University of Glasgow

Dowding, Sarah N. (2013) *The regulation of RNA polymerase III-mediated transcription by p53, AP-1 and JNKs.* PhD thesis.

http://theses.gla.ac.uk/4756/

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Glasgow Theses Service
http://theses.gla.ac.uk/
theses@gla.ac.uk

The Regulation of RNA Polymerase III-mediated Transcription by p53, AP-1 and JNKs

Sarah N. Dowding
MA (Cantab)

Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

Institute of Cancer Sciences
College of Medical, Veterinary and Life Sciences
University of Glasgow

December 2013

Summary

Cellular growth is the accumulation of mass by a cell. This is required in order to ensure the maintenance of cell size upon cell division. As the majority of the dry mass of a cell is protein, protein synthesis is essential for cellular growth. RNA Polymerase III (Pol III) is crucial for protein synthesis as it transcribes the genes encoding 5S ribosomal RNA and the tRNAs – key components of the translation machinery. As a consequence, Pol III plays an important role on cell growth and proliferation. Pol III-mediated transcription is highly regulated. Cellular stress usually results in decreased Pol III-mediated transcription, while increased transcription occurs following exposure of cells to mitogens. Understanding how Pol III is regulated is particularly important as loss of regulation is associated with cancer, where Pol III-associated transcription factors and Pol III products are frequently found highly expressed. In this thesis, the role of p53, AP-1 and c-Jun N-terminal kinases (JNKs) in the regulation of Pol III-mediated transcription in humans is examined.

p53 is induced in response to stress and modulates the expression of a vast array of target genes. p53 represses Pol III-mediated transcription by binding to the Pol III-specific transcription factor TFIIIB and inhibiting polymerase recruitment to Pol III-target genes. In Chapter Three, it is demonstrated that repression of Pol III-mediated transcription is not the universal response to p53 upregulation. Treatment with the chemotherapeutic drug doxorubicin induces p53 robustly, and results in rapid reduction in tRNA levels but this is not dependent upon the presence of p53. Indeed, it is demonstrated that stress does not always result in the repression of Pol III-mediated transcription. Exposure of cells to ultraviolet light leads to an increase in tRNA levels. This demonstrates the complexity of the regulation of Pol III-mediated transcription in response to stress.

Together, c-Jun and c-Fos produce one form of the transcription factor AP-1. AP-1 mediates cell proliferation and cell death in response to mitogenic stimuli and stress via the binding and activation of target genes. c-Jun and c-Fos have been found binding Pol III target genes. In Chapter Four, their occupancy at tRNA genes is examined in detail and found to be associated with active genes. While AP-1 binding motifs are found at a subset of tRNA genes, this is not found to be associated with c-Jun or c-Fos occupancy. Instead, recruitment to tRNA genes

may occur via the association with Pol III-specific transcription factors that is observed.

JNKs can regulate Pol III-mediated transcription by altering the level of the Pol III-specific transcription factor TFIIIB. JNKs also phosphorylate and activate c-Jun and other transcription factors, such as STAT1 and ATF2, found at Pol III target genes. The role of these transcription factors at tRNA genes has not been determined. However, in Chapter Five, it is demonstrated that JNKs may regulate Pol III-mediated transcription independently of regulating TFIIIB levels. Treatment of U2OS cells with a JNK inhibitor results in rapid reduction of tRNA levels resulting from reduced expression of tRNA genes. However, the levels of TFIIIB subunits TBP and Brf1 are unaffected. It is examined whether this effect may occur through a JNK target at tRNA genes or via another possible route, such as the phosphorylation of the Pol III machinery by JNK.

Data in Chapters Four and Five suggest c-Jun and JNK may positively regulate Pol III-mediated transcription. This is consistent with their activation by mitogens, as more Pol III products are required for increased cell growth and proliferation.

In conclusion, the data highlights the many pathways that converge to regulate Pol III-mediated transcription, and the complexity that arises as a consequence of this.

Table of Contents

List of Figures

List of Tables

Acknowledgements

Firstly, I would like to thank The Beatson Institute and Cancer Research UK for providing funding and resources for my research.

Ann Hedley, Damian Graczyk and Tony McBryan have kindly helped me with the bioinformatics analysis in my project.

I am grateful to Bob White for giving me a position in his lab and for the advice and help he has provided me with.

I thank Kevin Ryan for his unfailing support and confidence in my ability, and for welcoming me into his lab.

I am so grateful to all the members of R05, M06 and R20 for the welcome they gave me following my lab move, it made the transition much easier.

Thanks to all the other Beatson PhD students.

Mum, Dad and Mark. Thanks for always being at the other end of the phone, no matter what. And thanks for reminding me of the most important things in life. To you three, I am eternally grateful.

There would be no thesis in which to include an acknowledgements page without the following people: Tracy Berg, Joanna Birch, Kirsteen Campbell, Cassie Clarke, Damian Graczyk, Dritan Liko, Louise Mitchell, Elena Ruengeler, Sarah Slater and Dhaval Varshney. You have been a veritable fount of knowledge, help, friendship and cake. Thank you.

Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Sarah Dowding

Abbreviations

aa	amino acid
ADH	alcohol dehydrogenase
Ala	alanine
AP-1	activator protein 1
Arg	arginine
ARPP P0	acidic ribosomal phosphoprotein P0
Asn	asparagine
Asp	aspartic acid
Cdk	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
CK2	casein kinase II
coIP	co-immunoprecipitation
CRE	cAMP-response element
Cys	cysteine
DEN	diethylnitrosamine
DSB	double stranded break
EBV	Epstein-Barr virus
Gln	glutamine
Glu	glutamic acid
Gly	glycine
HAT	histone acetyltransferase
HCC	hepatocellular carcinoma
His	histidine
Ile	isoleucine
IVT	*in vitro* transcription
JNK	c-Jun N-terminal kinase
Leu	leucine

Lys	lysine
MAPK	mitogen-activated protein kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MEF	mouse embryonic fibroblast
Met	methionine
MKK	mitogen-activated protein kinase kinase
MMS	methyl methanesulfonate
MPA	mycophenolic acid
mRNA	messenger RNA
PBS	phosphate buffered saline
Phe	phenylalanine
PIC	pre-initiation complex
PLA	proximity ligation assay
Pol I	RNA Polymerase I
Pol II	RNA Polymerase II
Pol III	RNA Polymerase III
Pro	proline
RB	retinoblastoma protein
RE	response element
rRNA	ribosomal RNA
RTD	rapid tRNA degradation
SelCys	selenocysteine
Ser	serine
SINE	short interspersed nuclear element
snRNA	small nuclear RNA
SRP	signal recognition particle
TBP	TATA binding protein
TF	transcription factor
Thr	threonine

TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRE	TPA-responsive element
tRNA	transfer RNA
Trp	tryptophan
TSS	transcription start site
Tyr	tyrosine
Val	valine

1 Introduction

1.1 Transcription in eukaryotes

Transcription is essential to convert information encoded in a genome to functional products. It is the process by which DNA is 'read' and an RNA copy is made. RNA is then translated to protein, which is required for all cellular processes, or RNA may function directly without a protein intermediary. Together, transcription and translation are the key steps of protein synthesis. In eukaryotes, transcription usually starts at a transcription start site (TSS) at the beginning of a gene. This transcription start site is defined by the DNA sequence and, with the assistance of additional factors, is recognised by a DNA-dependent RNA polymerase. Once correctly positioned, the polymerase can initiate transcription. Following initiation, transcription moves into the elongation phase, catalysing the incorporation of ribonucleotides complementary to the DNA being read, and is finally terminated at a defined site. The RNA product is then released from the polymerase. RNAs encoding proteins (mRNAs) are then transported from the nucleus to the cytoplasm where they can be translated. mRNAs are translated by the ribosome, where transfer RNAs (tRNAs) decode the mRNA to allow the incorporation of amino acids in the correct order to produce the protein.

Prokaryotes have a single, multisubunit RNA polymerase. In eukaryotes, where the task of transcription has been divided, three major RNA polymerases (RNA Polymerases I, II and III) have evolved. These all contain subunits homologous to each of the five subunits of the bacterial RNA polymerase (Werner and Grohmann, 2011). RNA Polymerase II (Pol II) transcribes all protein-coding genes and most microRNA and snRNA (small nuclear RNA) genes [reviewed in (Baumann et al., 2010)]. RNA Polymerase I (Pol I) targets multiple copies of a single gene only, the ribosomal RNA gene that encodes 45S pre-rRNA which is then processed to produce three of the four RNA components of the ribosome, 28S, 18S and 5.8S [reviewed in (Russell and Zomerdijk, 2005, McStay and Grummt, 2008)]. RNA Polymerase III (Pol III) produces the fourth ribosomal RNA (5S rRNA), transfer RNAs (tRNAs) and a range of other short non-coding RNAs [reviewed in (White, 2002, Schramm and Hernandez, 2002)]. In plants, two additional nuclear polymerases have been identified, RNA Polymerases IV and V (Haag and Pikaard,

2011). These are related to RNA Polymerase II and produce non-coding RNA transcripts involved in gene silencing (Haag and Pikaard, 2011). In addition, eukaryotic cells contain a RNA polymerase responsible solely for transcription of the mitochondrial genome [reviewed in (Arnold et al., 2012)].

1.2 Pol III targets and their roles in the cell

The majority of genes transcribed by Pol III can be described as 'housekeeper genes'. They play essential roles in the cell including translation, RNA processing and protein localisation. In total, their transcription is estimated to account for over 10% of transcription in the nucleus (White, 2001). However, the exact amount will vary depending upon the state of the cell. Pol III-transcribed genes are located throughout the human genome. All genes transcribed by Pol III encode non-coding RNAs, RNAs that do not encode a protein but are themselves structurally or catalytically functional. The majority of these RNAs are less than 300nt in length (White, 2008). Below the Pol III products are described, focussing on those that we will encounter again later in this thesis.

1.2.1 tRNAs

tRNAs are essential components of the translation machinery, providing the link between the mRNA and the growing polypeptide. Their complex secondary structure is often visualised as a 'cloverleaf' structure (Figure 1.1). Further folding results in an L-shape tertiary structure. The anticodon loop contains the three bases that form the anticodon. This recognises a complementary codon in mRNA. The correct amino acid is loaded onto the acceptor stem of the tRNA by a tRNA synthetase. tRNAs are between 75 and 90bp in length and there are 516 genes encoding functional tRNAs in the human genome (as annotated in the Genomic tRNA Database, hg18 (Chan and Lowe, 2009)). tRNAs are found on every chromosome except the Y chromosome. They can be divided into groups based on the amino acid they bind and the anticodon they contain. For example, a tRNA with the anticodon AAG and a tRNA with the anticodon CAG are of the same isotype as they both bind leucine. However, they are from different isoacceptor classes as they have different anticodons and thus recognise different codons (CUU and CUG respectively). There are tRNA genes representing 51 isoacceptor classes in the human genome. The multiple genes present for

many isoacceptor classes add further complexity. These genes can have different internal sequences (despite having the same anticodon). Transcription of tRNA genes results in pre-tRNAs that require much additional processing to be functional. This includes removal of the 5' and 3' flanking sequences, addition of CCA to 3' end and modification of multiple residues (Hopper and Phizicky, 2003). Some tRNA genes also have introns, adding an additional splicing step to their processing.

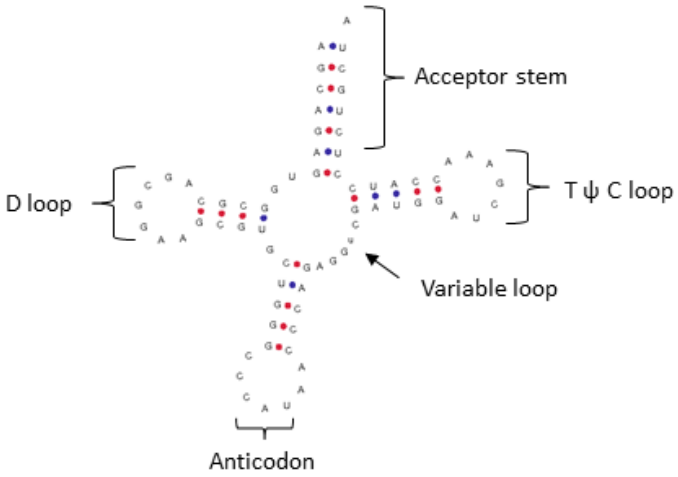

Figure 1.1 The tRNA secondary structure

Schematic of the secondary structure of a human $tRNA_i^{Met}$. The functional domains include the acceptor stem which binds to the amino acid and the anticodon which recognises the complementary codon in the mRNA. Predicted tRNA structure from Genomic tRNA Database (Chan and Lowe, 2009)

1.2.2 5S ribosomal RNA

5S rRNA is a component of the large subunit of the ribosome, and is thus required in equimolar quantities to the other ribosomal RNAs, 28S, 18S and 5.8S (produced by Pol I) (Phillips and McConkey, 1976). The ribosomal RNA carries out most of the structural and functional role of the ribosomes, while ribosomal proteins stabilise the ribosome core (Moore and Steitz, 2002). In humans, there are approximately 200-300 5S rRNA genes present in tandem repeats (Lander et al., 2001, Stults et al., 2008). They are highly conserved and have proved difficult to map accurately due to their repetitive nature (She et al., 2004). A role for 5S rRNA in ribosome biogenesis stress signalling has recently been identified (Li and Gu, 2011, Donati et al., 2013).

1.2.3 U6 snRNA

U6 is one of the small nuclear RNAs (snRNAs) that form the RNA portion of the spliceosome, a ribonucleoprotein complex that is required to remove introns from mRNAs ('splicing') (Will and Lührmann, 2011). The other four small nuclear RNAs in the spliceosome, U1, U2, U4 and U5, are products of Pol II. Nine full-length U6 genes exist in the human genome (Domitrovich and Kunkel, 2003).

1.2.4 7SL

7SL RNA forms the scaffold of the signal recognition particle (SRP) to which the six protein subunits of the SRP bind (Walter and Blobel, 1982). The SRP recognises the signal peptide in newly synthesised polypeptides and facilitates their insertion into the endoplasmic reticulum [reviewed in (Nyathi et al., 2013)]. As such, it is essential for ensuring correct localisation of many proteins. Three 7SL genes are present in the human genome, all located on the long arm of chromosome 14.

1.2.5 SINEs

Short interspersed nuclear elements (SINEs) are DNA sequences derived from Pol III-transcribed genes. These elements became dispersed throughout the genomes of mammals via retrotransposition. The most abundant SINEs in humans are Alu family, derived from the 7SL gene (Jelinek et al., 1980). Over 1 million Alus are present in the human genome (Lander et al., 2001). Their number means that they are capable of sequestering a large pool of the Pol III machinery. Under normal conditions, Alus are transcribed at low levels, and this has been observed to increase following stress (Paulson and Schmid, 1986, Liu et al., 1995, Rudin and Thompson, 2001). Alus were originally considered to be 'junk' DNA, with no function. They have now been shown to have roles in the heat-shock response, in the regulation of alternative splicing, in translational control and in disease (Mariner et al., 2008, Sorek et al., 2002, Chu et al., 1998, Kaneko et al., 2011). Alus are unique to primates, however other SINEs are found in other organisms. For example, B1 and B2 elements, in mice, are derived from a 7SL and a tRNA gene respectively (Labuda et al., 1991, Daniels and Deininger, 1985).

1.2.6 Other Pol III-transcribed cellular genes

There are many other Pol III-transcribed genes, in addition to those described above. H1 RNA is part of RNase P, a particle required for processing of the 5' end of pre-tRNAs (Bartkiewicz et al., 1989). rRNA processing requires RNase MRP which contains with RNAse MRP RNA, a Pol III product (Gold et al., 1989). Another Pol III product is 7SK. 7SK regulates the elongation step of Pol II-dependent transcription via its interaction with the elongation factor P-TEFb (Nguyen et al., 2001, Yang et al., 2001). Vault RNAs are also Pol III transcribed. They form part of the large ribonucleoprotein vault complexes that are implicated in multidrug resistance (Mossink et al., 2003).

1.2.7 Virus-encoded Pol III-transcribed genes

Several viral genes have evolved to exploit the Pol III transcription machinery. These include those encoding VA-1 which is found in all adenovirus serotypes and EBER1 and EBER2 of the Epstein-Barr virus (EBV). VA-1 is approximately 160 nucleotides long and promotes the translation of viral mRNA in late stages of viral infection, when it is highly expressed (Thimmappaya et al., 1982). The VA-1 promoter has been frequently used as a reporter for *in vitro* Pol III activity studies. EBER1 and EBER2 are highly expressed following EBV infection of B lymphocytes (Arrand and Rymo, 1982). Like VA-1, EBER1 and EBER2 contribute to translation of the viral mRNA, subverting translation of host cell mRNA (Rosa et al., 1981).

1.3 Mechanism of Pol III-mediated transcription

Like all transcription, RNA Polymerase III-mediated transcription can be divided into initiation, elongation and termination steps. Initiation is dependent upon a promoter sequence to recruit Pol III-specific transcription factors, which can in turn recruit the polymerase. Elongation is the process of transcribing the remainder of the gene, which in Pol III is very short and hence this is not a lengthy step. Termination of transcription occurs when the polymerase reaches a short run of thymine residues.

1.3.1 Promoter types

Pol III-transcribed genes in humans can be divided into three groups based on promoter type [reviewed in (White, 2002, Schramm and Hernandez, 2002, Dieci et al., 2007)]. The promoter sequences of type 1 and 2 promoters are within the transcribed region (Figure 1.2). Type 2 promoters are most common as they are found in all tRNA genes (except $tRNA^{SelCys}$) and other genes, including 7SL and VA-1. The type 2 promoter consists of conserved A and B boxes located downstream of the transcription start site (TSS), typically separated by 30-40bp. 5S genes are the only genes with type 1 promoters, which also consist of pair of conserved motifs, A box and C box, with an additional sequence, the intermediate element (IE), between them. In contrast, type 3 promoters, such as that of U6, are more similar to protein-coding genes with an external promoter upstream of the TSS which includes a TATA-box, a proximal sequence element (PSE) and a distal sequence element (DSE). Variations exist within these three types, for example the EBER2 gene has a TATA box and A and B boxes.

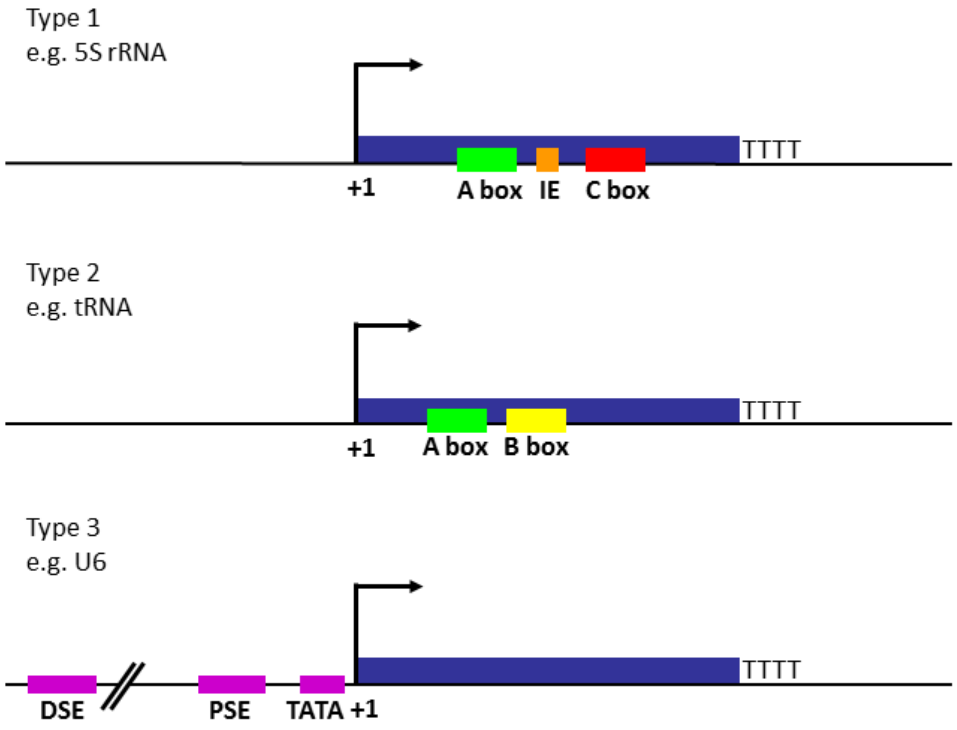

Figure 1.2 Promoter types of Pol III target genes

The diagram above illustrates the main promoter types of genes transcribed by Pol III. Some variations on these promoter types have been described. Transcribed region of gene indicated by blue box. +1 denotes the transcription start site. TTTT represents the site of termination (the number of thymine residues is variable). Approximate positions of promoter elements are shown as coloured boxes with element name stated beneath. IE = intermediate element, DSE = distal sequence element, PSE = proximal sequence element, TATA = TATA box. Adapted from (Schramm and Hernandez, 2002).

1.3.2 Pre-initiation complex formation

The promoter structure of a gene determines how the Pol III-specific basal transcription factors are recruited. Basal transcription factors, together with the polymerase, form the pre-initiation complex (PIC) at the transcription start site [reviewed in (Schramm and Hernandez, 2002, White, 2002)]. PIC formation is essential for transcription to begin.

At type 1 and 2 promoters, PIC formation requires the recognition of the internal promoters by TFIIIC, a multisubunit basal transcription factor (Figure 1.3). In type 1 promoters, this recognition is aided by the binding of the zinc-finger protein TFIIIA to the intermediate element. Once TFIIIC is positioned on the DNA, it positions TFIIIB just upstream of the TSS. TFIIIB then recruits Pol III to the transcription start site. In type 3 promoters, a variant TFIIIB (see below), binds at the TATA box upstream of the transcription start site and a multisubunit complex, $SNAP_c$, binds at the PSE. Together they recruit Pol III. Oct-1 and STAF bind at the distal sequence element (DSE) and promote PIC formation.

TFIIIB All Pol III target genes require TFIIIB for successful polymerase recruitment. *In vitro* transcription can be driven from a minimal PIC of TFIIIB and Pol III alone at artificial promoters (Kassavetis et al., 1990), highlighting TFIIIB's central role in Pol III-mediated transcription. TFIIIB is a complex of three independent subunits, TBP, Bdp1 and Brf1 or Brf2 (Figure 1.4). TBP, the TATA-binding protein, is required for the activity of Pols I, II and III. However, binding to a TATA box is not always required. A TBP mutant that cannot bind the TATA box is still functional in Pol III-mediated transcription (Bryant et al., 1996). Brf1, TFIIB-related factor 1, is, as its name suggests, related to the Pol II basal transcription factor TFIIB. At genes with type III promoters, Brf2 is present instead of Brf1 in TFIIIB (Teichmann et al., 2000, Schramm et al., 2000). Similar to Brf1, it has an N-terminal zinc binding domain and a core domain related to TFIIB (Schramm and Hernandez, 2002). However, it lacks two conserved domains present in the C-terminus of Brf1. The third subunit of TFIIIB is Bdp1, B double prime 1. In yeast, it is only loosely bound to the stable TBP-Brf1/2 complex in the absence of a DNA template (Huet et al., 1994).

