially prevent accumulation of 'free' 5S RNP and prevent activation of p53. Therefore, if the kinase involved in this phosphorylation could be identified, this would present a potential therapeutic target for ribosomopathy patients.

This project also demonstrated that a small fragment of the 28S rRNA (helix 83-85) can bind to RPL11 *in vitro*. This fragment of the 28S rRNA has been shown to bind to RPL11 in the same region as MDM2 (Zheng et al., 2015). Therefore, it would be beneficial in the future to attempt to introduce the rRNA fragment into human cell lines to see whether it has the ability to block RPL11-MDM2 interactions. This could be an interesting therapeutic as, if it could be targeted specifically to the nucleoplasm, it would promote MDM2-mediated p53 degradation in patients with ribosomopathies without affecting ribosome production.

Furthermore, despite the fact that RPL5 and RPL11 depletion does not activate p53 (Sloan et al., 2013a), severe truncations of RPL5 and RPL11 (resulting in haploinsufficiency) have been reported in patients with DBA (Singh et al., 2014, Cmejla et al., 2009, Delaporta et al., 2014). In this project, RPL5 depletion was shown to induce cell cycle defects, with cells accumulating in G2/M. This was in agreement with previous studies that haploinsufficiency of RPL5 results in a G2/M arrest (Singh et al., 2014). This suggests that the mechanism of DBA pathogenesis in patients with RPL5, and presumably RPL11, mutations differ from that of the other ribosomal proteins. Therefore, patients harbouring these mutations would not benefit from targeting of p53. This is similar to what is seen in cancer as p53 is lost or mutated in a large proportion of cancers (Muller and Vousden, 2013), upregulation of p53 signalling in cancer patients may not be an effective treatment. Several of the ribosomopathies have a predisposition to cancer and RPL10 and RPL5 mutations have been described in patients with AML. Therefore, a greater understanding of the pathogenesis of these diseases and 5S RNP signalling could help to provide novel treatments for cancer. Further investigation into the mechanism behind the G2/M arrest upon RPL5 depletion would help to discover novel therapeutic targets. This could be performed using a gene expression array in conjunction with depletion of RPL5 or RPL11 to identify changes in the expression of cell cycle regulators.

6.6 Conclusions

This work has shown the complex nature of regulation of the 5S RNP in p53 signalling. It was shown that helices 83-85 of the 28S rRNA could bind *in vitro* to RPL11 and could be potentially used to prevent RPL11-MDM2 interactions. Three ribosomal proteins were identified: RPL7, RPL18, and RPL21, which appear to be required for the integration of the 5S RNP into the ribosome and, if depleted, cause 5S RNP-dependent p53 induction and cell cycle arrest. These findings also demonstrate a role for SRSF1 as a potential regulator of the 5S RNP-p53 pathway and show that, whilst PRAS40 is potentially required for the p53 response from RPL5 and RPL11 expression, it does not interact with the intact 5S RNP. Furthermore, phosphorylation of the RPL5 has been identified as a potential key regulator of the complex. Mimicking phosphorylation of Y30 on RPL5 prevents integration of the 5S RNP into the ribosome but it was also shown that phosphorylation of RPL5 may be required for p53 activation. Further

analysis will be required to fully understand the regulation of the 5S RNP via SRSF1, PRAS40 and post-translational modifications.

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