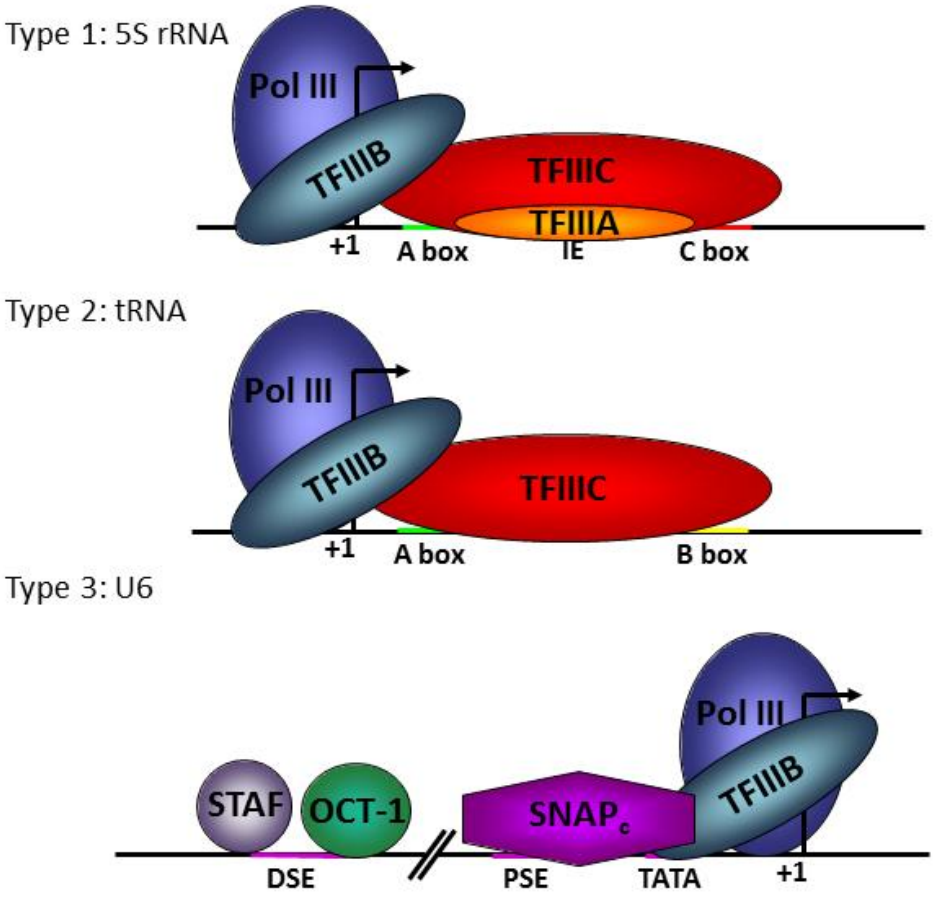

Figure 1.3 Pre-initiation complex formation at three Pol III promoters

Different Pol III pre-initiation complexes (PIC) form at the three promoter types. At type 1 promoters, TFIIIA binds to the promoter which includes the intermediate element (IE) and promotes TFIIIC recruitment. TFIIIB is then recruited, followed by recruitment of the polymerase. At type 2 promoters, TFIIIC binds directly to promoter sequence. This is followed by TFIIIB and Pol III recruitment as at type 2 promoters. At type 3 promoters, TFIIIB binds the TATA box (TATA) and the $SNAP_c$ complex binds the proximal sequence element (PSE). Pol III is then recruited to the transcription start site (+1). Oct-1 and STAF bind at the distal sequence element (DSE) and promote PIC formation.

TFIIIC TFIIIC consists of six subunits, TFIIIC220, TFIIIC110, TFIIIC102, TFIIIC90, TFIIIC63 and TFIIIC35 (Figure 1.4). Unlike TFIIIB, it has no direct relatives at other polymerases. It consists of two main subdomains, each recognising the A and B box, linked by a flexible linker (TFIIIC90) (Schramm and Hernandez, 2002). These subdomains contain TFIIIC220 which binds the B box and TFIIIC63 which binds the A box. TFIIIC102, TFIIIC90 and TFIIIC63 interact with TFIIIB. TFIIIC220, TFIIIC110 and TFIIIC90 have intrinsic histone acetyltransferase (HAT) activity which may contribute to gene activity (Hsieh et al., 1999a, Hsieh et al., 1999b, Kundu et al., 1999).

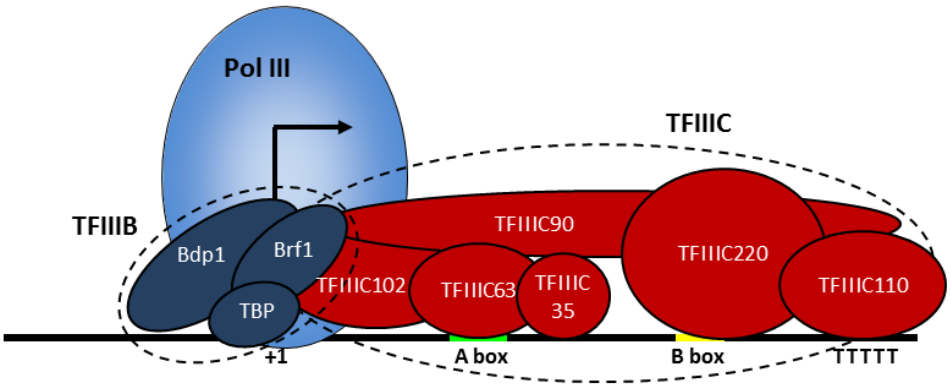

Figure 1.4 The subunits of TFIIIB and TFIIIC in the pre-initiation complex

The Pol III-specific transcription factors TFIIIB (dark blue) and TFIIIC (red) consist of multiple subunits with different binding and functional properties. Their relative positions at a type 2 promoter are depicted above. TFIIIC63 and TFIIIC220 bind to the A and B box motifs respectively. TFIIIC110 is associated with the terminator sequence (TTTTT) while TFIIIC90 acts as a flexible linker bridging the two subdomains of TFIIIC. TFIIIB subunits TBP, Brf1 and Bdp1 are positioned upstream of TFIIIC and recruit Pol III.

Recent ChIP-seq analysis, chromatin immunoprecipitation followed by deep sequencing of the associated chromatin, has provided data for Pol III machinery occupancy at all annotated Pol III target genes in the human genome (Oler et al., 2010, Raha et al., 2010, Moqtaderi et al., 2010, Barski et al., 2010, Canella et al., 2012). It confirms PIC formation models at different promoter types, as determined originally *in vitro*, are correct. For example, Brf1 and Brf2 occupancy is mutually exclusive, Brf1 occupying genes with type 1 and type 2 promoters and Brf2 at type 3 promoters (Moqtaderi et al., 2010, Oler et al., 2010). In *Saccharomyces cerevisiae* nearly all annotated Pol III target genes are occupied by Pol III (Moqtaderi and Struhl, 2004, Roberts et al., 2003, Harismendy

et al., 2003). However, in humans, many Pol III target genes are not occupied by Pol III, for example, in HeLa cells only approximately 50% of tRNA genes have Pol III present (Oler et al., 2010). TFIIIB occupancy and Pol III occupancy are highly correlated indicating TFIIIB recruitment is the rate-limiting step in PIC formation (Oler et al., 2010, Moqtaderi et al., 2010).

1.3.3 Initiation

Upon formation of the pre-initiation complex, transcription may begin. The polymerase unwinds the double stranded DNA, forming a transcription bubble in which the template strand is accessible (White, 2002). The polymerase then 'reads' the template strand, incorporating ribonucleotides to begin a RNA molecule of complementary sequence. While TFIIIB initially ensures Pol III is positioned correctly at the TSS, Pol III is released from TFIIIB as the transcription bubble moves along the gene (Kassavetis et al., 1990).

1.3.4 Elongation and termination

In comparison with PIC and transcription bubble formation, the rate-limiting steps in Pol III-mediated transcription, elongation and termination are very short and have minimal impact on the rate of Pol III-mediated transcription (White, 2002). Unlike Pols I and II, elongation requires no accessory factors, maybe because the transcribed region is so short (White, 2002). The end of the region to be transcribed is marked by a short run of thymine residues in the non-template strand of the DNA at which the polymerase pauses. The mechanism of termination has only recently been determined (Nielsen et al., 2013). Once paused, Pol III backtracks until it reaches a hairpin in the newly synthesised RNA, at which point the RNA is released. The polymerase is then recycled back to the promoter, allowing repeated rounds of transcription that occur more quickly than the initial round (Dieci et al., 2013).

1.4 Regulation of Pol III-mediated transcription

Cellular growth, or the accumulation of mass, is required with each cell cycle in order to maintain cell size. This includes the duplication of organelles, which is required to ensure two functional daughter cells are produced. The majority of a cell's dry mass is protein and cell growth is directly proportional to protein synthesis (Baxter and Stanners, 1978). Consequently, cell proliferation is also proportional to protein synthesis, except in cases where cell growth and proliferation are uncoupled, such as in terminally differentiated neurons and muscle cells (Conlon and Raff, 1999). In order to increase the rate of cell growth, as occurs following mitogenic signals, more protein synthesis is required. This depends upon increased ribosome biogenesis, thus regulation of rRNA synthesis (by Pol I and Pol III) is important for cell growth (Camacho et al., 1990, Ruggero and Pandolfi, 2003). Other Pol III products also play key roles in protein synthesis, including the tRNAs in translation and U6 and MRP RNA in mRNA and rRNA processing respectively. An increase in Pol III activity is, therefore, required for cell growth. Similarly, when growth is arrested, as occurs in times of reduced nutrient availability and other stresses, continued Pol III-mediated transcription would waste vital resources. It is well established that serum induces Pol III-mediated transcription and reduced nutrients and mitogenic signals have the opposite effect (Clarke et al., 1996, Johnson et al., 1974, Mauck and Green, 1974). Pol III activity also changes during the cell cycle, with reduced activity during mitosis and early G1 (Gottesfeld et al., 1994, White et al., 1995).

In the past, the regulation of Pol III-mediated transcription has been considered to modulate the transcription of all Pol III target genes in unison, based upon the environment the cell finds itself in. This was a reasonable assumption to make, as the Pol III products have related functions and, as described below, several mechanisms by which they are regulated target factors shared by all, such as TFIIIB. However, with the advent of microarray, ChIP-seq and RNA-seq, it has become apparent that more specific regulation of Pol III target genes may also occur (White, 2011). Differences in the promoter structure (and consequently the basal transcription factors involved) may account for this to some extent. However, it does not tell the whole story. In *S. cerevisiae* all 186 tRNA genes are occupied by Pol III, suggesting they are being actively transcribed (Harismendy

et al., 2003, Roberts et al., 2003, Moqtaderi and Struhl, 2004). However, in human cells approximately half are occupied by Pol III at any one time and the tRNA expression profiles vary between cell types (Raha et al., 2010, Moqtaderi et al., 2010, Oler et al., 2010, Barski et al., 2010, Dittmar et al., 2006).

The regulation of Pol III-mediated transcription has been the subject of many studies because aberrant cell growth is associated with several diseases including, most notably, cancer. In cancer cells, normal control over cell proliferation is overcome, allowing cells to divide with reduced restraint. For this uncontrolled division to persist, cell growth is also required. Pol III products have been found upregulated compared to normal samples in many tumours. Winter et al. found increased tRNA levels in ovarian tumour samples, while Chen et al. found 7SL upregulated in 31 of 39 breast cancer tumours when compared to normal tissue (Winter et al., 2000, Chen et al., 1997). Staining of a tumour microarray for $tRNA_i^{Met}$, the tRNA that binds the initiator methionine required to begin translation, showed that it was at high levels in breast tumours, lymphoma and other tumour types (Noor Nam, unpublished data). Many regulators of Pol III-mediated transcription, as described below, are deregulated during cancer development. This may account for the high levels of Pol III products which contribute to the increased cell growth and proliferation in cancer cells compared to normal cells (White, 2008). It is currently unclear whether high Pol III products are a cause or a consequence of cancer. Further work to understand the regulation of Pol III-mediated transcription may provide a solution to this. It will certainly contribute significantly to our understanding of cell growth and related diseases.

1.4.1 Mechanisms of regulating Pol III-mediated transcription

Pol III-mediated transcription is predominantly regulated at the initiation step as this is the rate-limiting step in transcription. Unlike Pol II target genes, the short length of Pol III genes and the lack of requirement for specific elongation factors mean the elongation step an inefficient target for regulation (Shilatifard, 2004). Regulation at the initiation step occurs through a variety of mechanisms and can involve many mediators.

1.4.1.1 Regulation of Pol III transcription machinery levels

Pol III-mediated transcription requires sufficient levels of the specific Pol III transcription factors and the polymerase itself. As such, these levels must be modulated in accordance with the growth status of the cell. Producing the machinery when it is not required would be a large drain on the cell's resources and, conversely, a lack of these proteins will impede cell growth. Increased TFIIIB and TFIIIC levels are frequently associated with increased Pol III activity (White, 2004). Increased levels of TFIIIC and TFIIIB subunits are found in cells infected with Epstein Barr Virus (EBV) and hepatitis B respectively (Felton-Edkins et al., 2006, Wang et al., 1995). Both of these viruses result in increased cell proliferation and Pol III activity. Likewise, high TFIIIB and TFIIIC protein levels are found in many tumours. Winter et al. found higher TFIIIC protein levels in ovarian carcinomas compared to normal tissue samples from the same patient (Winter et al., 2000). Pol III-specific transcription factor levels can be altered by changes in their gene expression or by changes in the stability of the protein. p53 induction has been shown to result in degradation of Brf1 in some cell types (Eichhorn and Jackson, 2001). High TFIIIB and TFIIIC mRNA levels are found in a wide range of cancers. These include colorectal and breast tumours where Brf1 and TFIIIC220, respectively, were found significantly upregulated compared to healthy tissue samples [(Zhao et al., 2004, Hong et al., 2010) data mined from Oncomine]. Increased TFIIIB expression has also been found to correlate with more aggressive disease, for example, in two studies, Brf1 expression has been shown to be significantly higher in prostate cancer metastases than in primary prostate tumours [(Grasso et al., 2012, Varambally et al., 2005) data mined from Oncomine]. In addition, recent studies showed that c-Jun N-terminal kinases (JNKs) can regulate the expression of all three TFIIIB subunits (Zhong et al., 2007, Zhong and Johnson, 2009). Clearly, regulating TFIIIB and TFIIIC levels has the potential to contribute to the overall regulation of Pol III-mediated transcription.

1.4.1.2 Phosphorylation of Pol III transcription machinery

TFIIIB can be phosphorylated by multiple kinases. Phosphorylation alters its ability to interact with other components of Pol III machinery in pre-initiation complex formation. For example, ERK, a mitogen-activated protein kinase

(MAPK), phosphorylates Brf1 in response to serum (Felton-Edkins et al., 2003) (Figure 1.5). This phosphorylation was shown to contribute to the subsequent activation of Pol III-mediated transcription, possibly by increasing TFIIIB's affinity for TFIIIC. Plk1 and CK2 also regulated Pol III activity by phosphorylating TFIIIB (Hu et al., 2004, Fairley et al., 2012). Phosphorylation is a faster mechanism of relaying a signal than alteration of protein levels and may be important in fine-tuning Pol III activity in response to changes in cellular environment.

1.4.1.3 Regulator binding to Pol III transcription machinery

The third mechanism by which initiation of Pol III-mediated transcription is regulated is through regulatory proteins binding to the Pol III machinery (Figure 1.5). As with phosphorylation, TFIIIB is the main target of these regulators, probably due to its rate-limiting role in initiation. Regulators act by either sequestering machinery away from Pol III target genes or altering interactions between components of the PIC while present at the gene.

Retinoblastoma protein (RB) RB is a global repressor of the level of Pol III-mediated transcription (White et al., 1996, Gjidoda and Henry, 2013). RB can bind to TFIIIB, preventing its recruitment to Pol III target genes by inhibiting its interaction with TFIIIC (Larminie et al., 1997, Chu et al., 1997). RB binding also prevents TFIIIB interaction with Pol III. This, along with RB interaction with $SNAP_C$, allows RB to repress genes with the type III promoters that do not recruit TFIIIC (Hirsch et al., 2000). RB activity is regulated through its phosphorylation by cyclin-dependent kinase/cyclin complexes, cdk4/cyclin D and cdk2/cyclin E. Phosphorylation of RB reduces its affinity for TFIIIB, thus Pol III-mediated transcription increases at the end of G_1 (Scott et al., 2001). TFIIIB can also be bound and repressed by RB-related proteins p107 and p130 (Sutcliffe et al., 1999).

p53 p53 represses Pol III-mediated transcription (Chesnokov et al., 1996, Cairns and White, 1998). TFIIIB is bound by p53, which prevents TFIIIB's recruitment to Pol III target genes by interfering with its interaction with TFIIIC (Chesnokov et al., 1996, Cairns and White, 1998, Crighton et al., 2003). Like RB, p53 prevents TFIIIB from interacting with Pol III, ensuring the repression of target genes, like

U6, that do not recruit TFIIIC (Crighton et al., 2003). p53 regulation of Pol III-mediated transcription is described in more detail below.

c-Myc c-Myc interacts with TFIIIB and binds at Pol III target genes (Gomez-Roman et al., 2003). When bound at these genes, c-Myc recruits the histone acetyltransferase (HAT) GCN5 and promotes gene expression (Kenneth et al., 2007). Histone acetylation is associated with a chromatin environment permissive for Pol III-mediated transcription (White, 2011). It is currently unclear whether c-Myc promotes the recruitment of TFIIIB or vice versa. c-Myc is activated in response to mitogenic factors and induces cell growth and proliferation through regulating expression of many target genes (Grandori et al., 2000). During proliferation, Pol III products, such as 5S rRNA and tRNAs, are required at higher levels to meet demands for increased protein synthesis and c-Myc activity may contribute to these higher levels.

Maf1 Maf1 was originally identified as a global negative regulator of Pol III-mediated transcription in *S. cerevisiae* (Pluta et al., 2001, Upadhya et al., 2002). This function is conserved in mammals, where Maf1 activity is associated with reduced PIC formation at Pol III target genes (Reina et al., 2006, Johnson et al., 2007, Goodfellow et al., 2008). Maf1 associates with Pol III and TFIIIB and can be found at Pol III target genes (Moir and Willis, 2013). When Maf1 is phosphorylated, it is no longer able to repress Pol III-mediated transcription. Maf1 is the target of multiple kinases and phosphatases, including mTORC1 (Shor et al., 2010, Kantidakis et al., 2010). This contributes to the regulation of Pol III-mediated in response to nutrient availability.

1.4.2 Transcription-independent regulation of Pol III products

The most efficient route to regulate Pol III product levels is by regulating their transcription. However, they may also be manipulated by transcription-independent mechanisms. The levels of Pol III products may be altered via changes in their stability. The rapid tRNA degradation (RTD) pathway contributes to tRNA levels in *S. cerevisiae* (Wichtowska et al., 2013). At present, no equivalent pathway has been identified in humans. Transcripts produced by Pol III require processing to produce functional products. In tRNAs, this is extensive (Hopper and Phizicky, 2003). Perturbation of any processing step will reduce the

level of functional products and, consequently, has the potential to disrupt cell growth.

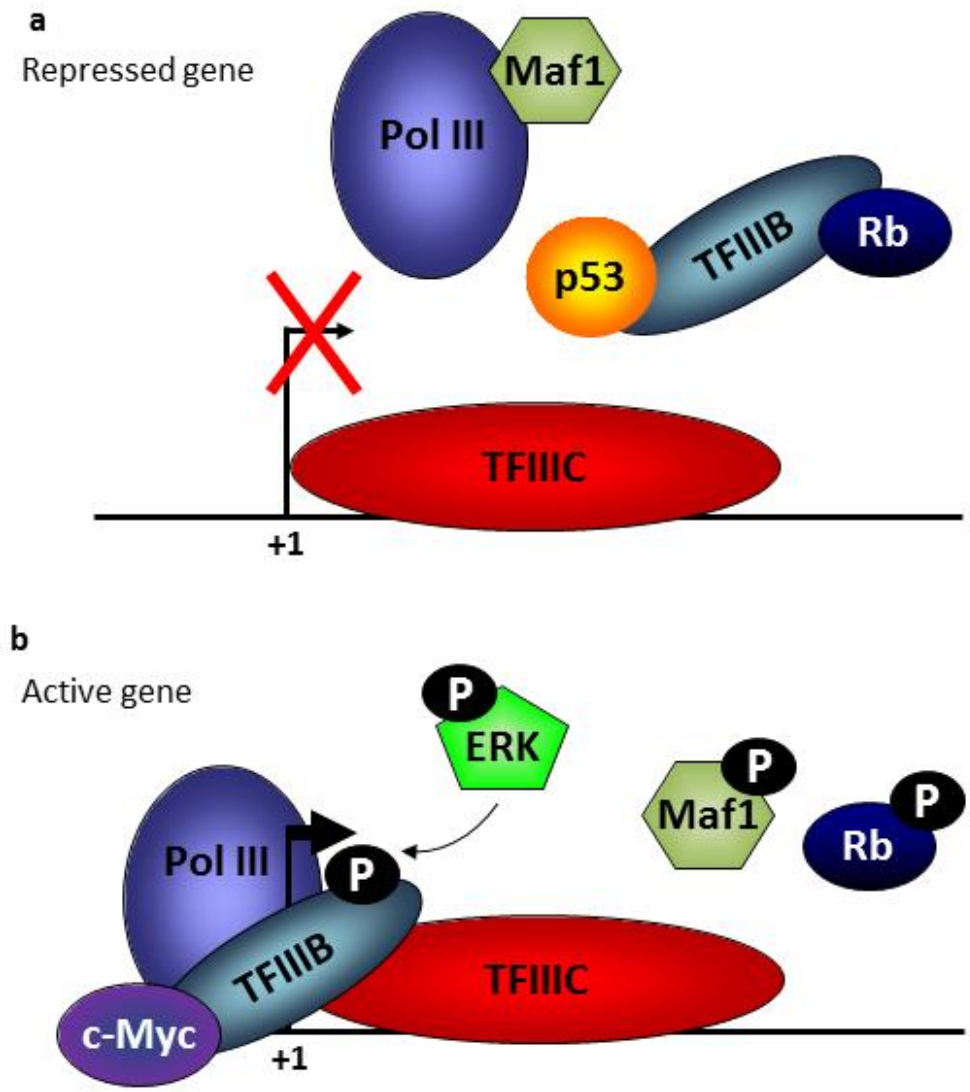

Figure 1.5 Regulators of Pol III-mediated transcription

The diagram above illustrates how regulators can act at a gene with a type 2 promoter. For simplicity, not all known regulators are included. Repression or activation can be mediated by a single regulator. a) Repression of a gene can be mediated by p53, RB or Maf1. p53 and RB prevent pre-initiation complex formation by binding to TFIIIB, preventing its recruitment by TFIIIC. Unphosphorylated Maf1 binds Pol III, preventing its recruitment to the transcription start site (+1). b) Activation of a gene can be mediated by c-Myc, ERK or inactivation of repressors. c-Myc binds at promoters, interacting with TFIIIB and promoting transcription. ERK phosphorylates TFIIIB, probably increasing its affinity for TFIIIC. Phosphorylation of Maf1 and RB prevent their association with Pol III and TFIIIB respectively. Adapted from (White, 2008).

1.4.3 Comparing regulation of Pols I, II and III

p53, RB and c-Myc regulate transcription by Pols I, II and III. This shared regulation may have evolved to allow tight regulation of ribosome biosynthesis and core housekeeping processes (White, 2008). Despite this shared regulation, it was thought that the majority of Pol II transcriptional regulators were specific to Pol II target genes (Raha et al., 2010). However, over the past few years, many transcription factors normally associated with Pol II genes, including c-Jun, c-Fos, STAT-1 and Elk1, have been found at Pol III target genes (Raha et al., 2010, Oler et al., 2010, Zhong et al., 2011). Their function at Pol III target genes remains to be determined.

Recently, it has been shown that chromatin at Pol III target genes has similar modification patterns to that at Pol II target genes (Barski et al., 2010, Oler et al., 2010). As at Pol II-transcribed genes, acetylated histones are associated with active transcription and deacetylation is associated with repression of transcription. Many histone methylation events are also shared between Pol II- and Pol III-transcribed genes. It is unclear at the moment whether the activity of Pol III target genes is a cause or a consequence of the chromatin environment (White, 2011).

In addition, Pol II itself has been found bound upstream of many Pol III target genes (Raha et al., 2010, Oler et al., 2010, Barski et al., 2010). While not required for Pol III-mediated transcription, it may contribute to a permissive chromatin environment. Together, these findings hint at a further complexity to regulation of Pol III-mediated transcription than is currently understood.

1.5 p53

p53 is a transcription factor that activates and represses a vast range of genes in many functional classes, including Pol III-transcribed genes. Initially identified in 1979, p53 was soon found to be a key figure in the cell's response to stress and, due to its role in protecting the cell from accruing DNA damage, it was dubbed 'the guardian of the genome' (Lane, 1992). Upon stress, p53 can induce cell cycle arrest, senescence or apoptosis (Figure 1.6). p53's role, however, extends beyond this; it has been identified to regulate DNA repair, cellular metabolism, and remodelling of the extracellular matrix, among other functions (Vousden and Prives, 2009). p53 is activated by stimuli including DNA damage, hypoxia, oncogenic stress and ribonucleotide depletion (Horn and Vousden, 2007). It is particularly well studied due to its role as a tumour suppressor. The loss or mutation of p53, or the disruption of other p53 pathway components, is required for the development of many tumours in humans (Lozano and Elledge, 2000). Many p53 mutations found in cancer are so-called 'gain of function' mutations, converting p53 from a tumour suppressor to an oncogene (Muller and Vousden, 2013).

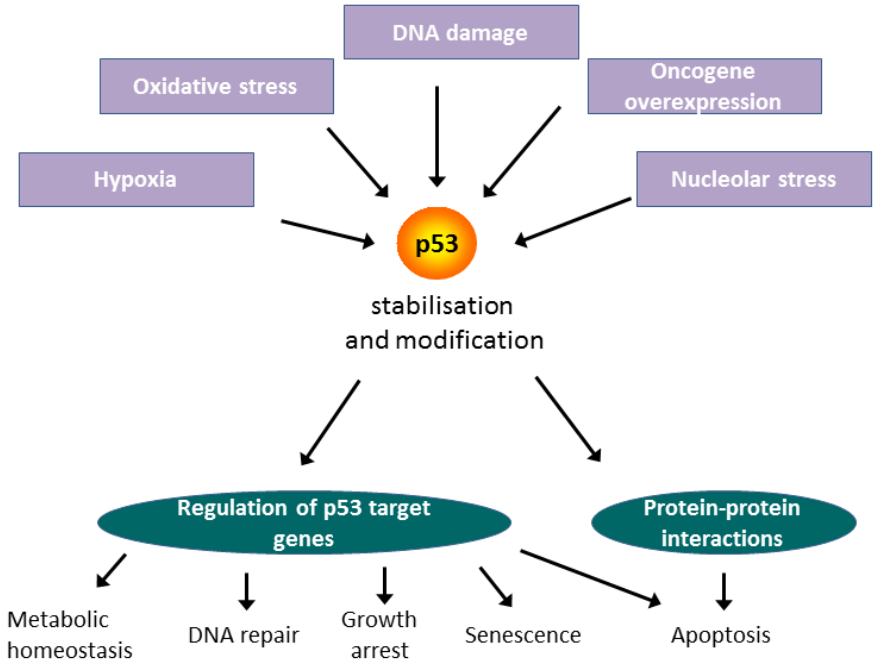

Figure 1.6 The p53 pathway

The p53 pathway is depicted above, in a simplified form. Some of the stresses capable of inducing p53 activity are highlighted in purple. These result in the stabilisation and post-translational modification of p53 by a range of mechanisms (not shown). p53 mediates responses including growth arrest and apoptosis via the routes shown in green. Adapted from (Levine and Oren, 2009).

1.5.1 Structure and regulation of p53

The transcriptional activity of p53 is mediated primarily by interaction of the transcription activation domains (TAD I and II) at p53's N-terminus with other proteins (Ko and Prives, 1996, Laptenko and Prives, 2006). The core DNA-binding domain, between residues 98 and 303, recognises and binds specific DNA motifs present upstream of many target genes. p53 binds as a tetramer which is formed via its oligomerisation domain located towards its C-terminus (aa 323-363).

p53 activity is regulated primarily through its stability and post-translational modification (Kruse and Gu, 2009). Normally in the cell, p53 is maintained at a low level by MDM2 which binds and ubiquitinates it, marking it for proteasomal degradation. Upon stress, this degradation pathway can be overcome by the inhibition of MDM2. p53 can be phosphorylated, acetylated and methylated at multiple sites in all protein domains (Meek and Anderson, 2009, Dai and Gu, 2010). The exact post-translational modifications that occur depend upon the stimulus and can alter p53 stability, activity and the target genes it associates with. For example, p53 is phosphorylated at Serine 15 by ATM and ATR following DNA damage, decreasing p53's association with MDM2 and increasing its association with its coactivator p300/CBP (Shieh et al., 1997, Lambert et al., 1998). Also, phosphorylation of p53 at Serine 46 is required for induction of proapoptotic genes but not involved in regulating cell cycle genes (Oda et al., 2000).

1.5.2 p53 response

p53 is stabilised following stress, whose identity and strength contribute to determining the outcome of p53 induction (Horn and Vousden, 2007, Murray-Zmijewski et al., 2008). p53 mediates this outcome by differential regulation of target genes.

1.5.2.1 Cell cycle arrest

Transient cell cycle arrest allows time for repair before DNA replication and mitosis, thus preventing the transfer of damaged DNA to daughter cells. p53 induces cell cycle arrest primarily through activating transcription of its target gene *CDKN1A*, which encodes for the cell cycle regulator p21 (Vogelstein et al.,

2000). p21 binds to Cyclin E/Cdk2 and Cyclin D/Cdk4 complexes, preventing them from phosphorylating RB. Unphosphorylated RB can bind to E2F transcription factors, preventing them from activating the expression of their target genes. These genes are required for cell cycle progression. Thus, when p21 is present, the cell cycle remains arrested in G1. p53 can also induce G2 arrest by p21 activation. p21 represses Cdc2 activity, which is required for progression into mitosis. GADD45 and 14-3-3, whose genes are also direct targets of p53, also contribute to G2 arrest (El-Deiry, 1998).

1.5.2.2 Senescence

Senescence, an irreversible cell cycle arrest, can also be mediated by p53. As in transient cell cycle arrest, p21 is key to this p53-induced response (Brown et al., 1997). Senescence can be induced by stress, including irreparable DNA damage and oncogene overexpression. Indeed, evidence suggests that induction of senescence may be a key tool in p53's ability to suppress tumour development following oncogene activation (Vousden and Prives, 2009).

1.5.2.3 Apoptosis

In some instances, p53 causes the cell to undergo apoptosis (a form of programmed cell death). This may occur if repair of DNA damage is not successful or if the stress inducing p53 is particularly strong. p53 directly induces the expression of many pro-apoptotic factors, including Puma, Noxa, Bax and Bid (Fridman and Lowe, 2003). In addition, p53 can repress transcription of pro-survival factors Bcl-2, Bcl-xL and Survivin. p53 can also regulate apoptosis independently of transcription, binding to Bak and promoting cytochrome C release from the mitochondria (Yee and Vousden, 2005).

1.5.3 Mechanism of regulating transcription

p53 activates or represses the transcription of its target genes. Several different pathways for this have been identified (Beckerman and Prives, 2010). However, the mechanisms by which p53 regulates its target genes are not fully understood. This is especially true of gene repression.

1.5.3.1 Gene activation

Nearly all genes directly activated by p53 have a p53 response element (p53 RE) nearby. This DNA motif usually consists of two 'half sites' separated by a spacer region of 0-21 base pairs. The half site is 5'-RRRCWWGYYY-3' where R is a purine, W is adenine or thymine and Y is a pyrimidine (el-Deiry et al., 1992). p53 binds at this site and recruits basal transcription factors and coactivators to induce transcription (Laptenko and Prives, 2006). Coactivators include the histone acetyltransferases (HATs) CBP, p300 and PCAF which can acetylate local histones making the environment more conducive to transcription.

1.5.3.2 Gene repression

While it is a paradigm mediator of transcription activation, p53 represses many genes via several mechanisms (Beckerman and Prives, 2010, Rinn and Huarte, 2011). In those genes with a p53 RE, p53 may recruit negative regulators to the promoter. For example, at Nanog and c-Myc genes, HDAC1 is recruited via p53's interaction with mSin3a, thus resulting in an environment less conducive to transcription (Ho et al., 2005, Lin et al., 2005). Alternatively, the binding of p53 to its binding motif can prevent/displace the association of positive regulators with their binding motifs nearby. However, many repressed genes do not have a p53 RE nearby. Repression of these genes may occur due to loss of transcriptional activators to p53-bound genes, so-called 'squelching'. Alternatively in these cases, p53 may interact with other proteins present at the genes and recruit negative regulators. For example, p53 has been shown to interact with NF-Y, a transcription factor, at the cyclin B2 promoter and recruit HDAC1 (Imbriano et al., 2005). As described below, the mechanism by which p53 represses Pol III-mediated transcription is different again.

1.5.4 Regulation of Pol III-mediated transcription by p53

1.5.4.1 Establishing p53 as a regulator of Pol III-mediated transcription

A range of evidence supports a role for p53 in Pol III regulation. MEFs from p53 knockout mice have higher Pol III activity than MEFs from their wild type littermates (Cairns and White, 1998). In fibroblasts from humans with Li Fraumeni syndrome, an inherited mutation in p53, Pol III activity is higher than

in individuals without a mutation (Stein et al., 2002a). A direct effect of p53 on Pol III-mediated transcription was established using *in vitro* and cellular assays. In *in vitro* transcription assays (IVTs), addition of p53 was found to repress expression of Pol III target genes (Chesnokov et al., 1996, Cairns and White, 1998). These genes were from all three Pol III promoter types, including tRNA, 5S, U6, Alu and VA-1, establishing p53 as a general repressor of Pol III-mediated transcription. Similarly, p53 overexpression in p53-null cells repressed VA-1 expression and this effect could be overcome by cotransfection with plasmids encoding E6 or Mdm2, known inhibitors of p53 (Stein et al., 2002a). Several mutated forms of p53 have lost the ability to repress Pol III-mediated transcription (Stein et al., 2002a). Indeed, the most common p53 mutation in human tumours, R175H, converts p53 from a repressor to an activator of VA-1 transcription in SAOS2 cells (Stein et al., 2002a). Interestingly, p53 with a R175P mutation shows reduced ability to repress Pol III-mediated transcription but can still cause growth arrest (Stein et al., 2002a, Crook et al., 1994). This demonstrates that Pol III-repression is not a secondary result of p53-induced cell cycle arrest.

1.5.4.2 Mechanism

p53 is known to interact with TBP at Pol II promoters (where TBP is a subunit of TFIID) (Ko and Prives, 1996). This interaction also appears to be important for the regulation of Pol III-mediated transcription by p53. p53 interacts with TFIIIB, preventing the recruitment of TFIIIB to TFIIIC at Pol III target genes (Chesnokov et al., 1996, Cairns and White, 1998, Crighton et al., 2003). p53 does not bind to Pol III-transcribed genes, with the exception of U6 (Gridasova and Henry, 2005). By binding to TFIIIB, p53 also prevents TFIIIB's interaction with Pol III (Crighton et al., 2003). This may account for p53's ability to repress U6 and other genes with type 3 promoters. Alternatively, repression of this subset of Pol III target genes may occur through a different mechanism. p53 presence correlates with HDAC occupancy of these genes and p53 can also interact with another component of the PIC at type 3 promoters, $SNAP_C$ (Gridasova and Henry, 2005).

p53 mutant studies have shed further light onto the p53-TFIIIB interaction. These show that both N and C termini of p53 are required to repress Pol III activity at genes with type 2 promoters (Stein et al., 2002b). This supports the hypothesis

that p53 interacts with TBP in TFIIIB, as these regions of p53 are required for p53-TBP interactions (non Pol III-specific) (Horikoshi et al., 1995). Unexpectedly for a repression mechanism that does not appear to require p53 binding at target gene promoters, point mutations in the DNA binding domain prevent Pol III repression (Stein et al., 2002a).

Eichhorn and Jackson showed that Brf1 is degraded following p53 induction in the human fibroblast cell line TR9-7, suggesting an additional route by which p53 may regulation Pol III-mediated transcription (Eichhorn and Jackson, 2001).

1.5.4.3 Regulation of p53 regulation of Pol III-mediated transcription

It is unclear whether p53 can always repress Pol III-mediated transcription or only under certain conditions, for example when it is modified at a specific site. The former is supported by the observation that Pol III activity is higher in p53-null MEFs than wild type MEFs (Cairns and White, 1998). This suggests that basal levels of p53 (without a stress-induced modification) are sufficient to repress Pol III. However, while oncogenic stress induces p53-dependent repression of Pol III-mediated transcription (Morton et al., 2007), DNA damage by methyl methanesulfonate (MMS) induces p53 but the repression of Pol III-mediated transcription observed is independent of p53 (J. Morton and R. White, unpublished). This suggests that repression of Pol III-mediated transcription may not be a universal response to p53 induction. This is examined further in Chapter Three.

1.6 AP-1

AP-1 is a dimeric transcription factor with a complex array of roles in proliferation, apoptosis, differentiation and invasion. Its activity is regulated by multiple upstream factors, which are activated in response to extracellular and intracellular signals including growth factors, cytokines and stress (Shaulian and Karin, 2001). Most notable of these upstream factors are the mitogen-activated protein kinases (MAPKs). However, other regulators include casein kinase II (CK2) and glycogen synthase kinase 3 (GSK-3) (Boyle et al., 1991, Karin and Hawkins, 1996). Upon activation, AP-1 regulates transcription of an array of target genes by RNA Polymerase II (Shaulian and Karin, 2002). Among these are the genes encoding the components of TFIIIB, TBP and Brf1 (Fromm et al., 2008, Zhong et al., 2011). This provides the potential for AP-1 to modulate Pol III-mediated transcription. Recent studies have also identified the presence of AP-1 at many Pol III-transcribed genes, suggesting AP-1 may not be a Pol II-specific regulator of transcription (Raha et al., 2010, Zhong et al., 2011).

1.6.1 AP-1 structure

AP-1 (activator protein 1) consists of two proteins from the JUN, FOS, ATF and MAF families. The JUN family members are c-Jun, JunD and JunB. The FOS family includes c-Fos, FosB and Fra-1. ATF2 is a member of the ATF family. AP-1 composition varies depending upon the levels of these proteins in the cell and the ability of the proteins to interact with one another (Table 1.1) [reviewed in detail in (Chinenov and Kerppola, 2001)]. Not all combinations of proteins are possible, for example c-Jun can form a heterodimer with c-Fos (c-Jun/c-Fos) and a homodimer (c-Jun/c-Jun) but a c-Fos/c-Fos dimer is not possible. AP-1 proteins each contain a basic leucine zipper (bZIP) domain through which dimerization occurs (Landschulz et al., 1988). Dimerization brings the DNA binding domains of the proteins together, allowing them to bind at target genes (Ellenberger et al., 1992). Binding usually occurs at AP-1 binding motifs located upstream of target genes. AP-1 binding motifs include the TPA-responsive element (TRE, TGACTCA) and the cAMP-response element (CRE, TGACGTCA) sites. The binding strength of AP-1 to these sites depends upon the dimer composition of AP-1 (Table 1.1). TRE is the most common binding site for the c-

Jun/c-Fos dimer, while CRE is the most common for c-Jun/ATF2 (Nakabeppu et al., 1988, Hai and Curran, 1991).

Once bound at target genes, gene expression is regulated through the transactivation domains of the AP-1 proteins. AP-1 proteins can undergo post-translational modification. For example, the phosphorylation of c-Jun at Serine 63 and Serine 73 in its transactivation domain by c-Jun N-terminal kinase (JNK) promotes its transactivational function (Hibi et al., 1993, Derijard et al., 1994). The transcriptional activity of c-Fos and ATF2 is also enhanced by phosphorylation (Chen et al., 1993, Chen et al., 1996, Gupta et al., 1995). Exactly how AP-1 modulates the transcription of its target genes is not fully defined. Its ability to interact with basal transcription machinery and coactivators, including TBP and p300/CBP, may contribute to regulation (Ransone et al., 1993, Arias et al., 1994). Moreover, c-Jun has recently been shown to recruit the nucleosome remodelling and histone deacetylation (NuRD) repressor complex to the gene encoding stem cell marker lgr5 in a phosphorylation-dependent manner (Aguilera et al., 2011).

AP-1 dimer		AP-1 binding motif preference
c-Jun	c-Jun	TRE>CRE
	JunB	
	JunD	
	c-Fos	
	FosB	
	Fra1	
	ATF2	CRE>TRE
c-Fos	c-Jun	TRE>CRE
	JunB	
	JunD	
	ATF2	CRE>TRE

Table 1.1 AP-1 dimers and their binding preferences

The table above shows some of the proteins that can form AP-1 dimers with c-Jun or c-Fos. Their preference for binding to specific AP-1 binding motifs is described in the third column. TRE = TPA-responsive element, CRE = cAMP-response element. For simplicity, AP-1 proteins not discussed in the main text are not included. Adapted from (Eferl and Wagner, 2003)

1.6.2 AP-1 function

The cellular outcome of AP-1 activity is dependent upon which of its target genes it regulates. This is determined by dimer composition (itself determined by levels of the AP-1 proteins), post-translational modification of the AP-1 proteins and the binding motifs present at the target genes. While binding motifs are constant at a single gene, protein levels and post-translational modification are subject to regulation by multiple upstream pathways and, thus, AP-1 is regulated in response to the cellular environment (Shaulian and Karin, 2001). For example, c-Fos expression is upregulated by ERK following 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment, while JNK phosphorylates c-Jun in response to UV (Whitmarsh et al., 1995, Hibi et al., 1993, Derijard et al., 1994). However, it is the combination of proteins making up the AP-1 dimer that appears to be most important in determining the target gene recognised (van Dam and Castellazzi, 2001). The multiple possible dimer combinations account for the complexity of AP-1 function, for example it has both pro- and anti-proliferative roles (Shaulian and Karin, 2002). c-Jun and c-Fos have undergone the most extensive functional analysis and it is these AP-1 factors whose function will be addressed here, as they have been identified at tRNA genes and other Pol III-transcribed genes (Raha et al., 2010). Historically, despite their known requirement to form a dimer, each protein's function has been studied separately due to the difficulty in determining the role of a transient dimer.

1.6.2.1 Proliferation

AP-1 dimers have both pro- and anti-proliferative effects dependent primarily upon their composite proteins. c-Jun has pro-proliferative effects. c-Jun null mouse embryonic fibroblasts (MEFs) are unable to proliferate (Schreiber et al., 1999, Wisdom et al., 1999). As part of AP-1, c-Jun can activate Cyclin D1, allowing the G1-S phase transition (Wisdom et al., 1999). It also inhibits p53 expression and, thus, prevents p21-induced arrest (Schreiber et al., 1999). The role of JunB and JunD in proliferation is less well defined. However, evidence suggests JunB has a negative impact on proliferation, while the impact of JunD is context-dependent (Eferl and Wagner, 2003). c-Fos null fibroblasts are able to proliferate normally, as are FosB null fibroblasts (Brusselbach et al., 1995, Gruda et al., 1996). However, when both c-Fos and FosB are depleted, proliferation is

significantly reduced (Brown et al., 1998). This suggests an ability to functionally compensate for one another to some degree.

1.6.2.2 Apoptosis

AP-1 has both pro– and anti-apoptotic activity. c-Jun can positively regulate Bim, a pro-apoptotic protein, and it may be this that contributes to c-Jun's ability to drive apoptosis in neuronal cells (Whitfield et al., 2001). Apoptosis may also be induced by Fas ligand (FasL), expression of which can be induced by the c-Jun/c-Fos dimer (Kasibhatla et al., 1998). On the other hand, c-Jun can repress p53 activity and thus prevent p53-induced apoptosis (Schreiber et al., 1999).

1.6.2.3 Other functions

AP-1 activity contributes to many other cellular processes including angiogenesis, development, invasion and differentiation. They have been described in detail elsewhere (Jochum et al., 2001, Shaulian and Karin, 2002, Ozanne et al., 2006).

1.6.3 AP-1 and cancer

With functions in a number of processes known to contribute to cancer development upon deregulation, it is not surprising that AP-1 is implicated in tumour development (Young et al., 2003, Jochum et al., 2001). But with its complex role in these processes, its role in cancer is equally complex. c-Jun and c-Fos were first identified due to their constitutively active mutants found in the Finkel–Biskis–Jinkins osteosarcoma virus and avian sarcomavirus 17 respectively, v-Jun and v-Fos (Vogt, 2002). These proteins are capable of the transforming the infected cell. c-Jun and c-Fos also have oncogenic capacity. c-Jun activation is important in skin and liver tumour development, while c-Fos is required for osteosarcoma development in mice (Young et al., 1999, Eferl et al., 2003, Wang et al., 1991, Grigoriadis et al., 1993). c-Fos deletion cannot prevent development of skin tumours in a mouse tumour model, but it does prevent progression to malignancy (Saez et al., 1995). c-Fos also functions as a tumour suppressor. Rhabdomyosarcomas arise when c-Fos is deleted in p53 null mice, possibly resulting from impaired apoptosis (Fleischmann et al., 2003). JunB has

an anti-oncogenic capacity that could derive from its competition with c-Jun (Eferl and Wagner, 2003).

1.7 JNKs

The c-Jun N terminal kinases (JNKs) are one subfamily of the mitogen-activated protein kinase (MAPK) family (Johnson and Lapadat, 2002). The MAPK family also includes the ERK and p38 subfamilies. MAPKs are one component of signalling cascades by which extracellular stimuli can be relayed through the cell to influence cellular processes. These processes include proliferation, cell growth, differentiation, migration and apoptosis. The MAPKs are highly conserved from yeast to humans, highlighting their important role in cellular function (Widmann et al., 1999). JNKs can regulate Pol III-mediated transcription by regulating the expression of TFIIIB subunits (Zhong et al., 2007, Zhong and Johnson, 2009, Zhong et al., 2011).

1.7.1 Structure and regulation of JNKs

The human genome contains three JNK genes. JNK1 and JNK2 are expressed ubiquitously, while JNK3 expression is limited to the brain, heart and testis (Mohit et al., 1995). The functional roles of JNK1 and JNK2 have not been fully differentiated. It seems they have some shared roles, particularly in development, and some protein specific roles (Bode and Dong, 2007, Chen et al., 2009). JNK1 and JNK2 are each expressed as four different isoforms through alternative splicing, adding further complexity to JNK activity within the cell (Gupta et al., 1996). JNK1α1 and JNK1ß1 are both 46kDa and share the same final exon but each has a different exon 6 (one has exon 6α and the other exon 6ß). JNK1α2 and JNK1ß2 are 54kDa and share an alternative final exon, but again differ in exon 6. JNK2 undergoes similar splicing. The importance of different isoforms has not been determined. JNKs are activated by mitogenic factors, cytokines and stress signals and influence proliferation, development and apoptosis through phosphorylation of a range of targets (Davis, 2000, Bogoyevitch and Kobe, 2006). Stimuli include TNFα and UV light (Rosette and Karin, 1996). JNK kinase activity requires its ATP-binding pocket, catalytic loop and substrate binding site. JNKs are activated by dual phosphorylation at a Thr-X-Tyr motif in the activation loop of JNK by MKK4 (mitogen-activated protein

kinase kinase 4) and MKK7 (Lawler et al., 1998). These kinases specifically phosphorylate JNK and can be activated by multiple MAPKKKs (MAPK kinase kinase) (Figure 1.7) (Davis, 2000). MAPKKK activity is stimulus-dependent. Some stimuli may activate MAPKKKs that can phosphorylate MKK4/7, while other stimuli may not activate these MAPKKKs. Hence, JNK activation is dependent upon the stimuli present.

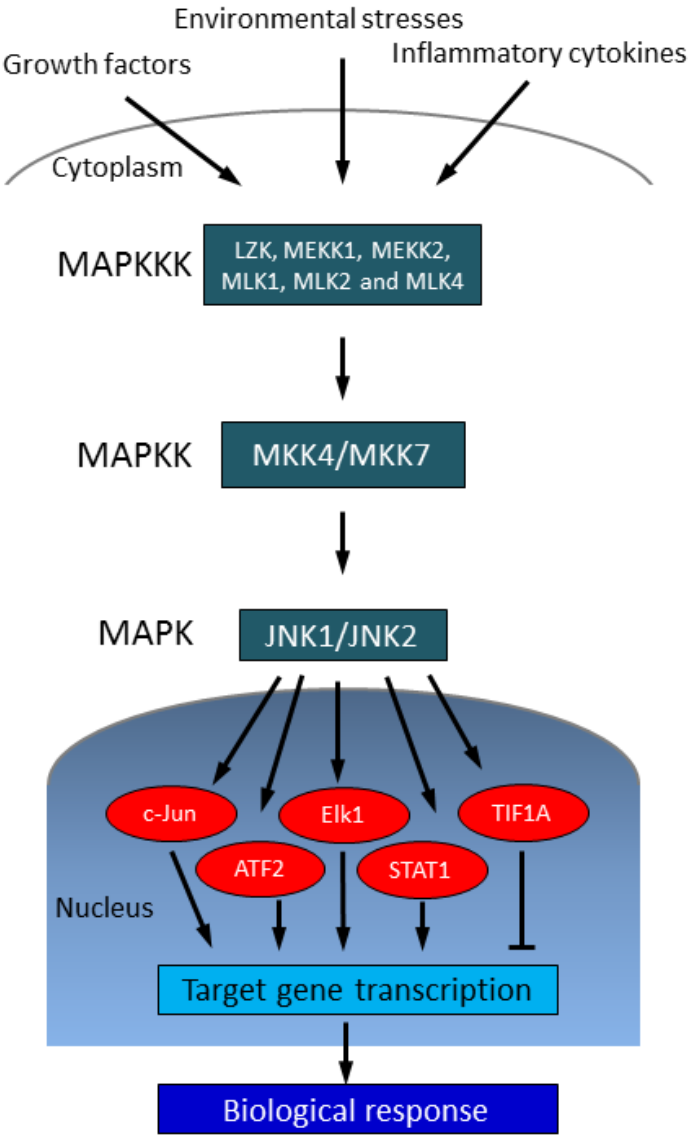

Figure 1.7 The JNK pathway

The diagram above illustrates the JNK pathway, from extracellular stimuli to biological response, in simplified form. Extracellular signals activate MAPKKKs (intermediate pathway not shown). These phosphorylate MKK4 and MKK7, resulting in the phosphorylation of JNKs. Once activated, JNK phosphorylates target proteins, including c-Jun and ATF2. The activity of the target protein is altered and can, in turn, alter the transcription of its target genes. This mediates the biological response. Possible biological responses include proliferation and apoptosis. Adapted from (Wagner and Nebreda, 2009)

1.7.2 JNK substrates

JNK has many phosphorylation targets, including both nuclear and cytoplasmic proteins, that have roles in many processes. They have been reviewed in detail elsewhere (Bogoyevitch and Kobe, 2006). Highlighted below are those that have known links with Pol III-mediated transcription.

1.7.2.1 c-Jun

JNKs were first identified because of their ability to phosphorylate c-Jun (Hibi et al., 1993, Derijard et al., 1994). Phosphorylation occurs at serines 63 and 73 and threonines 91 and 93 and a JNK binding domain in c-Jun is required for phosphorylation to occur (Hibi et al., 1993, Derijard et al., 1994, Morton et al., 2003). Phosphorylation of c-Jun at serines 63 and 73 promotes its ability to activate a range of target genes, many involved in cell proliferation and apoptosis (see Section 1.6.2). JNK1 and JNK2 appear to have different roles in regulating c-Jun. JNK1 is most efficient at phosphorylating c-Jun, while JNK2 has been described as a negative regulator of c-Jun (Gupta et al., 1996, Sabapathy et al., 2004). It promotes c-Jun degradation when bound to c-Jun in its unphosphorylated form (Sabapathy et al., 2004). Jun family members JunD and JunB are also JNK substrates (Yazgan and Pfarr, 2002, Li et al., 1999). Like c-Jun, phosphorylation increases their transcriptional activity.

1.7.2.2 ATF2

ATF2 is another AP-1 protein targeted for phosphorylation by JNK. It is phosphorylated at Thr69 and Thr71 in its transactivation domain, enhancing its transactivational activity (Gupta et al., 1995). As part of an AP-1 dimer, ATF2 plays an important role in the regulation of cell development, proliferation and death (Lau and Ronai, 2012).

1.7.2.3 p53

p53 can be phosphorylated by JNK at Thr81 in rat cells (Buschmann et al., 2001). This modification stabilises p53 and, thus, upregulates its activity. The authors suggest that phosphorylation at this site is required for p53 stabilisation. More

recently, JNK1 has been shown to negatively regulate p53 (Tafolla et al., 2005). The function of p53 is described in detail above (Section 1.5)

1.7.2.4 c-Myc

JNK phosphorylates c-Myc at Ser61 and Ser71 (Noguchi et al., 1999). This phosphorylation is associated with an increase in c-Myc-mediated apoptosis and appears to be primarily carried out by JNK1 (Noguchi et al., 1999). c-Myc contributes to apoptosis in addition to its key role in inducing proliferation (Grandori et al., 2000).

1.7.2.5 Elk1

Elk1 is a member of the Ets family of transcription factors (Sharrocks, 2001). Two sites in it transactivation domain, Ser383 and Ser389, are phosphorylated by JNK (Whitmarsh et al., 1995, Yang et al., 1998). Phosphorylation increases the transcriptional activity of Elk1, whose targets genes include TFIIIB subunits TBP and Brf1 (Zhong et al., 2007, Zhong and Johnson, 2009).

1.7.2.6 STAT1

JNK activation can also result in the phosphorylation and activation of STAT1 at Ser727 (Zhao et al., 2005). Another member of the STAT family member, STAT3, is phosphorylated by JNK (Zhang et al., 2001). STAT proteins are activated in response to growth factors and cytokines and contribute to the regulation of many cellular processes including differentiation, proliferation and apoptosis (Calo et al., 2003).

1.7.2.7 TIF-IA

TIF-IA is a Pol I-specific transcription factor. It binds to the TBP-containing factor SL1 on the promoter of 45S rRNA gene and recruits Pol I (White, 2008). Following JNK activation by stress, JNK phosphorylates TIF-IA at Thr200 (Mayer et al., 2005). This reduces TIF-IA's interaction with SL-1 and Pol I, resulting in reduced Pol I-mediated transcription and, thus, reduced rRNA levels (Mayer et al., 2005).

1.7.3 JNK and cancer

JNK mutations are uncommon in cancers, however JNK activation has been found in several human tumour types, including in breast cancer, osteosarcoma and hepatocellular carcinoma (HCC) (Wang et al., 2003, Papachristou et al., 2003, Hui et al., 2008, Chang et al., 2009). They play a complex role in cancer, acting in both positive and negative roles depending on cancer type (Bode and Dong, 2007). In HCC, two independent studies found that more than 50% of HCC samples had upregulated JNK1 (but not JNK2) activity (Hui et al., 2008, Chang et al., 2009). Knockout of JNK1 in mice results in reduced size and number of chemically-induced mouse liver tumours, compared to the wild type (Hui et al., 2008). In addition, JNK1 was recently identified as an important kinase in tobacco-induced lung cancer in mice (Takahashi et al., 2010). Conversely, JNK1 may have a tumour suppressive role in TPA-induced skin carcinogenesis, where JNK1-/- mice are more susceptible to tumour development than their WT or JNK2-/- littermates (She et al., 2002). JNK activity may be required for c-Jun's transforming activity, for example JNK activation and c-Jun phosphorylation have been demonstrated to be required for transformation by oncogenic Ras in mouse models (Smeal et al., 1991, Behrens et al., 2000).

1.8 PhD Objectives

Regulation of Pol III-mediated transcription is essential to ensure a cell has sufficient Pol III products and to prevent the transcription of Pol III target genes from consuming too much of the cell's resources. 5S rRNA and tRNAs contribute significantly to protein synthesis, alongside other Pol III products. Protein synthesis is essential for cell growth and, consequently, cell proliferation. Pol III-mediated transcription is regulated by many proteins, however how these regulators work is not fully understood and further regulators remain to be identified. Understanding the regulation of Pol III-mediated transcription is given further importance by the increase in Pol III-mediated transcription that is associated with cancer.

The aim of this thesis was to investigate the regulation of Pol III-mediated transcription by three proteins, all with a known association to cancer. In Chapter Three, the role of p53 in regulating Pol III activity in response to DNA damage is investigated. Chapter Four analyses the occupancy of c-Jun and c-Fos at tRNA genes and investigates whether they may contribute to Pol III-mediated transcription. In Chapter Five, the objective was to determine whether JNK may directly regulate Pol III-transcribed genes, independently of its ability to regulate TFIIIB levels.

2 Materials and Methods

2.1 Cell culture

U2OS cells (a human osteosarcoma cell line) were grown in Dulbecco's Modified Eagle Medium (DMEM) (4.5g/l glucose, 110mg/l sodium pyruvate) (Gibco, Life Technologies) supplemented with 10% foetal bovine serum (FBS) (PAA Laboratories), 20mM L-Glutamine (Gibco, Life Technologies), 100U/ml penicillin and 100µg/ml streptomycin (Pen Strep, Life Technologies) in a humid incubator at 37°C with 5% CO_2. NARF2 cells were provided by Prof. Gordon Peters, London Research Institute. These are U2OS cells expressing human $p14^{ARF}$ from an IPTG-regulated promoter (Stott et al., 1998). NARF2 were maintained in the supplemented media described above, with 300µg/ml G418 sulphate solution (Formedium) and 150µg/ml Hygromycin B (Life Technologies) to maintain selection. U2OS-pRS-Scr, U2OS-pRS-p53, RKO-pRS-Scr, RKO-pRS-p53, MCF7-pRS-Scr and MCF7-pRS-p53 were provided by Prof. Kevin Ryan, Beatson Institute (Crighton et al., 2006, Crighton et al., 2007). These cells stably express non-silencing shRNA or an shRNA targeting p53 under a constitutive promoter. Reselection was carried out approximately once a month by addition of 1ug/ml puromycin to the media. c-Jun-null mouse embryonic fibroblasts (MEFs) stably expressing c-Jun or c-Jun mutants (c-Jun S63/73A, c-Jun Δ284-286, v-Jun, TAM67-GFP) from a pBabe vector were provided by Prof. David Gillespie, Beatson Institute (MacLaren et al., 2004) and cultured in supplemented media as described above.

Passaging of cells was carried out every 2-3 days. Media was removed and replaced with 0.25% trypsin (Gibco) in PBS-EDTA to disengage cells from plate. Once this was complete, trypsin was quenched by addition of fresh media. Cells were then transferred to new tissue culture dishes.

Cryofreezing of cells was carried out for long-term storage. Trypsinised cells were pelleted and resuspended in 1ml freezing media (70% FBS, 20% DMEM without supplements, 10% dimethyl sulfoxide (DMSO)) per near confluent T75 tissue culture flask. Cells were transferred to cryo-vials in a cryo-freezing container containing room temperature isopropanol. This was placed in at -80°C overnight. Vials were then transferred to a liquid nitrogen freezer. To thaw

cells, they were placed at 37°C. Cells were then added to fresh media and pelleted by centrifugation. The supernatant was discarded and pellet was resuspended in fresh media.

2.1.1 UV exposure

Cells were grown to approximately 80% confluency in tissue culture dishes, then washed with PBS. PBS was removed and plates placed in UV irradiator without lids. 50J/m^2 UVB (312nm) (UV Crosslinker XL-1000, Spectronics Corporation) or 50J/m^2 UVC (254nm) (UV Crosslinker CL-E508, UVITEC Cambridge) was used. Fresh media was added directly after UV exposure. Cells were harvested 8 hours later.

2.1.2 Chemical compound addition

IPTG (Isopropyl ß-D-1-thiogalactopyranoside) (Sigma) was dissolved in sterile water to 1M and stored at 4°C. ARF expression was induced in NARF2 cells by treatment with 1mM IPTG for 24 hours. Doxorubicin (Adriamycin, Merck Millipore) was dissolved in H_2O to stock concentration of 10mg/ml. Final concentrations used as described in text. SP600125 (JNK Inhibitor II, Merck Millipore) was dissolved in DMSO to make a 20mM stock solution and stored at -20°C. Used at final concentration as described in the text. JNK-IN-8 (JNK inhibitor XVI, Merck Millipore) was dissolved in DMSO to make a 100mM stock solution and stored -20°C. Before treatment of cells, it was further diluted in DMSO for each concentration required, so that 3µl of DMSO was added per 1ml of media.

2.1.3 Transient transfection

5x10^5 U2OS cells were plated in 10cm dishes 48 hours before transfection. pEGFP-C3 (empty vector) and pEGFP-C3-TAM67 were transfected using Lipofectamine 2000 (Life Technologies). 20µl of Lipofectamine 2000 and 5µg of plasmid were incubated separately in 1.5ml warm DMEM (no supplements) for 5 minutes at room temperature. These were then combined and incubated for a further 20 minutes to allow complex formation. The media on the plates of cells to be transfected was replaced with 7ml of DMEM (10% FBS, 1% L-glutamine, no antibiotics). The transfection complex containing media was then added. 24

hours later GFP was observed in 50-80% of cells and cells were harvested for protein.

2.2 Protein extract preparation

Cells were washed twice with cold PBS. They were then scraped into a small volume of PBS, transferred to a microcentrifuge tube and centrifuged to pellet cells (2min, 400*g*, 4˚C). The pellet was resuspended in low salt microextraction buffer (20mM HEPES, 150mM NaCl, 0.2mM EDTA, 25% glycerol, 1x Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific), 0.5% Triton X-100) of approximately equal volume to the pellet and incubated on ice for 10 minutes. The sample was then freeze-thawed 3-4 times to mechanically disrupt the cells. The extracts were then centrifuged at 16,000*g* at 4˚C for 15 minutes to pellet any cell debris. Supernatant was collected. The protein concentration of the cell extract was determined by spectrophotometry (Biophotometer, Eppendorf) using Bio-Rad Protein Assay reagent (Bio-Rad). A standard curve was produced using BSA and protein extract absorbance was compared to this to determine concentration. Extracts were used for western blot or immunoprecipitation experiments.

2.3 SDS-PAGE gel electrophoresis

Protein samples were separated according to size using SDS-polyacrylamide gel electrophoresis. 12% vertical polyacrylamide gels (resolving gel: 375mM Tris pH 8.8, 0.1% SDS, stacking gel: 125mM Tris pH6.8, 0.1% SDS) were poured and run with mini-Protean gel system (Bio-Rad). Electrophoresis was performed in SDS running buffer (76.8mM glycine, 10mM Tris pH8.3, 0.1% SDS). Alternatively, 12% or 4-12% Novex Bis-Tris precast gels were used with Novex NuPAGE SDS-PAGE Gel System (Life Technologies). For these gels, electrophoresis was carried out in 1x MOPS SDS Running Buffer (Life Technologies).

10µg of protein extract was denatured by addition of 5µl of 4x SDS-PAGE loading buffer (125mM Tris pH6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.04% bromophenol blue) and heating for 2 minutes at 100˚C. The sample was then loaded into gel and electrophoresis performed at 180V until required separation, as determined by Full-Range rainbow ladder RPN800E (GE Healthcare).

2.4 Western blotting

Protein was transferred from polyacrylamide gel to nitrocellulose membrane using BioRad wet transfer system. Transfer was carried out in transfer buffer (76.8mM glycine, 10mM Tris pH8.3, 20% methanol) at 4°C for 2 hours at 100V or overnight at 30V. Nitrocellulose was then blocked with blocking reagent, TBS-T (32.5mM Tris, 150mM NaCl, 0.2% Tween) with 5% milk or 5% BSA (when probing for phosphorylated proteins) for a minimum of 30 minutes. Primary antibody (Table 2.1) was added in 1% milk or 5% BSA for a minimum of 1 hour at room temperature. Blots were then washed three times with TBS-T to remove unbound antibody. An appropriate HRP-labelled secondary antibody (Dako) was added for a minimum of 1 hour at room temperature. For Western blots carried out with immunoprecipitated samples, HRP-labelled light chain specific-secondary antibodies (Jackson) were used. Following incubation with secondary antibody, membrane was washed with TBS-Tween (3x5 minutes, 2x15 minutes) and TBS (1x 5 minutes). The presence of secondary antibody was visualised by enhanced chemiluminescence using Western Lightening ECL reagents (Perkin Elmer), followed by exposure of membranes to autoradiograph film.

Protein recognised	Supplier	Antibody	Dilution
HSP70	Santa Cruz Biotechnology	sc-24	1:2000
Tubulin	Sigma-Aldrich	T-9026	1:10,000
Actin	Santa Cruz Biotechnology	sc-1615	1:5000
p53	Santa Cruz Biotechnology	sc-126	1:1000
Phospho-p53(Ser9)	Cell Signalling Technology	#9288	1:1000
Phospho-p53(Ser15)	Cell Signalling Technology	#9284	1:1000
Phospho-p53(Ser20)	Cell Signalling Technology	#9287	1:1000
Phospho-p53(Ser46)	Cell Signalling Technology	#2521	1:1000
Phospho-p53(Ser392)	Cell Signalling Technology	#9251	1:1000
Acetyl-p53 (Lys305)	Biolegend	614102	1:1000
Acetyl-p53 (Lys373)	Millipore	06-916	1:50,000
Acetyl-p53 (Lys382)	Cell Signalling Technology	#2525	1:1000
Acetyl-p53 (Lys386)	Abcam	ab52172	1:500
$p14^{ARF}$	Non-commercial (K. Vousden)	4037	1:1000
Brf1	Non-commercial (R. White)	482	1:1000
Phospho-Brf1 (Thr145)	Non-commercial (R. White)	SK2839	1:1000
TBP	Abcam	ab818	1:3000
TFIIIC110	Santa Cruz Biotechnology	sc-81406	1:1000
c-Jun (DNA binding domain)	Santa Cruz Biotechnology	sc-44	1:1000
c-Jun (aa1-79)	Santa Cruz Biotechnology	sc-1694	1:1000
c-Jun (N-terminal)	Cell Signalling Technology	#9165	1:1000
Phospho-c-Jun (Ser 63)	Santa Cruz Biotechnology	sc-822	1:1000
Phospho-c-Jun (Ser 63/73)	Santa Cruz Biotechnology	sc-16312	1:1000
c-Fos	Santa Cruz Biotechnology	sc-447	1:1000
c-Fos	Santa Cruz Biotechnology	sc-52	1:1000
JNK	Santa Cruz Biotechnology	sc-474	1:1000
JNK	Cell Signalling Technology	#9252	1:1000
Phospho-JNK (Thr183/Tyr185)	Cell Signalling Technology	#9251	1:1000

Table 2.1 Primary antibodies used for western blot analysis

2.5 Protein Immunoprecipitation

The method of immunoprecipitation chosen was dependent whether cross-linking of antibody to beads was required.

2.5.1 IP with crosslinking

30µl of Protein A or G coated magnetics beads (Dynabeads, Life Technologies) were incubated with 5µg of antibody or appropriate IgG to a total volume of 255µl in PBS/NP-40 (PBS, 0.015% NP-40) for a minimum of 1 hour at room temperature. The beads were then washed three times with conjugation buffer (20mM Sodium Phosphate, 0.15M NaCl (pH 7-9)) and incubated in 150µl of 5mM BS^3 (bissulfosuccinimidyl suberate, Thermo Scientific) for 30 minutes at room temperature to crosslink antibody to beads. The crosslinking reaction was then quenched by addition of 7.5µl of Tris(pH 7.5). Subsequently, the beads were washed 3 times with PBS/NP40 and resuspended in 200µl PBS/NP40. 250-500µg of protein and 100x protease and phosphatase inhibitor cocktail (PPIC, to a final concentration of 1x, Fisher Scientific) were added. Following 3 hour incubation on a rotating wheel at 4°C, beads were washed with PBS/NP40 4 times. Bound protein was eluted by incubation in 2xSDS-PAGE loading buffer at 80°C for 5 minutes in shaking incubator. Samples were then applied to magnet (to separate the beads) and supernatant removed to fresh tubes. 10% of input protein extract was taken and an equal volume of 4x SDS-PAGE loading buffer was added followed by heating at 80°C for 5 minutes in shaking incubator. IP and input samples were then heated at 100°C for 2 minutes and analysed by gel electrophoresis and western blotting.

2.5.2 IP without crosslinking

When no crosslinking was required, 5µg of primary antibody (Table 2.2) was incubated with 250-500µg protein extract and PPIC (to 1x concentration) for 3 hours at 4°C on rotating wheel. 30µl Protein A or G magnetic beads (Dynabeads, Life Technologies) were washed 3 times with PBS/NP40 (PBS, 0.015% NP-40) and blocked in 50µl 1mg/ml BSA (made up in PBS/NP-40) for 15 minutes. Blocking buffer was then removed and antibody/protein complexes were added to beads. Following 2 hour incubation at 4°C on rotating wheel, beads were washed 4 times with PBS/NP40 and the bound protein was eluted by incubation in 2xSDS-

PAGE loading buffer at 80°C for 5 minutes in shaking incubator. 10% of input protein extract was taken and an equal volume of 4x SDS-PAGE loading buffer was added followed by heating at 80°C for 5 minutes in shaking incubator. IP and input samples were then heated at 100°C for 2 minutes and analysed by gel electrophoresis and western blotting.

Protein recognised	Supplier	Antibody
IgG (mouse)	Sigma-Aldrich	
IgG (rabbit)	Sigma-Aldrich	
c-Jun (DNA binding domain)	Santa Cruz Biotechnology	sc-44
TFIIIC110	Santa Cruz Biotechnology	sc-81406
GFP	Life Technologies	A6455
JNK	Santa Cruz Biotechnology	sc-474

Table 2.2 Antibodies used for immunoprecipitation

2.6 RNA extraction

Cells were washed 2-3 times with cold PBS and scraped into 1ml TRIzol (Life Technologies) (per 10cm plate). Samples in TRIzol were then snap frozen and stored at -80°C until time of RNA extraction. At this time, samples were thawed at room temperature and 200µl chloroform was added per 1ml TRIzol. Sample was vortexed thoroughly and allowed to stand briefly before centrifugation at 16,000*g* for 15min at 4°C. Of the resulting three phases of liquid, the top phase was carefully removed to a fresh eppendorf tube. To this, 0.5ml isopropanol was added (per 1ml of TRIzol) and mixed gently by inverting tube several times. The sample was then centrifuged for 10min at 16,000*g* at 4°C and supernatant was discarded. The remaining pellet was washed with 1ml 75% ethanol. The tube was then centrifuged for 5min at 9000*g* at 4°C. The ethanol was removed and pellet allowed to air dry. As soon as pellet went transparent, it was resuspended in 20-50µl of DEPC-treated water and placed at 55°C in shaking incubator for 15 minutes to ensure complete rehydration. RNA was quantified using a Nanovue spectrophotometer (GE Healthcare) and stored at -80°C.

2.7 cDNA preparation

Two alternative reverse transcription methods were used to prepare cDNA from RNA. SuperScript III (Life Technologies) was used for Figure 3.4. QuantiTect Reverse Transcription kit (Qiagen) was used for all other reverse transcription.

SuperScript III 200ng of RNA and 2µl of 10x hexanucleotide mix (Roche) were combined and total volume made up to 25µl with nuclease-free water. This was then incubated at 80°C for 10min and transferred directly onto ice. 8µl First Strand Buffer (Life Technologies), 4µl 0.1M DTT, 2µl 10mM dNTP mix and 1µl SuperScript III reverse transcriptase (RT) were then added and the reaction mixture was incubated at 50°C for 1 hour. RT inactivation was carried out by incubation at 70°C for 15min. Alongside, cDNA was also prepared from 400ng of RNA (to analyse whether reaction was quantitative). To determine whether the RNA was contaminated with genomic DNA, reactions lacking RT were also carried out. cDNA was stored at -20°C.

Quantitect Reverse Transcription Reverse transcription was carried out according to manufacturer's instructions. 10ng of RNA was used for each sample. In addition, reverse transcription of 20ng and 5ng of RNA was carried out to test whether the reaction was quantitative. No RT controls were used to check for genomic DNA contamination of RNA samples. cDNA was stored at -20°C.

2.8 Chromatin Immunoprecipitation

Cells were grown to about 80% confluency in 10cm dishes and crosslinking of chromatin was carried out by addition of formaldehyde to the media to a final concentration of 1% and incubation for 7-10 minutes at room temperature. To quench crosslinking reaction, glycine was added to the final concentration of 0.125M at room temperature for 5 minutes. Plates were placed on ice and most of the media was discarded. Cells were scraped into remaining media while maintained on ice. Cells in the resulting suspension were then pelleted by centrifugation at 400*g* for 5 minutes at 4°C. This pellet was washed by resuspension in PBS + 0.5% NP-40, followed by centrifugation. This wash step was repeated once more and pellet was snap frozen and stored at -80°C until required.

Cell pellet was thawed and washed once with PBS + 0.5% NP-40 and spun down (400*g* for 5 minutes at 4°C). Pellet was then resuspended in cold high salt buffer (PBS, 1M NaCl, 0.5% NP-40) and incubated for 30 minutes on ice. This was then centrifuged at 400*g* for 5 minutes at 4°C and the resulting pellet washed once with PBS + 0.5% NP-40 as above. It was then resuspended in low salt buffer (LSB) (0.1M NaCl, 10mM Tris, 1mM EDTA, 0.1% NP-40) and incubated for 30 minutes on ice. The cells were then pelleted, supernatant discarded and resuspended in 600µl LSB. This suspension was then passed through a 26G needle three times. The volume was then made up to 2.7ml with LSB and was mixed with 300µl of 20% (w/v) N-laurylsarcosine sodium salt. The suspension was then transferred to a 50ml Falcon tube containing 40 ml LSB/100mM sucrose, carefully to ensure the suspension sat at the top. This was then centrifuged at 4000g for 10 min at 4°C. Following this, the pellet was resuspended in 2ml TE (10mM Tris pH8, 1mM EDTA) and passed through another sucrose cushion. The pellet was then resuspended in 2ml TE and mixed with 200µl of 11x NET buffer (550mM Tris pH7.4, 1.65M NaCl, 5.5mM EDTA, 5.5% NP-40). This was then sonicated at 4°C in a water bath sonicator (Biorupter, Diagnode) for 10 minutes (30sec on/30sec off). Ice was replaced and this was repeated a further two times. The resulting suspension was centrifuged at 16000*g* for 15min at 4°C to pellet the cellular debris.

The supernatant (containing sonicated chromatin) was aliquoted evenly into microcentrifuge tubes, according to the number of IPs to be carried out. 10% of the volume of an aliquot was taken for the 10% input sample. 2-5µg of antibody (Table 2.3) was added to each tube and they were incubated overnight at 4°C on a rotor wheel. The following day, 50µl protein G sepharose beads (for Pol III and histone ChIPs) or 30µl protein G magnetic beads (other ChIPs) were washed three times in 1xNET buffer (made up with TE) and added to each IP. These were incubated for 2 hours at 4°C. The beads were then washed twice with cold RIPA buffer (50mM Tris-HCl pH8.0, 150mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40), twice with cold LiCl buffer (10mM Tris-HCl pH8.0, 250mM LiCl, 0.5% deoxycholate, 0.5% NP-40, 1mM EDTA) and twice with cold TE. For elution from sepharose beads, the beads were incubated in 400µl of TE/1%SDS for 10 minutes at room temperature. 10% input was made up to 400µl with TE/1%SDS. 20µg of RNase A (Life Technologies) was added to all samples and they were then

incubated at 37°C for 1 hour. Proteinase K was added to the concentration of 0.125mg/ml and samples incubated at 42°C overnight. DNA was purified using Qiagen PCR Purification kit (according to manufacturer's instructions). For elution from magnetic beads and DNA purification (adapted from (Nelson et al., 2006)), 50µl of 10% Chelex 100 (BioRad) was mixed with beads or 10% input and heated at 95°C for 15 minutes. Samples were cooled and treated with 0.125mg/ml Proteinase K for 30 minutes at 55°C in heated shaker. Samples were then heated to 95°C for a further 10 minutes to inactivate Proteinase K. Samples were made up to 250µl total volume with H_2O (Milli-Q purified), vortexed for 10 seconds and centrifuged at 4°C for 1min at 12000*g*. 100µl of supernatant was carefully removed to a fresh tube and the remaining supernatant and pellet was discarded. All ChIP samples were stored at -20°C before analysis by qPCR.

Protein recognised	**Supplier**	**Antibody**
TAF I 48	Santa Cruz Biotechnology	sc-6571
RPC155 (Pol III subunit)	Non-commercial (R. White)	1900
Brf1	Non-commercial (R. White)	127
TBP	Abcam	ab818
Histone H3	Abcam	ab1791
HDAC1	Santa Cruz Biotechnology	sc-7872
HDAC2	Santa Cruz Biotechnology	sc-7899
Acetyl-histone H3 (pan)	Millipore	06-599
Phospho-histone H3 (Ser10)	Santa Cruz Biotechnology	sc-8656
JNK	Santa Cruz Biotechnology	sc-474

Table 2.3 Antibodies used in chromatin immunoprecipitation

2.9 qPCR

Gene expression analysis (RT-qPCR) and ChIP analysis (qPCR) were carried out in 96 well plates using C1000 Thermocycler CFX96 Real Time System (Bio-Rad).

Total reaction volume per well of 10µl. For gene expression analysis, 5µl PerfeCTa SYBR Green Fast Mix (Quanta BioSciences), 0.25µl of 10µM forward primer, 0.25µl of 10µM reverse primer, 0.5µl cDNA and 4µl Milli-Q filtered H_2O. For ChIP analysis, 0.5µl of DNA was used after purification by Qiagen Purification kit or 2.5µl of sample was used after purification by Chelex (H_2O volume altered to maintain 10µl reaction volume). For gene expression analysis, a two-step protocol was used, 5 min at 95°C followed by 35 cycles of [95°C for 30sec, 60°C (58°C for c-Jun primers) for 20sec]. For ChIP analysis, a three step protocol was used, 5 min at 95°C followed by 35 cycles of [95°C for 30 sec, 60°C for 20 sec, 72°C for 10 sec]. Samples were tested in duplicate and a standard curve made with combined samples (cDNA analysis) or input DNA (ChIP analysis) was used to determine efficiency of the PCR reaction in each sample. A well containing no sample (no template control, NTC) was included for each primer set to test for primer dimer formation. Data was collected and analysed in BioRad CFX Manager software (Version 2.1.1022.0523). Further analysis was carried out in Microsoft Excel. In gene expression analysis, the average starting quantity (SQ) of duplicates was determined and normalised to the average of loading control (ARPP P0). For ChIP samples, the signal was quantified as percentage of signal in input. % input was calculated with the formula [(IP/input)x100] – [(TAF_I48 IP/input)x100].

Target cDNA	Primer sequences
ARPP P0	F GCACTGGAAGTCCAACTACTTC R TGAGGTCCTCCTTGGTGAACAC
$tRNA^{Leu}$	F ATGGCGAGTGGTCTAAGG R ACCAGAAGACCCGAACACAG
$tRNA^{Tyr}$	F CCTTCGATAGCTCAGCTGGT R CGACCTAAGGATGTCCACAAAT
$tRNA^{Ile}$	F GGCGGCCGGTTAGCTCAG R CCCGTACGGGGATCGAAC
$tRNA^{iMet}$	F AGAGTGGCGCAGCGGAAG R GAGGATGGTTTCGATCCATC
$tRNA^{Pro}$	F GCTCGTTGGTCTAGGGGT R CTCGTCCGGGATTTGAAC
5S rRNA	F GGCCATACCACCCTGAACGC R CAGCACCCGGTATTCCCAGG
Brf1	F TGGCCCACGTGATTGCTGCC R CAGCAGGTGCGCAAAGCGTG
TBP	F CTCAGGGTGCCATGACTCCCG R TTGTTGTTGCTGCTGCTGCCTTTG
Bdp1	F GATCAGAGCAAGGAAGGCAAGCCAGAAG R CAACTGAAGATGGTGAATTATCTACTACAG
c-Jun	F CCCCAAGATCCTGAAACAGA R CCGTTGCTGGACTGGATTAT

Table 2.4 Primer sequences for RT-qPCR

Target gene	Primer sequences
$tRNA^{Leu}$	F ATGGCGAGTGGTCTAAGG R ACCAGAAGACCCGAACACAG
$tRNA^{Tyr}$	F CCTTCGATAGCTCAGCTGGT R CGACCTAAGGATGTCCACAAAT
$tRNA^{Val}$	F TGTAGCTCAGTGGTAGAGC R GAAACGAAATGTTCAACGATAG
$tRNA^{Thr}$	F GTGGCCAAGTGGTAAGGCGTC R ACCCGACTTCCCCACAGCC
$tRNA^{Leu}$ (inactive)	F CTTGAAACTTGCCCCAGTCA R TTCGCGTACTTTTTAAATGCTG

Table 2.5 Primers sequences used in qPCR analysis of ChIP samples

2.10 Cell Cycle Analysis

Following treatment, cells were trypsinised (approximately $2.5x10^5$ cells per treatment) and transferred to 15ml Falcon tube. They were then centrifuged at 180*g* for 5 minutes at room temperature and washed once in PBS. Next, the cell pellet was resuspended in 300µl PBS and 700µl of 100% ethanol was added dropwise while vortexing. Then placed at -20°C for a minimum of 1hr. Once fixed, tubes centrifuged directly from freezer. Cell pellet was then washed once in PBS and resuspended in 300µl of PBS containing 10µg/ml propidium iodide (PI) and 250µg/ml RNase A. Incubated in dark at room temperature for 30 minutes. Samples were then stored at 4°C until analysis by flow cytometry on a BD Biosciences FACSCalibur. Cell cycle profiles determined using Cell Quest Pro (BD Bisosciences.

2.11 Bioinformatics and data analysis

ChIP-seq data downloaded from GEO database, accession number: GSE19551(Raha et al., 2010) and GSE25533 (Tiwari et al., 2011)
ChIP-seq data downloaded from: http://genome.ucsc.edu/cgi-bin/hgTrackUi?db=hg18&g=wgEncodeYaleChIPseq (Moqtaderi et al., 2010)

List of tRNAs in the human genome from the Genomic tRNA Database (http://lowelab.ucsc.edu/GtRNAdb/) (Chan and Lowe, 2009)

ChIP-seq data mined by Damian Graczyk. All ChIP-seq peaks mined had a p-value of less than 0.05. TRE/CRE and CCAAT motif searches carried out by Ann Hedley. Upon receiving the data from ChIP-seq and motif search, I carried out all of the data analysis myself.

All data analysis was carried out using Microsoft Excel. All graphs were created using Microsoft Excel, with the exception of Venn diagrams which were created using Google Charts. All error bars represent standard deviation from the mean. Student T-test was used to determine significance where indicated. Chi-squared test was used to analyse the association between binding motifs and/or protein occupancy at tRNA genes.

3 Regulation of RNA Polymerase III-mediated transcription by p53

3.1 Introduction

p53 was first shown to regulate Pol III-mediated transcription over a decade ago (Chesnokov et al., 1996, Cairns and White, 1998). Since then, the mechanism for this repression has been determined: p53 sequesters TFIIIB, preventing its recruitment to the promoter, as described in Chapter 1 (Cairns and White, 1998, Crighton et al., 2003). However, little is understood about when p53 regulates Pol III-mediated transcription following p53 activation *in vivo*. How is p53's regulation of Pol III-mediated transcription regulated? In a cell, p53 can be activated by many different stimuli and can result in a range of different outcomes including cell cycle arrest, DNA repair, senescence, apoptosis, induction of autophagy and changes in metabolism (Vogelstein et al., 2000, Vousden and Prives, 2009). It is possible that repression of Pol III-mediated transcription may be beneficial in some intended outcomes of p53 activation but not others. For example, after cell cycle arrest, Pol III repression may contribute to the slowing of growth that is required to maintain cell size when division stops. In contrast, upon induction of apoptosis there may be little selection for the repression of Pol III-mediated transcription because the cell is going to die anyway. In this chapter, I will address whether repression of Pol III–mediated transcription is a universal response to p53 activation or is dependent upon the activating signal and the desired outcome.

The clearest example of p53 repression of endogenous targets of Pol III as part of a stress response was described by Morton and colleagues in 2007 (Morton et al., 2007). They showed that induction of ARF in U2OS osteosarcoma cells resulted in reduced tRNA expression and this was dependent upon ARF's ability to induce p53 activity. ARF induction is a model for oncogenic stress, as ARF is the central mediator of the oncogenic stress response (Lowe and Sherr, 2003). ARF (otherwise known as $p14^{ARF}$ in humans and $p19^{ARF}$ in mice) is induced through increased expression and stabilisation by E2F1, Myc and Ras overexpression (Gil and Peters, 2006, Sherr, 2006). Once induced, ARF binds to MDM2 and prevents it from binding p53. p53 is, therefore, no longer targeted for degradation and thus p53 is stabilised. ARF is central to p53 induction that occurs following

oncogenic stress. Morton and colleagues found that E2F1 overexpression resulted in Pol III repression and attributed this to the activation of p53 by ARF. Upon oncogenic stress, p53 causes cell cycle arrest and may induce senescence or apoptosis (Sherr, 2006). This prevents a cell's continued proliferation following the damage that may be inflicted by oncogene overexpression.

Hypoxia, ribonucleotide depletion and DNA damage can all result in Pol III repression (Ernens et al., 2006, Crighton et al., 2003, Gridasova and Henry, 2005). In rat cardiomyocytes, hypoxia (1% oxygen) did not result in p53 induction and repression of Pol III-mediated transcription was attributed to the increased binding of RB, and reduced binding of c-Myc and ERK, to TFIIIB (Ernens et al., 2006). Ribonucleotide depletion was carried out by mycophenolic acid (MPA) treatment, which prevents guanine synthesis, inducing p53 by causing nucleolar stress (Sun et al., 2008). Repression of Pol III-mediated transcription was observed following MPA treatment of U2OS cells (T. Kantidakis and R. White, unpublished). However, in cells in which p53 had been knocked down, MPA had the same Pol III repressive effect, suggesting p53 was not required for the repression observed. Methyl methanesulfonate (MMS) causes DNA damage by methylation of DNA (Wyatt and Pittman, 2006). This damage induces p53 in HeLa cells and suppresses Pol III-mediated transcription (Crighton et al., 2003). However, the repression of Pol III-mediated transcription is independent of p53 (J. Morton and R. White, unpublished). DNA damage is also induced by UV exposure (Latonen and Laiho, 2005). Interestingly, Gridasova and Henry found that the Pol III products U6 and 5S rRNA showed different responses to UVC exposure, 5S levels increased and U6 levels decreased in the MCF7 breast cancer cell line upon exposure to UVC (Gridasova and Henry, 2005). Further investigation suggested that these changes may be p53 dependent. As Pol III transcribes U6 and 5S rRNA from different promoters, this provides an intriguing hint that p53 regulation may occur by different mechanisms at different Pol III promoter structures. It also provides the first indication that p53 may repress Pol III-mediated transcription *in vivo* following a stimulus other than oncogenic stress. Clearly, repression of Pol III-mediated transcription is a frequent outcome of stress. However, activation of p53 does not always lead to p53-induced repression of Pol III-mediated transcription.

The physiological outcome of p53 induction is the result of the integration of many factors influenced by the nature and magnitude of the stress and the cellular environment (Murray-Zmijewski et al., 2008, Espinosa, 2008). p53 interacts with many proteins that contribute to its activity (Beckerman and Prives, 2010). It is also targeted for multiple post-translational modifications which can alter its stability and its ability to interact with cofactors and DNA (Meek and Anderson, 2009, Dai and Gu, 2010). Thus, in two different cell types, the same stress can cause different outcomes to p53 induction. Conversely, two different stimuli can induce the same outcome to p53 induction. Pol III repression by p53 has been studied in a diverse range of cell types so far. It is therefore difficult to conclude if differences in the effect of p53 on Pol III-mediated transcription are due to inherent differences in the stimuli involved or due to differences in cell type. For this reason, in this chapter, the response of Pol III-mediated transcription to stress is examined in U2OS only. U2OS were chosen as they are the parental cell of the ARF-inducible (NARF2) cells in which repression of Pol III-mediated transcription following ARF induction was shown to be p53 dependent and they have a functional p53 pathway (with the exception of ARF) (Morton et al., 2007).

The conflicting results from MMS and UVC treatment suggest that the type of DNA damage inflicted may play a role in determining how Pol III-mediated transcription is regulated (Crighton et al., 2003, Gridasova and Henry, 2005). p53 induction by DNA damage occurs via two main pathways, however cross-talk is likely to exist (Vogelstein et al., 2000). In one, double-stranded DNA breaks cause ATM induction and this induces p53 via Chk2. Other types of DNA damage, such as DNA crosslinking, induce p53 via ATR and Chk1. ATM and ATR induce different post-translational modifications of p53, allowing the outcome of p53 induction to be tailored to the stimulus (Meek, 2009). As p53 represses Pol III-mediated transcription by interacting with TFIIIB, we hypothesise that a likely route by which this interaction may be regulated is through the post-translational modification of p53. In this model, upon stress, p53 is induced but this only causes repression of Pol III-mediated transcription if a specific post-translational modification of p53 occurs, promoting its interaction with TFIIIB. In this chapter, the post-translational modification of p53 is examined following

several stresses to determine if there is a correlation between modification and p53's ability to repress Pol III-mediated transcription.

In this chapter, the effect of UVC and doxorubicin on Pol III-mediated transcription is examined. UVC was chosen in order to extend the study by Gridasova and Henry, in which they show that U6 and 5S rRNA respond differently to $50J/m^2$ UVC treatment (Gridasova and Henry, 2005). UVC (<280nm) is absorbed by the DNA resulting in the formation of pyrimidine photodimers. These, in turn, can result in double-stranded breaks (Latonen and Laiho, 2005). UV can also induce DNA-independent effects on the cell, such as through the activation of JNK and p38 kinases. Doxorubicin (Adriamycin) was chosen as it is a common chemotherapeutic drug that causes robust p53 induction. It is a topoisomerase inhibitor and, like UV, causes double-stranded breaks. The concentration of doxorubicin used was chosen as it resulted cell cycle arrest rather than cell death. p53 activation will often result in cell cycle arrest following DNA damage, allowing the cell time to repair damage before damage is transferred to the daughter cells. Cell death may be induced following stronger damage. Repression of Pol III-mediated transcription will contribute to the repression of protein synthesis following cell cycle arrest (Ruggero and Pandolfi, 2003). Whether regulation of Pol III-mediated transcription is advantageous during cell death is unclear.

In this chapter, the effect of two alternative DNA damage stimuli on Pol III-mediated transcription and on p53 induction is examined. The post-translational modification of p53 that occurs following these treatments is compared to those following ARF induction to examine whether any of these modifications may contribute to p53's ability to repress Pol III-mediated transcription. Whether basal levels of p53 may also regulate Pol III-mediated transcription is also addressed.

3.2 Results

3.2.1 Doxorubicin causes p53-independent repression of tRNA transcription

To examine the cellular response to DNA damage, U2OS expressing a scrambled (Scr) or p53 shRNA were treated with 200ng/ml of doxorubicin. This is a low dose, resulting in no detectable cell death by the time of harvest (data not shown). Cells were harvested 1 hour and 6 hours after treatment. A large increase in p53 levels was observed in the Scr shRNA expressing cells after 6 hours, as determined by western blot (Figure 3.1). Phosphorylation of p53 at Serine 15 was also detectable by this time point. This modification is indicative of activation of the DNA damage response. In the cells expressing p53 shRNA, basal levels of p53 were lower and p53 levels, while increasing upon doxorubicin treatment, did not reach the levels seen in the Scr shRNA-expressing cells (Figure 3.1). Likewise, little p53 phosphorylated at serine 15 was detectable. Treatment of U2OS Scr shRNA cells with doxorubicin resulted in a 50-80% reduction in $tRNA^{Tyr}$, $tRNA^{Leu}$ and $tRNA_i^{Met}$ levels compared to control treated (Figure 3.2). The reduction in tRNA levels was also observed in p53 shRNA-expressing cells when treated with doxorubicin, suggesting that repression of tRNA levels following doxorubicin treatment is independent of p53 (Figure 3.2).

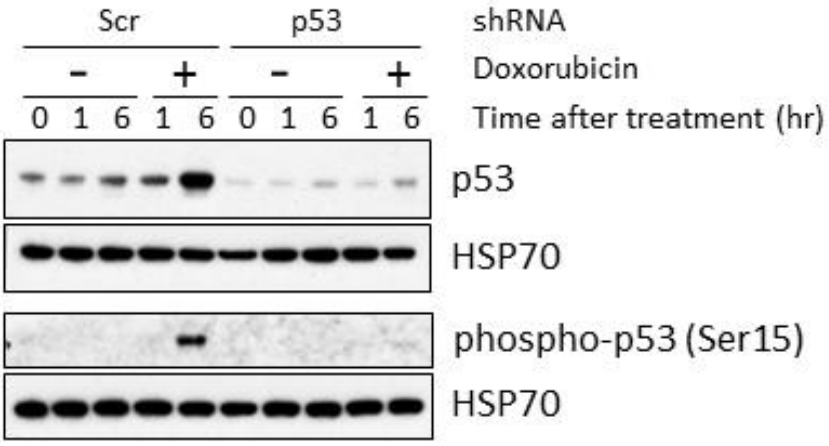

Figure 3.1 Doxorubicin treatment increases p53 protein levels

Analysis by western blot of p53 and phospho-p53(Ser15) protein levels in U2OS stably expressing shRNA targeted to a scrambled sequence or p53, and treated with 200ng/ml doxorubicin or H_2O. Cells were harvested 1hr or 6hr post-treatment. HSP70 used as a loading control. n=1

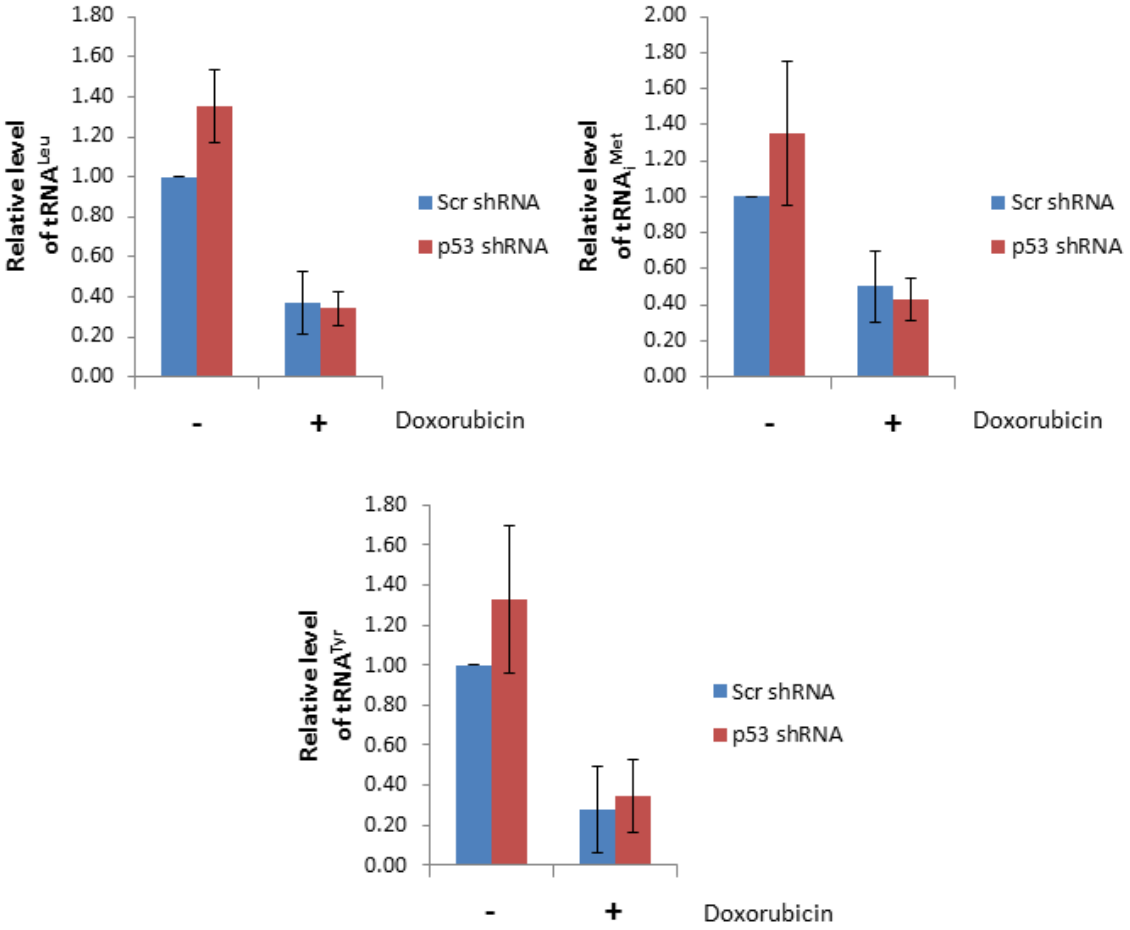

Figure 3.2 A non-lethal dose of doxorubicin results in p53-independent repression of tRNA transcription

Analysis by RT-qPCR of $tRNA^{Leu}$, $tRNA_i^{Met}$ and $tRNA^{Tyr}$ levels in U2OS stably expressing shRNA targeted to a scrambled sequence or p53, and treated with 200ng/ml doxorubicin or H_2O. Cells were harvested 6hr post-treatment. Expression was normalised to ARPP P0 mRNA levels. n=3

3.2.2 UVC upregulates Pol III-mediated transcription

U2OS cells were exposed to 50J/m^2 UVC and were harvested for protein and RNA eight hours later. This resulted in minimal increase in p53 levels, according to western blot analysis (Figure 3.3, Figure 3.5). However, the proportion of p53 phosphorylated at Serine 15 rose dramatically (Figure 3.3). UVC treatment resulted in a small but significant increase in $tRNA_i^{Met}$, $tRNA^{Leu}$ and 5S rRNA levels (Figure 3.4a). This is consistent with the increase in 5S rRNA levels observed in MCF7 cells following UVC treatment (Gridasova and Henry, 2005). Interestingly, levels of mRNA for the TFIIIB subunits, TBP, Brf1 and Bdp1, are reduced following UVC treatment (Figure 3.4b). This is not reflected in the protein levels of TBP and Brf1, which appear to remain unchanged (Figure 3.3). In future studies, it will be interesting to examine whether the increased tRNA and 5S rRNA levels are dependent upon p53. If they are, they are unlikely to be due to p53's established repressive effect on Pol III-mediated transcription through binding to TFIIIB.

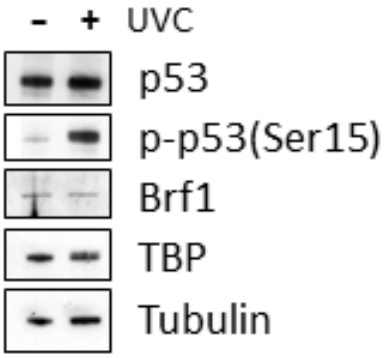

Figure 3.3 Exposure to UVC induces p53 activation

Analysis by western blot of p53, phospho-p53(Ser15), Brf1 and TBP protein levels in U2OS (stably expressing scrambled shRNA) 8hr after exposure to 50J/m^2 UVC. Tubulin used as a loading control. n=1

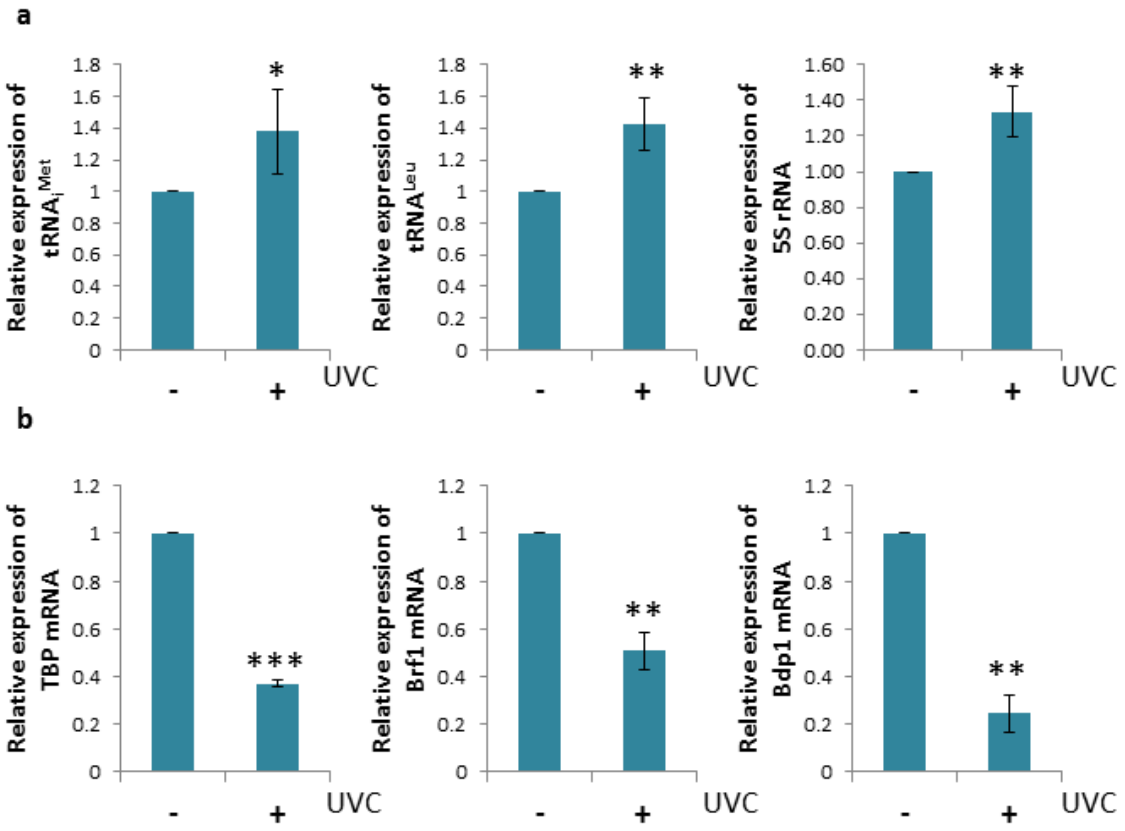

Figure 3.4 Exposure to UVC induces Pol III-mediated transcription

Analysis by RT-qPCR of a) $tRNA_i^{Met}$, $tRNA^{Leu}$ and 5S rRNA levels (n=4) and b)TBP, Brf1 and Bdp1 mRNA levels (n=3) in U2OS (stably expressing scrambled shRNA) 8hr after exposure to 50J/m^2 UVC. The expression was normalised to ARPP P0 mRNA levels. *p<0.05, ***p<0.01 ***p<0.001

3.2.3 p53 modification following oncogenic stress and DNA damage

I hypothesised that p53 repression of Pol III-mediated transcription may require a specific post-translational modification of p53, allowing it to interact with TFIIIB. A difference in modification status could explain why p53 represses Pol III following oncogenic stress but not doxorubicin treatment. Therefore, western blotting was employed to probe for different post-translational modifications of p53 following UVC treatment and doxorubicin treatment in U2OS and ARF induction in NARF2 cells. The NARF2 cells were induced with IPTG according to the protocol of Morton et al. (Morton et al., 2007). Induction of ARF and increased p53 levels were detectable following IPTG treatment (Figure 3.5b). In addition, reduced $tRNA^{Tyr}$ and 5S rRNA levels were detected by semi-quantitative RT-PCR following ARF induction (data not shown). Cells were also treated with UVB ($50J/m^2$), however the effect of UVB on Pol III-mediated transcription remains to be determined. Doxorubicin and ARF induction resulted in very similar total p53 levels, while UVC-exposed cells only showed weak p53 induction (Figure 3.5a). An increase in phosphorylation of p53 at Serine 9 and Serine 15 was apparent in UVB, UVC and doxorubicin treated cells compared to their respective controls (Figure 3.5a). A slight increase in Serine 15 phosphorylation and no increase in Serine 9 phosphorylation was detectable in NARF2 cells when ARF was induced. Phosphorylation at Serine 20 and Serine 46 was detectable at low levels but did not change with ARF induction. Slight increases in Serine 20 and Serine 46 phosphorylation occurred in both UVB and UVC treated cells compared to control. While, with doxorubicin treatment Serine 46 phosphorylation was slightly reduced. Phosphorylation at Ser20 does not change with doxorubicin treatment. Interestingly, an increase in phosphorylation at Serine 392 occurred with all treatments. It was highest following doxorubicin treatment, while p-p53(Ser392) levels were lower following ARF induction and lower still following UV exposure.

Acetylation of p53 was also examined. Acetylation of p53 at Lysine 382 was undetectable in NARF2 cells (Figure 3.6). Acetylation at Lysine 305, Lysine 373 and Lysine 386 was detectable and may have reduced slightly with ARF induction. Only acetylation at Lysine 382 changed with doxorubicin treatment. Following treatment, an additional band was detectable below the band present

in the untreated cells. This is indicative of acetylation, as proteins usually migrate faster when acetylated. There may be a slight increase in acetylation at all sites examined following both UVB and UVC treatment. However as this is very subtle, further examination is necessary. Overall, changes in p53 modification were very limited compared to changes in total p53 levels observed, with the exception of phosphorylation at Serines 15 and 392. In particular, there was no evidence of p53 modification in ARF-induced cells that distinguishes it from the other treatments and could account for p53's ability to repress Pol III-mediated transcription following this treatment. This does not rule out the possibility that modification of p53 is required for p53 to interact with TFIIIB and, as a result, repress Pol III-mediated transcription. This remains an interesting hypothesis for future studies.

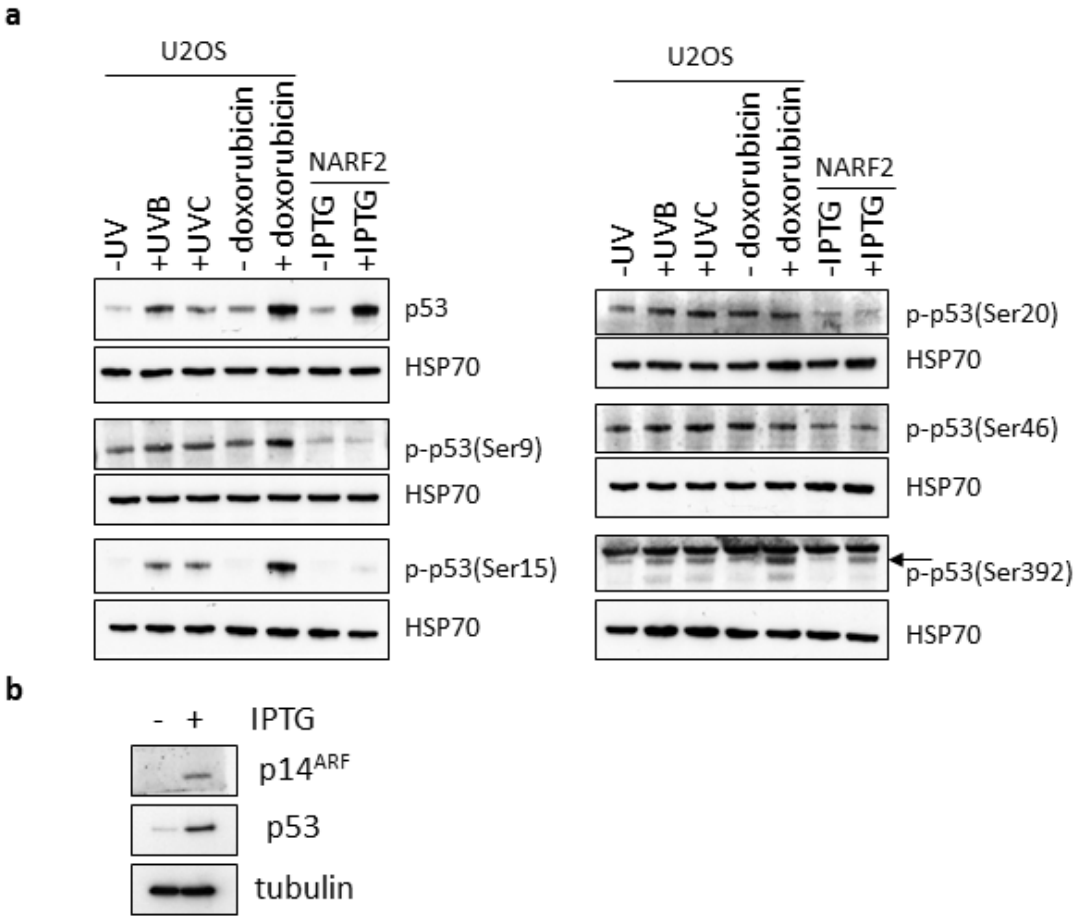

Figure 3.5 Analysis of p53 phosphorylation following DNA damage and ARF-induction

a) Analysis by western blot of total p53 protein level and levels of p53 phosphorylated at Ser9, Ser15, Ser20, Ser46 or Ser392 in U2OS, following 50J/m^2 UVB (harvested 8hr post-treatment), 50J/m^2 UVC (harvested 8hr post-treatment), 580ng/ml doxorubicin or H_2O (harvested 24hr post-treatment), and NARF2, -/+ 1mM IPTG to induce expression ARF (harvested 24hr post-induction). HSP70 used as a loading control. n=1 b) Analysis by western blots of p14^{ARF} and p53 protein levels in NARF2 cells following induction by IPTG for 24 hours. Tubulin used as a loading control.

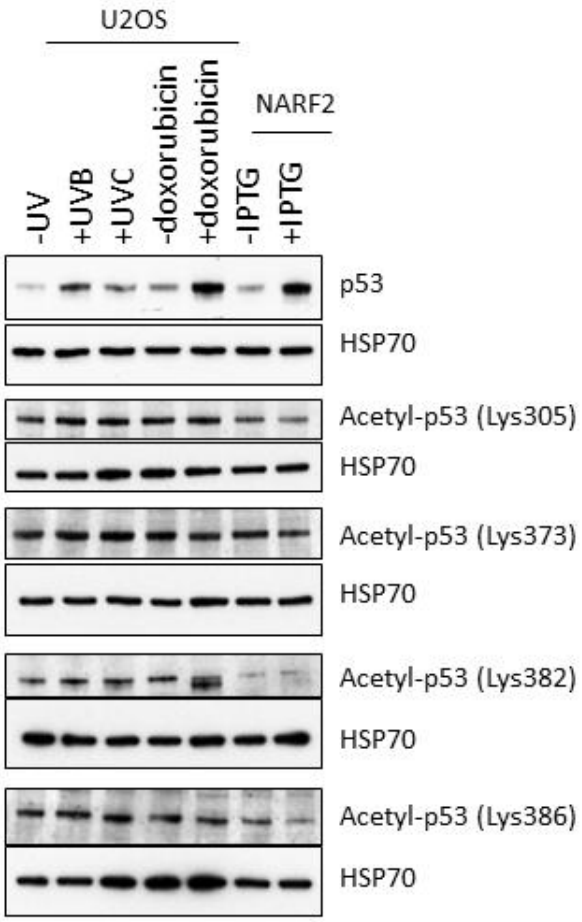

Figure 3.6 Analysis of p53 acetylation following DNA damage and ARF-induction

Analysis by western blot of total p53 protein level and levels of p53 acetylated at Lys305, Lys373, Lys382 or Lys386 in U2OS U2OS, following 50J/m^2 UVB (harvested 8hr post-treatment), 50J/m^2 UVC (harvested 8hr post-treatment), 580ng/ml doxorubicin or H_2O (harvested 24hr post-treatment), and NARF2, -/+ 1mM IPTG to induce expression ARF (harvested 24hr post-induction). HSP70 protein level is a loading control. n=1

3.2.4 Basal p53 levels do not influence tRNA levels in RKO and MCF7 cell lines

Having examined the role of p53 in regulating Pol III-mediated transcription under stress conditions, I wanted to determine whether basal levels of p53 could influence Pol III activity. To examine this, RKO (colon cancer cell line with wild-type p53) and MCF7 (breast cancer cell line with wild type p53) cells stably expressing a scrambled shRNA or a shRNA targeting p53 were cultured and harvested. Western blotting showed that p53 shRNA expression resulted in reduced p53 levels in both cell types (Figure 3.7). However, no significant differences in $tRNA^{Leu}$, $tRNA^{Ile}$ and $tRNA_i^{Met}$ levels were observed between cells expressing the scrambled and p53 shRNA, when examined by RT-qPCR (Figure 3.8). Returning to Figure 3.2, in untreated U2OS expressing the same shRNAs, $tRNA^{Tyr}$ and $tRNA_i^{Met}$ levels did not differ between shRNA. However, $tRNA^{Leu}$ levels were significantly higher in U2OS in which p53 had been knocked down, than in the Scr shRNA-expressing U2OS. Overall, these finding suggests that basal levels of p53 may regulate Pol III-mediated transcription but this regulation is likely to be subtle and, potentially, cell type dependent.

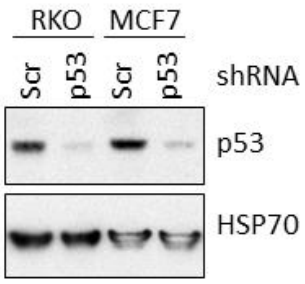

Figure 3.7 p53 protein level is reduced in cells stably expressing p53 shRNA

Analysis by western blot of p53 protein levels in RKO and MCF7 cells stably expressing shRNA targeted to a scrambled sequence or p53. HSP70 protein level is a loading control. n=3

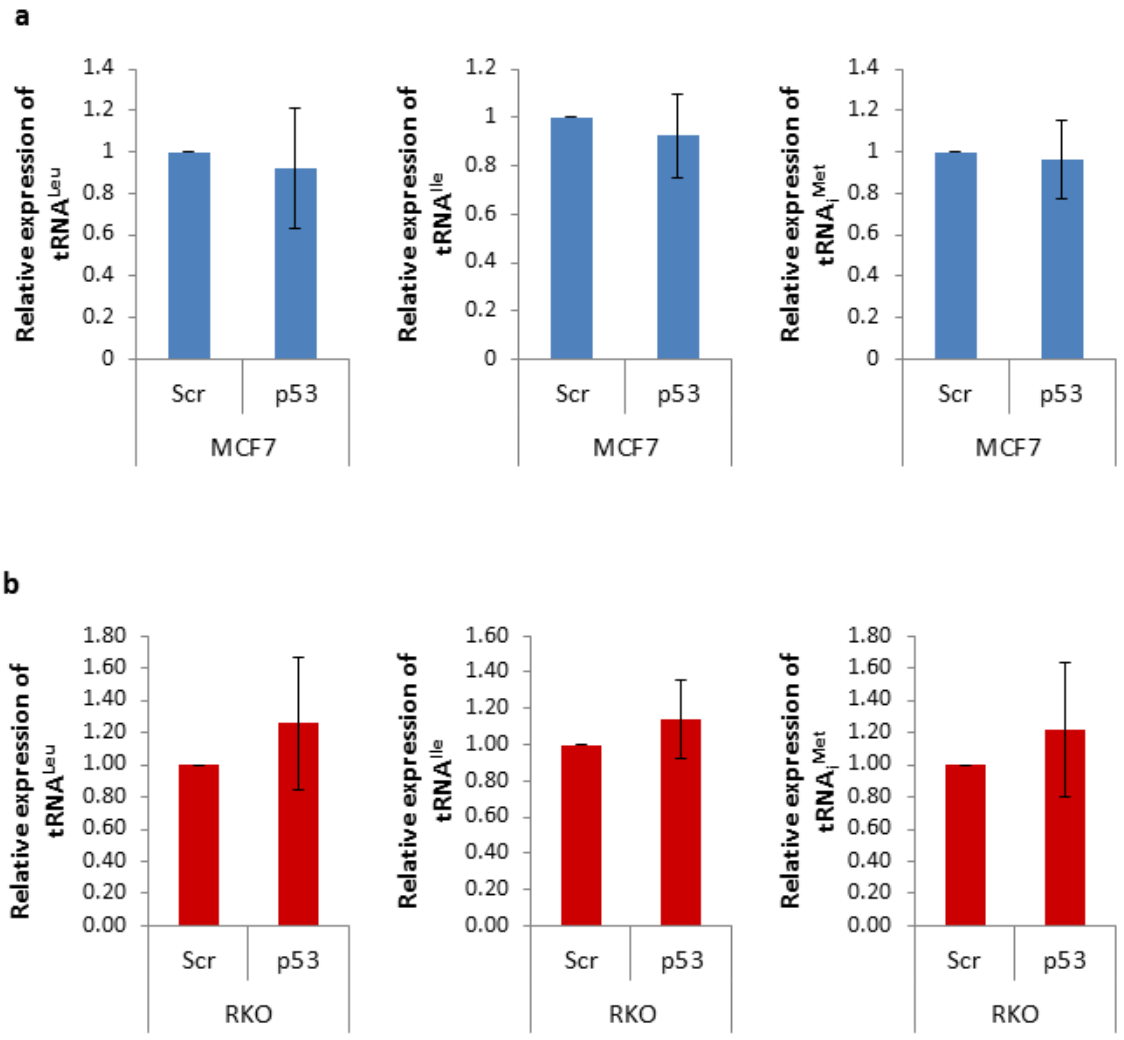

Figure 3.8 Stable knockdown of p53 in RKO and MCF7 cells does not alter tRNA levels

Analysis by RT-qPCR of $tRNA^{Leu}$, $tRNA^{Ile}$ and $tRNA_i^{Met}$ levels in a) RKO cells stably expressing shRNA targeted to a scrambled sequence or p53 and b) MCF7 cells stably expressing shRNA targeted to a scrambled sequence or p53. The expression was normalised to ARPP P0 mRNA levels. Student t-tests showed no significant differences. n=3

3.3 Discussion

Following stress, a cell usually undergoes arrest. Subsequently, it may re-enter the cell cycle, it may undergo senescence, or cell death may occur (Vousden and Prives, 2009). Cell cycle arrest allows the cell to recover and repair any damage done by the stress before the next cell division. When a cell arrests, it is essential that cell growth (accumulation of cellular mass) is also arrested in order to maintain a constant cell size. Ribosome biogenesis is rate limiting for growth and protein synthesis is key to cell growth as the majority of dry cellular mass is protein (Ruggero and Pandolfi, 2003). It has been demonstrated that repression of Pol III activity is part of this cell growth arrest (Goodfellow and White, 2007). This is not a surprising finding as Pol III-mediated transcription is responsible for producing 5S rRNA, tRNAs and other important components of the protein synthesis machinery (Dieci et al., 2007). In mammals, p53 is the key initiator of cell cycle arrest and cell death in response to stress (Vogelstein et al., 2000). p53 can also repress Pol I and Pol III-mediated transcription which together produce the RNA components of the ribosome (Chesnokov et al., 1996, Cairns and White, 1998, Budde and Grummt, 1999). However, as demonstrated in this chapter, repression of Pol III activity in response to stress does not always require p53. Furthermore, the results following UVC treatment demonstrate that Pol III repression may not be a universal response to stress.

Gridasova and Henry found that 5S rRNA transcription increased upon exposure of MCF7 cells to $50J/m^2$ of UVC (Gridasova and Henry, 2005). I found that this also occurs in U2OS cells (Figure 3.4a). And tRNA levels, which were not studied by Gridasova and Henry, also increased. It is interesting that similar effects are observed in 5S rRNA and tRNA levels as tRNA and 5S rRNA genes share a similar promoter structure (Type 2 and Type 1 respectively) (Figure 1.2). They bind TFIIIC and TFIIIB is then recruited. In contrast, U6 exhibited reduced levels following UVC exposure (Gridasova and Henry, 2005). This has a more disparate promoter structure (type 3) and TFIIIC is not part of the PIC at this promoter and the TFIIIB recruited contains Brf2 rather than Brf1. It is likely that targeting of a shared feature in PIC formation is responsible for the increases in 5S rRNA and tRNA levels seen. Gridasova and Henry found that overexpression of p53 in HeLa cells resulted in an increase in 5S rRNA levels. As a result, they concluded that the increase in 5S rRNA levels following UVC treatment in MCF7 cells was also

likely to be p53 dependent. However, the effects of UVC treatment and p53 overexpression are not comparable so this conclusion should be treated cautiously. Unfortunately, time restrictions prevented the determination of p53 dependency of the Pol III activity in the UVC treatment of U2OS cells. However, if p53 is required, it is unlikely to act through its known mechanism of regulating Pol III activity. In this mechanism, active p53 binds to TFIIIB and prevents its recruitment to target genes and, thus, reduces their transcription (Cairns and White, 1998, Crighton et al., 2003). Following UVC treatment, p53 is activated yet Pol III-mediated transcription increases. This could, for example, be a secondary effect of p53 induction rather than a direct effect. The increase in Pol III activity following exposure to UVC is interesting and could provide useful information about the regulation of Pol III activity following stress, given further study.

The increase in 5S rRNA and tRNA levels following UVC is not the expected outcome of cell cycle arrest, as upon cell cycle arrest a cell has reduced requirement for protein synthesis. The cell cycle status of U2OS following UVC requires further examination, however it is possible that this unexpected response is due to the non-physiological nature of the experiment. UVC does not penetrate the ozone layer except where the ozone layer has been damaged (Latonen and Laiho, 2005). Also, U2OS cells are bone-derived cells, which would never be exposed to UV under normal conditions. Nevertheless, UVC causes damage that can be caused by other treatments (including UVB) and thus activates appropriate pathways in response to this damage. However, there has been no evolutionary pressure to develop a suitable 'whole cell' response to UVC. UVC activates other pathways in addition to p53 and one of these could contribute to the regulation of Pol III-mediated transcription (Healy et al., 2013). For example, c-Jun N-terminal kinase (JNK) is activated by UV, allowing it to phosphorylate its targets, such as c-Jun (Bogoyevitch and Kobe, 2006). JNK activity is known to promote Pol III-mediated transcription and c-Jun also has a potential positive influence (see Chapters 4 and 5) (Zhong and Johnson, 2009, Raha et al., 2010). It is, therefore, possible that JNK activation may contribute to the increased Pol III products seen. Indeed, increased levels of phospho-c-Jun were observed following UVC treatment (data not shown). To determine if JNK could contribute, cells were pre-treated with a JNK inhibitor before exposing to

UVC. It was found that this prevented the increase in Pol III transcription seen after UVC treatment (data not shown). However, as JNK inhibitor alone resulted in a decrease in Pol III-mediated transcription in U2OS (Figure 5.5), these results were not conclusive. In future studies, it will be interesting to examine whether JNK contributes to the increase in Pol III-mediated transcription observed following UVC treatment.

Doxorubicin treatment results in substantial p53 activation and significant repression of Pol III-mediated transcription (Figure 3.1, Figure 3.2). However, unlike following ARF induction (Morton et al., 2007), these are not linked. p53 is not required for the repression of Pol III-mediated transcription following doxorubicin treatment (Figure 3.2). So, what may account for difference between oncogenic and DNA damage response with regards to p53's role in Pol III activity?

The first possibility I considered was that a specific p53 modification was required for its Pol III repression activity and this modification occurred following ARF induction but not doxorubicin treatment. The most obvious way a modification may affect p53's activity (with regard to Pol III) is by altering its interacting surface with TFIIIB. The most noticeable conclusion from Figure 3.5 and Figure 3.6 is that ARF induction results in very few post-translational modifications of p53, while following DNA damage they are more common. This is supported by the literature where ARF activity is generally ascribed to its ability to stabilise p53 (through ARF's ability to bind with MDM2) while modification of p53 is associated with DNA damage (Sherr, 2006). One exception to this, demonstrated in this chapter, is Ser392 phosphorylation. This has been described previously, where the authors concluded this phosphorylation was a universal outcome of p53 induction (Cox and Meek, 2010). Moreover, as it is observed following all treatments it is difficult to foresee how this would result in a response specific to ARF induction. Acetylation at Lysine 120 has been found to be associated with p53 activation by ARF (Mellert et al., 2007). Lysine 120 is in the DNA binding domain of p53 and this domain is required for p53 to regulate Pol III activity, despite DNA binding of p53 appearing to be unnecessary (Stein et al., 2002b). Given more time, it would be very interesting to examine whether this acetylation is required for Pol III regulation by p53. This could be most directly examined by looking at whether p53, mutated so it cannot be acetylated at this site, can bind to

TFIIIB, the described mechanism of action in Pol III repression. It would be best to investigate by this route as the acetylation may be necessary for other functions of p53 after stress. These could also impact Pol III-mediated transcription. This route of investigation will be more specific than previous studies that looked at the regions of p53 required for repression of Pol III activity (Stein et al., 2002b, Gridasova and Henry, 2005). The whole of p53, except for residues 13-19 and 364-392, is required for repression of Pol III-mediated transcription (Stein et al., 2002b). The only exception is for the U6 gene, where only the C-terminus of p53 is required for repression (Gridasova and Henry, 2005). In conclusion, these experiments did not demonstrate a likely candidate of a post-translational modification that promotes the interaction between p53 and TFIIIB. However, further studies may prove fruitful as there are many modifications that could not be examined in this chapter.

Another mechanism by which post-translational modification of p53 could regulate p53's regulation of Pol III should be considered. One of the modifications that occurs following DNA damage could prevent p53's interaction with TFIIIB, either by altering the interaction surface so that the interaction can no longer occur or because it promotes a stronger association with another protein. As Pol III-mediated transcription can be repressed independently of p53, as demonstrated following doxorubicin treatment, this would probably not be evolutionarily negative. This model of regulation deserves further examination.

Post-translational modification of p53 is not the only potential model that would result in p53 being able to regulate Pol III activity following some stresses but not others. I propose two alternative models: 1) a specific cofactor may be required and/or 2) a specific modification of TFIIIB may be required. p53 recruits cofactors, including histone acetyltransferases GCN5 and CBP/p300, to modify the activity of the target genes it binds to (Beckerman and Prives, 2010). It is not unreasonable to suppose that a cofactor is also involved in p53 sequestering of TFIIIB away from the Pol III machinery. The location of the p53-TFIIIB interaction in the cell is not known, but a cofactor could, for example, ensure that it is held in a particular location. Alternatively, a cofactor may be necessary to sterically block the binding of TFIIIB to TFIIIC or to modify TFIIIB to either allow the interaction with p53 or prevent the interaction with TFIIIC. To determine if a cofactor is present, one could immunoprecipitate TFIIIB (using an antibody recognising Brf1) and carry out mass spectrometry on the complexes pulled down with it. Post-translational modification of polymerase machinery is a common mechanism for modulating its activity. For

example, Brf1 is phosphorylated by ERK and CK2, promoting TFIIIB recruitment to Pol III target gene promoters. TFIIIB may undergo modification following ARF induction (but not DNA damage) allowing it to interact with p53.

Where possible, this study has attempted to remove the possibility of genetic background contributing to differences in the stress response. The ARF induction experiments by Morton et al. were carried out using NARF2 cells, U2OS with inducible ARF. I used U2OS cells for my DNA damage experiments to keep the background as similar as possible. However, these have been grown separately over time, so it is possible that genetic differences between the parental U2OS and the NARF2 cells may now exist. The only definitive difference is that U2OS cells do not express ARF. There is some evidence that ARF may be activated downstream of DNA damage (Khan et al., 2000). If this is the case, it may be that DNA damage can regulate p53-dependent repression of Pol III in cell types other than U2OS. Though U2OS are considered more 'normal' than many tumour cell lines, it would be beneficial to repeat these stress studies in untransformed, primary cell lines. This may give us more evidence about what happens to Pol III in an untransformed cell, as Pol III-mediated transcription is subject to regulation by many proteins that are modulated cancer.

Another possible reason for the disparity in the DNA damage and oncogenic stress responses is that a threshold p53 level may be required before p53 can have an impact on Pol III activity. This seems unlikely as total p53 levels are similar between doxorubicin-treated and ARF-induced cells (Figure 3.5a). The level of Pol III activity is a balance between positive and negative regulators. There do not appear to be more positive regulators active following doxorubicin treatment (which might increase the p53 activity threshold compared to oncogenic stress), as Pol III activity goes down following treatment. However, there is a possibility that different positive regulators are activated in each instance and, due to its mechanism of action, p53 is not able to overcome those induced by doxorubicin treatment. This hypothesis highlights a further possibility: that redundancy exists in the pathways regulating Pol III activity following stress. Maybe following doxorubicin treatment, p53 and another regulator act to repress Pol III transcription, and when p53 is knocked down the other regulator is able to act sufficiently alone. Yet this alternative regulator is not activated in response to ARF induction. To investigate this one would first

have to find this regulator and then repeat the doxorubicin experiment in its absence. The most probable candidates are Maf1 and RB. RB acts very much like p53, binding to TFIIIB and preventing its recruitment to Pol III target genes (White et al., 1996, Sutcliffe et al., 2000). Upon p53 induction, it is activated via the p53 target gene p21 and may therefore contribute to Pol III repression during cell cycle arrest following stress. Maf1 is also an interesting possibility. When unphosphorylated, it binds to Pol III, inhibiting Pol III-mediated transcription. In yeast, it is key to Pol III regulation following stress, but its role following stress has not been well studied in humans (Boguta, 2013, Moir and Willis, 2013). In addition, it will be interesting to examine whether p53 family members p63 and p73 may regulate Pol III activity. The complex response of Pol III-mediated transcription to p53 induction may be attributed to redundancy in the regulation of Pol III activity. If this is the case, it would demonstrate the importance of repressing Pol III activity following stress.

p53 is present in cells at low levels in non-stressed conditions and this basal level of p53 contributes to cellular function (Vousden and Prives, 2009). p53 null MEFs have higher Pol III product levels than the wild type control, suggesting that basal levels of p53 may contribute to regulation of Pol III activity. In this chapter, I addressed this further. Knockdown of p53 with a short hairpin RNA did not have an effect on Pol III product levels in RKO or MCF7 cells (Figure 3.8). And knockdown of p53 in U2OS cells only resulted in a significant increase in the level of one of the tRNAs examined (Figure 3.2). This suggests that if basal p53 does repress Pol III-mediated transcription, it may be a promoter-specific and cell type-specific effect. However, it is also possible that these cells have adapted to the reduced p53 levels. Pol III has many regulators and these may continue to act to ensure Pol III activity is at the optimal level, despite reduced levels of p53. An inducible hairpin system would be more suitable to determine basal p53's role. Pol III activity could be measured directly after p53 knockdown, before the cell has time to adapt. It will also be interesting to look in animal models, for example, when p53 knockout is induced in a particular organ of a mouse. At present, p53's role in the regulation of Pol III-mediated transcription in unstressed cells is unclear.

Further study of the p53-TFIIIB interaction will be key to understanding how p53 repression of Pol III-mediated transcription is regulated. I attempted to visualise

this interaction by co-immunoprecipitation (coIP) and by proximity ligation assay (PLA), but failed. This interaction is, however, shown by coIP by Crighton and colleagues (Crighton et al., 2003). We hypothesise that the p53-TFIIIB complex is only formed following stresses, such as ARF induction, that induce p53-dependent repression of Pol III activity. In this case, visualising this interaction would provide an accurate marker for whether Pol III was being repressed by p53. Alternatively, p53-TFIIIB complex formation may occur whenever p53 is induced, even when p53 is not required to repress Pol III activity, such as following doxorubicin treatment. This would suggest that further regulators are required for the Pol III repression, either to contribute additionally to the p53 repressive mechanism (such as by modifications or as a cofactor) or to repress Pol III by an alternative pathway. In either case, once we can visualise this interaction successfully, studies can be carried out to determine the domains of p53 and TFIIIB involved in this interaction. And, through studies in which this interaction is disrupted, we could determine whether Pol III repression by p53 contributes to p53's tumour suppressive function. If it did, this would be a fascinating discovery. p53 pathway function is lost in many tumours and Pol III activity is high in many cancers (White, 2008). This would suggest that high Pol III product levels may not merely be a consequence of cancer, but could contribute to tumour development.

The findings in this chapter, together with previously published and unpublished data, suggest that p53 may not be important in repressing Pol III-mediated transcription in response to DNA damage (Figure 3.9). Repression of Pol III does appear to be the standard response to most stresses, as predicted. It will be interesting to determine whether p53 repression of Pol III is associated with one cellular outcome in particular, be it cell cycle arrest, senescence or apoptosis. Looking at stresses beyond oncogenic stress and DNA damage will tell us more about the importance of Pol III regulation following cellular stress. Studying Pol III-mediated transcription following disruption of the ribosome biogenesis pathway may be particularly interesting as Pol III produces 5S rRNA, a key component of the ribosome, and 5S rRNA itself has been shown to contribute to the induction of p53 following disruption (Chakraborty et al., 2011, Donati et al., 2013) Overall, p53 repression of Pol III-mediated transcription appears to be particularly associated with the oncogenic stress response. This is particularly

interesting as evidence suggests that p53's tumour suppressor activity may be more dependent upon its oncogenic function than p53's ability to respond to DNA damage (Christophorou et al., 2006). It is, therefore, possible that regulation of Pol III-mediated transcription could be an important part of p53's wide spectrum of activities.

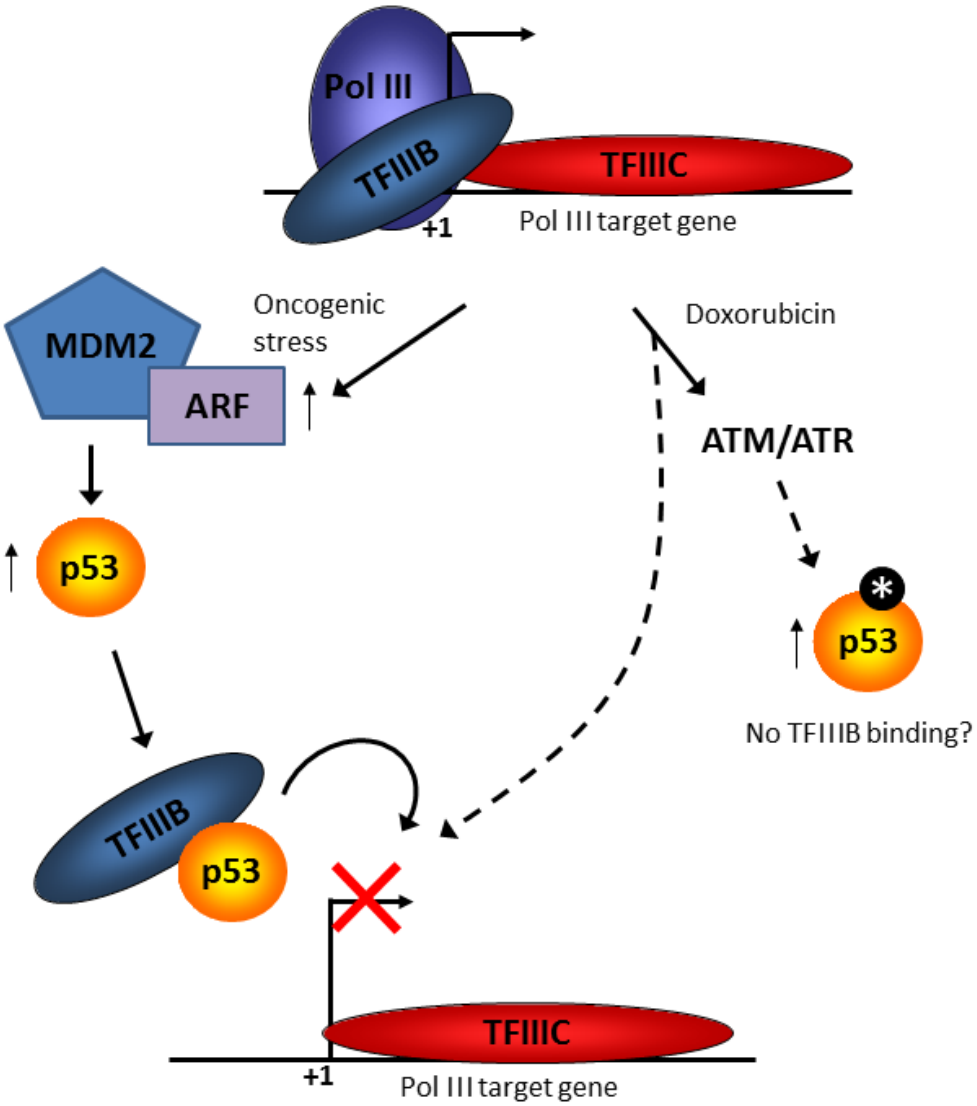

Figure 3.9 Model for regulation of Pol III-mediated transcription in response to stress

Stress often results in reduced Pol III-mediated transcription. Following oncogene overexpression, ARF is induced and binds to MDM2, stabilising p53 and resulting in p53-dependent repression of Pol III-mediated transcription. In the current model for this repression, p53 binds to TFIIIB preventing its recruitment to the Pol III target gene, and consequently preventing polymerase recruitment. Upon doxorubicin treatment, p53 levels rise, probably via the ATM/ATR DNA damage pathways, however the reduction in Pol III-mediated transcription that is observed occurs independently of p53. One possible model is that p53 cannot bind TFIIIB following doxorubicin treatment. The mediators of repression of Pol III-mediated transcription upon doxorubicin treatment have not been defined. Dashed lines indicate the existence of intermediate steps in the pathway.

4 Regulation of RNA Polymerase III-mediated transcription by AP-1

4.1 Introduction

Over the past decade it has become increasingly apparent that transcription by RNA Polymerases I, II and III can be regulated by shared factors (White, 2008). While initially unexpected, it provides an efficient mechanism for the regulation of translational machinery, where the products of all three polymerases are required in unison. p53, as described in Chapter 3, is one example. p53 represses 45S rRNA expression by inhibiting Pol I-mediated transcription and also regulates many Pol II target genes (Zhai and Comai, 2000, Vousden and Prives, 2009). And, by sequestering TFIIIB away from promoters and preventing the formation of a complete pre-initiation complex (PIC), active p53 reduces transcription of all Pol III target genes (Cairns and White, 1998, Crighton et al., 2003). However, 'shared' regulators can also be found interacting with the Pol III machinery at promoters. For example, c-Myc binds to TFIIIB and enhances transcription of Pol III-transcribed genes (Gomez-Roman et al., 2003). Recently, the employment of ChIP-seq technology has identified Ets1, Stat1 and the AP-1 proteins, c-Jun and c-Fos, at multiple Pol III target genes (Oler et al., 2010, Raha et al., 2010, Zhong et al., 2011). These transcription factors are more commonly associated with regulating Pol II-transcribed genes and their function at Pol III-transcribed genes, if they have one, is undetermined.

AP-1 is a dimeric transcription factor and AP-1 proteins include c-Jun, c-Fos and ATF2. It has been shown that AP-1 can regulate Pol III-mediated transcription via altering levels of Pol III machinery available (Zhong et al., 2011, Felton-Edkins et al., 2006). Ethanol treatment of HepG2-ADH cells (a hepatocellular carcinoma cell line stably-expressing alcohol dehydrogenase (ADH) to allow alcohol metabolism) results in the induction of $tRNA^{Leu}$, 5S rRNA and 7SL expression which appears to be dependent upon the increased TFIIIB levels that are also observed (Zhong et al., 2011). c-Jun binds at the promoters of TBP and Brf1 genes (Fromm et al., 2008, Zhong et al., 2011). Its occupancy increases between 2- and 4-fold following ethanol treatment (Zhong et al., 2011). The authors conclude that c-Jun is required for the increase in TBP and Brf1 mRNA levels following ethanol treatment. (Zhong et al., 2011). However, the mechanism of

this regulation has not been fully defined (including which AP-1 dimer is involved). Recent data from our lab has shown that transformation of TIFFs (telomerase-immortalised foreskin fibroblasts) by v-Fos expression results in a 4- to 8-fold increase in $tRNA_i^{Met}$ and $tRNA^{Tyr}$ when compared to TIFFs transfected with an empty vector (unpublished results, Joanna Birch). This may occur via the increase in TBP and TFIIIC220 that is also observed, however further investigation is required. Like the TBP gene, TFIIIC220 has an AP-1 binding site 81-88bp upstream of transcription start site (+1). This site is required for the higher level of TFIIIC220 mRNA seen in EBV (Epstein Barr Virus) infected HeLa cells compared to uninfected HeLas (Felton-Edkins et al., 2006). The higher mRNA level probably accounts for the higher TFIIIC220 protein level that, with other TFIIIC subunits, may contribute to the increased Pol III-mediated transcription seen in infected cells. ATF2 and, possibly, c-Jun bind at the TFIIIC220 gene (Felton-Edkins et al., 2006). This binding does not appear to be different between EBV-infected and uninfected cells. However, EBV infection can promote ATF2 phosphorylation by activating MAPK pathways and the authors suggest this increase in ATF2 phosphorylation may account for the increased TFIIIC220 expression. These examples demonstrate that Pol III-specific transcription factors are subject to upregulation by AP-1.

The presence of AP-1 at Pol III-transcribed genes suggests a further mechanism of regulating Pol III machinery. AP-1 often binds at its target genes via an AP-1 binding motif, such as TRE (TGACTCA) or CRE (TGACGTCA), situated nearby. The first example of AP-1 near Pol III-transcribed genes came from Alu elements, a class of short interspersed nuclear elements (SINEs) derived from a 7SL gene and transcribed by Pol III. A TRE motif is present upstream (-33) of the EPL Alu, an Alu that gave rise to a whole subfamily of Alus in primates (Chesnokov and Schmid, 1996). Upon deletion or mutation of this motif, the expression of this Alu is significantly reduced *in vivo* (Chesnokov and Schmid, 1996). While technically unable to explore this finding further *in vitro*, the authors did propose that the AP-1 site may have contributed to its ability to produce a whole subfamily of Alus where other Alus did not. Retrotransposition requires transcription of the gene, so genes that are more highly transcribed are more likely to be retrotransposed, a key advantage in producing an Alu subfamily. CRE motifs have been identified upstream of the 7SL gene and the EBER genes,

EBER1 and EBER2, from the Epstein-Barr virus that recruit Pol III machinery for their transcription in infected cells. The CRE motifs promote transcription of these genes *in vitro* and *in vivo* (Howe and Shu, 1989, Bredow et al., 1990, Felton-Edkins et al., 2006). The CRE motifs at the EBER genes were found to be occupied by ATF2 and ATF2 was not detected at 5S rRNA genes (which do not have CRE motifs) (Felton-Edkins et al., 2006). EBV infection activates MAPKs, promoting ATF2 phosphorylation and this may promote EBER gene transcription (Young and Rickinson, 2004, Felton-Edkins et al., 2006). More recently, ChIP-seq experiments have identified c-Jun and c-Fos at 76% and 86% of Pol III-bound genes respectively (Raha et al., 2010). The exact genes bound were not described in detail. In contrast, JunD, another c-Jun family member, was only present at 15% of Pol III-bound genes (Raha et al., 2010). Zhong and colleagues also identified c-Jun at a $tRNA^{Leu}$ gene and found the first evidence of dynamicity to this association (Zhong et al., 2011). Upon treatment of cells with ethanol, c-Jun occupancy and tRNA expression increased, although the requirement for c-Jun in increased tRNA expression was not proven.

In summary, AP-1 can regulate Pol III-mediated transcription by the regulation of Pol III basal transcription factor levels and may also contribute to Pol III activity directly by binding at Pol III target genes. In this chapter, the presence of AP-1 at Pol III target genes will be investigated further, with a specific focus on tRNA genes.

4.2 Results

4.2.1 c-Jun and c-Fos occupy tRNA genes

c-Jun and c-Fos are frequently present at Pol III target genes, as identified by Raha and colleagues in K562 cells (a human chronic myeloid leukaemia cell line) using ChIP-seq (Raha et al., 2010). This data was subsequently made publically available in the Gene Expression Omnibus (GEO) database (accession number GSE19551). We utilised this data for a more in-depth study of c-Jun and c-Fos occupancy at tRNA genes. Occupancy at 631 human tRNA genes was analysed (tRNA genes from hg18 Genomic tRNA Database (Chan and Lowe, 2009)). This total includes 109 predicted pseudogenes and 6 other tRNA of undetermined function. These were included in the analysis as they may help identify features of tRNA genes required for c-Jun and c-Fos recruitment. Of the 631 tRNA genes, c-Jun was found at 115 (18%) and c-Fos was found at 222 (35%) (Figure 4.1). Only three pseudogenes were occupied, two by c-Fos only and one by c-Jun and c-Fos. 86% of tRNA genes with c-Jun also had detectable c-Fos occupancy. The correlation of c-Jun and c-Fos binding at tRNA genes was found to be significant; a chi-squared test gave a p value of less than 0.001. This suggests that c-Jun and c-Fos may be present as a dimer.

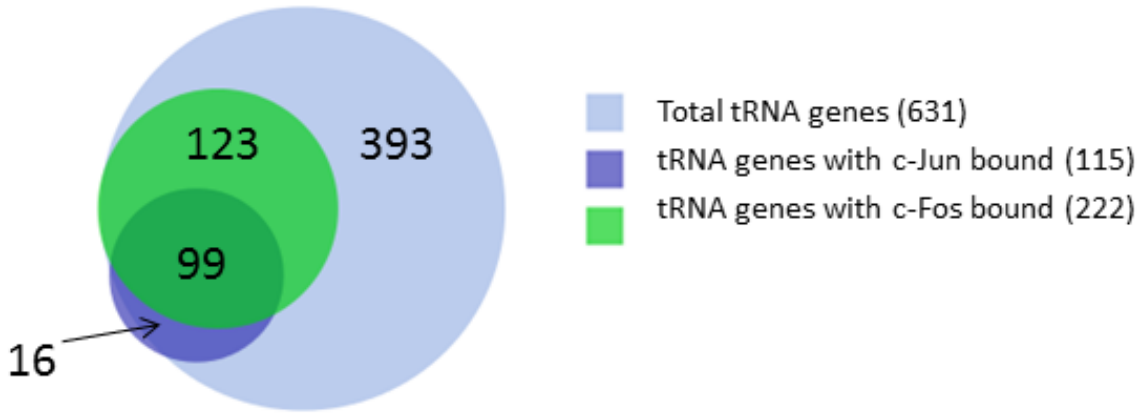

Figure 4.1 c-Jun and c-Fos occupy many tRNA genes

Venn diagram displaying the occupancy by c-Jun and c-Fos at tRNAs in K562 cells. ChIP-seq data mined from (Raha et al., 2010). Chi-squared test: $p<0.001$ for association between c-Jun and c-Fos binding.

To explore how widespread the occupancy of c-Jun and c-Fos is at tRNA genes, their presence was classified by tRNA isoacceptor. For example, $tRNA^{Leu}$ exists as five different isoacceptors, each with a different DNA anticodon but all charged with leucine. Most isoacceptors have multiple genes (Chan and Lowe, 2009). Figure 4.2 shows that for most isoacceptors, there is at least one gene with both c-Jun and c-Fos present. There are four isoacceptors at which only either c-Jun or c-Fos is present. There are no genes encoding $tRNA^{Ile}$-GAT, $tRNA^{Tyr}$-ATA and $tRNA^{Asn}$-ATT that have c-Jun and/or c-Fos present. However, c-Jun and c-Fos are present at genes that encode tRNAs specific for every amino acid.

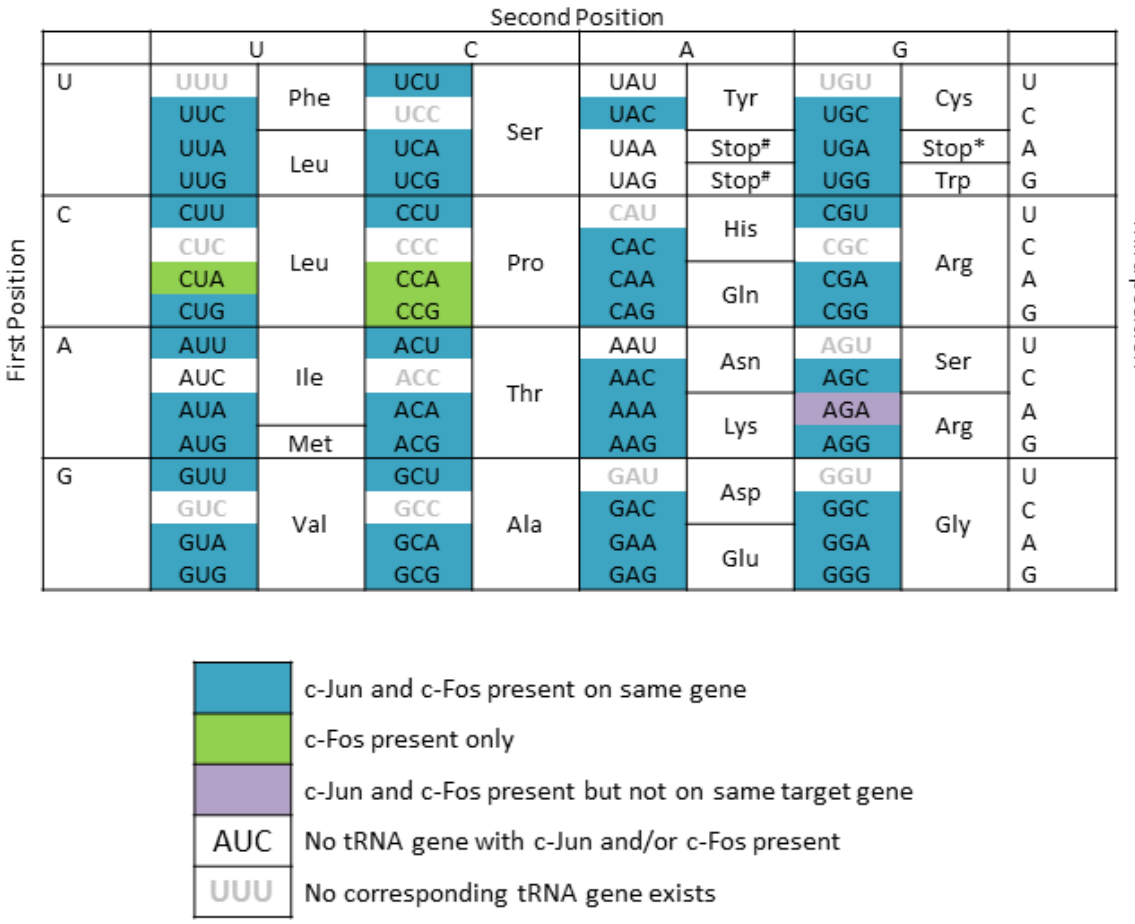

Figure 4.2 c-Jun and c-Fos are detected at genes encoding the majority of tRNA isoacceptors

Codon table highlighted according to c-Jun and c-Fos occupancy at genes encoding the tRNA with the complimentary anticodon. For example, if highlighted in blue, at least one gene for this isoacceptor class has both c-Jun and c-Fos present. *UGA - usually a stop codon, in a small number of proteins codes for selenocysteine. #Stop codon does not require tRNA. However, tRNA genes have been identified with the corresponding anticodons and are described as 'suppressor tRNAs'. ChIP-seq data mined from (Raha et al., 2010).

4.2.2 c-Jun and c-Fos occupancy correlates with occupancy of Pol III subunit RPC155

Presence of Pol III at tRNA genes is often used as a marker of actively transcribed genes. A ChIP-seq dataset from K562 cells is available for RPC155, a subunit of Pol III, from a different study (Moqtaderi et al., 2010). Of all tRNAs, 58% have Pol III bound (data displayed as Venn diagram Figure 4.3a). 53% of these Pol III occupied tRNA genes are also occupied by c-Jun and/or c-Fos (Figure 4.3b). Only 18% of tRNA genes occupied by c-Jun and/or c-Fos are not also occupied by Pol III. A Chi-squared test shows significant association between c-Jun/c-Fos occupancy and Pol III occupancy. However, 47% of Pol III-occupied genes do not have c-Jun and/or c-Fos bound. This suggests that, while c-Jun and c-Fos are associated with active tRNA genes, c-Jun and c-Fos recruitment is not simply a by-product of tRNA activity.

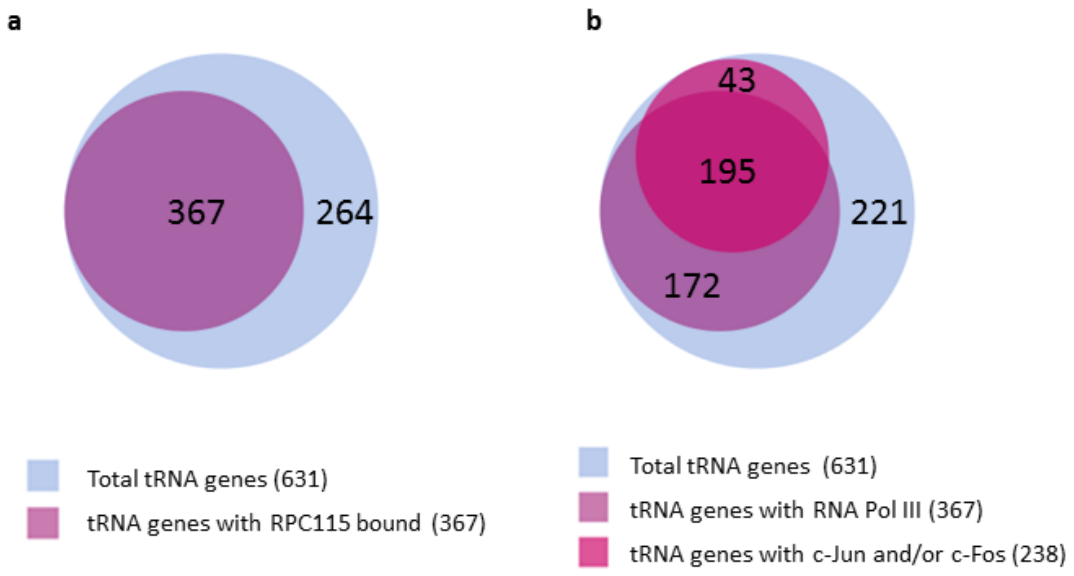

Figure 4.3 c-Jun and c-Fos occupancy correlates with Pol III occupancy

a) Venn diagram displaying the occupancy Pol III subunit RPC155 at tRNA genes in K562 cells. b) Venn diagram displaying the occupancy by RPC155 and c-Jun and c-Fos at tRNA genes in K562 cells. RPC155 ChIP-seq data from (Moqtaderi et al., 2010). c-Jun and c-Fos ChIP-seq data from (Raha et al., 2010). Chi-squared test gives $p<0.001$ for association between Pol III and c-Jun/c-Fos occupancy.

4.2.3 c-Jun and c-Fos occupancy at tRNA genes is not associated with the presence of a consensus AP-1 binding site nearby

To determine how c-Jun and c-Fos are recruited to tRNA genes, we examined the human genome for the incidence of AP-1 binding motifs TRE (TGACTCA) and CRE (TGACGTCA) within 1000bp and 250bp of tRNA genes. 12% of tRNA genes were found to have an AP-1 consensus site nearby (Figure 4.4) However, the presence of these sites at a gene was not associated with the occupancy of c-Jun and/or c-Fos (Figure 4.4a,b). Only 13% of tRNA genes with c-Jun and 9.5% with c-Fos also had an AP-1 motif nearby. This data suggests that c-Jun and c-Fos are not recruited to tRNA genes through binding to consensus binding motifs.

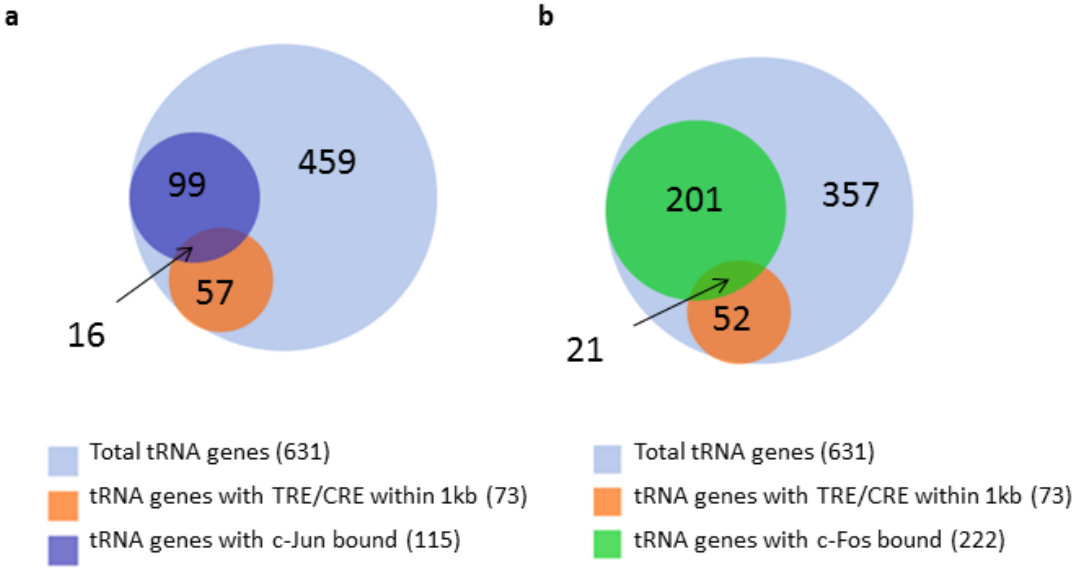

Figure 4.4 c-Jun and c-Fos occupy tRNA genes independently of AP-1 binding motifs

Venn diagram displaying tRNA genes with a TRE or CRE motif within 1kb and the occupancy by a) c-Jun and b) c-Fos at tRNAs in K562 cells. TRE/CRE incidence analysis in human genome carried out. ChIP-seq data mined from (Raha et al., 2010). Association between c-Jun binding and CRE/TRE motif presence and c-Fos binding and CRE/TRE presence is not significant according to Chi-squared analysis.

4.2.4 c-Jun associates with TFIIIC

To further explore the presence of c-Jun and c-Fos at tRNA genes, protein immunoprecipitation was employed to test for interactions with components of the Pol III pre-initiation complex. Coimmunoprecipitation of the TFIIIC subunit TFIIIC110 was detected when U2OS (a human osteosarcoma cell line) protein lysate was incubated with a c-Jun specific antibody, suggesting that c-Jun and TFIIIC can exist in the same protein complex (Figure 4.5a). This association was

confirmed in nuclear extract from HeLa cells (Figure 4.5b). In preliminary experiments, the reverse co-immunoprecipitation was also possible. c-Jun was associated with TFIIIC following immunoprecipitation using an anti-TFIIIC antibody (Figure 4.5c). Association with TFIIIC63 (another subunit of TFIIIC) was also detectable, however background level of antibody binding was high (data not shown). c-Jun interaction with the TFIIIB subunit Brf1 was also detected (data not shown). This data clearly demonstrates that c-Jun can associate with the Pol III machinery.

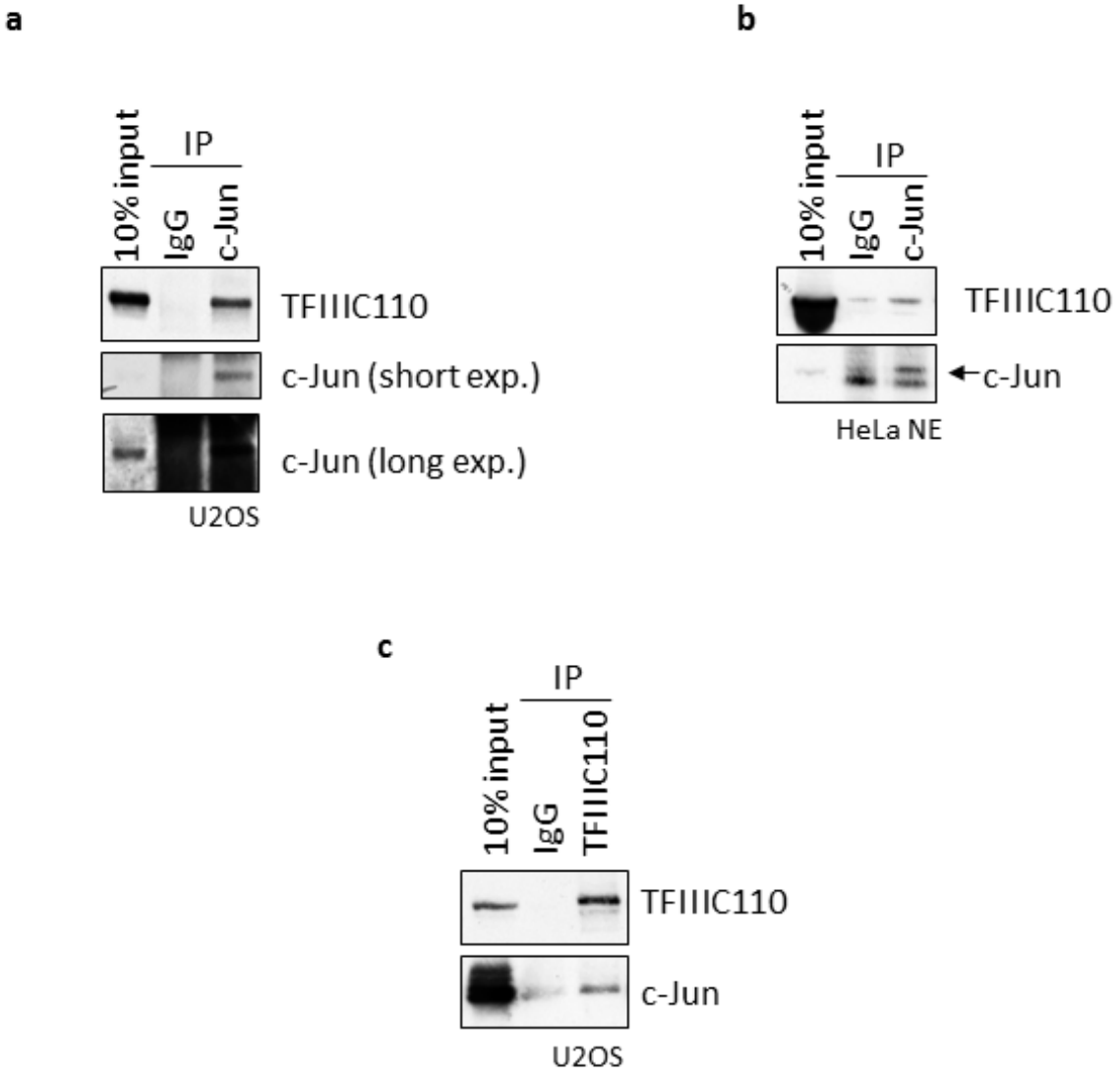

Figure 4.5 c-Jun associates with TFIIIC

Anti-c-Jun antibody used to immunoprecipitate c-Jun from a) U2OS whole cell protein extract and b) HeLa nuclear extract. Western blot analysis carried out on precipitate with antibodies against TFIIIC110 and c-Jun. c) Anti-TFIIIC110 antibody used to immunoprecipitate TFIIIC110 from U2OS whole cell protein extract. Western blot analysis carried out on precipitate with antibodies against TFIIIC110 and c-Jun. IgG used as control for non-specific binding.

4.2.5 c-Jun association with TFIIIC is independent of DNA

As both TFIIIC and c-Jun are known to bind DNA, it was important to determine whether their association may occur only via DNA rather than in a common protein complex. U2OS cell lysates were treated with DNase and then c-Jun was immunoprecipitated. As seen in Figure 4.6, the amount of TFIIIC110 co-immunoprecipitated in the DNase-treated sample (lane 6) was equal to that detected in the sample that was not subjected to DNase treatment (lane 5). Thus indicating that the association of c-Jun with TFIIIC110 does not occur through DNA but by protein-protein interaction.

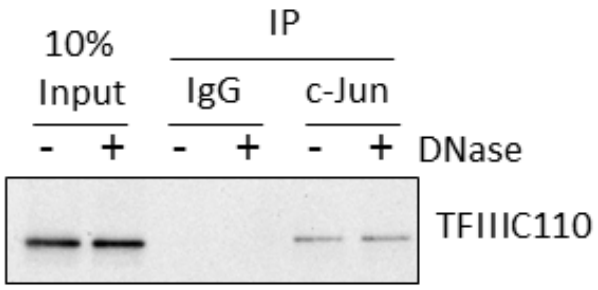

Figure 4.6 c-Jun association with TFIIIC is DNA-independent

U2OS protein extract incubated with DNase I (or mock) for 1hr at 37°C before immunoprecipitation with anti-c-Jun antibody. Followed by western blot analysis for TFIIIC110. IgG used as control for non-specific binding.

4.2.6 N-terminal deletion mutant of c-Jun (TAM67) can associate with TFIIIC

To begin to map the association between TFIIIC110 and c-Jun, an N-terminal deletion mutant of c-Jun, TAM67, was utilised. This mutant is missing amino acids 3-122 which includes the entire transactivation domain (Figure 4.7a). Lysates were made from U2OS cells that had been transiently transfected with a plasmid containing a gene encoding GFP-TAM67. Good protein expression was achieved as can be seen by comparing lanes 1 and 2 of Figure 4.7b. When GFP-TAM67 was immunoprecipitated using an antibody that recognises GFP, TFIIIC110 was present in the associated complexes (Figure 4.7b, lane 6). However, as GFP-

TAM67 can form a dimer with wild-type c-Jun, it is possible that this association is occurring via the full-length protein (Brown et al., 1993). To determine if the N-terminus of c-Jun is required for its interaction with the Pol III machinery, a c-Jun null cell line would be required. If c-Fos alone is capable of interacting with Pol III machinery then a c-Jun c-Fos double knockout cell line would be required. Alternatively, *in vitro* analysis could be carried out.

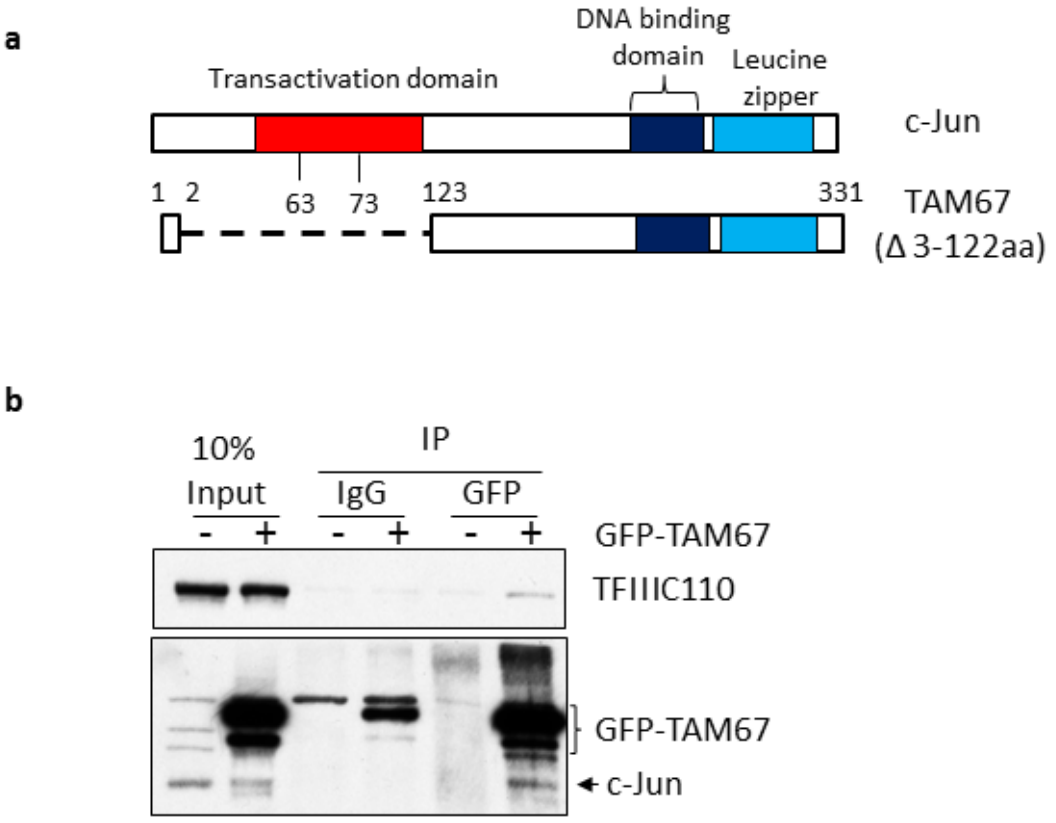

Figure 4.7 N-terminal deletion mutant of c-Jun (TAM67) can associate with TFIIIC

a) Diagram illustrating the domains of c-Jun (*top*) and TAM67 (*bottom*), a mutant of c-Jun lacking the N-terminus (including the transactivation domain). b) Anti-GFP antibody used to immunoprecipitate GFP-TAM67 from protein lysates from U2OS harvested 24 hours after transfection with pEGFP-C3-TAM67 or empty vector. Western blot analysis carried out on precipitate with antibodies against TFIIIC110 and c-Jun. Endogenous c-Jun (arrow) and GFP-TAM67 are detectable (brackets). IgG used as control for non-specific binding.

4.2.7 c-Fos may associate with TFIIIC

As c-Jun and c-Fos are both found at tRNA genes it seemed likely that they are present together in the form of an AP-1 heterodimer. On the basis of the experiments with c-Jun, as described above, it would be predicted that c-Fos would also associate with TFIIIC110. Indeed, preliminary data shows c-Fos can associate with TFIIIC110 in U2OS lysate (Figure 4.8).

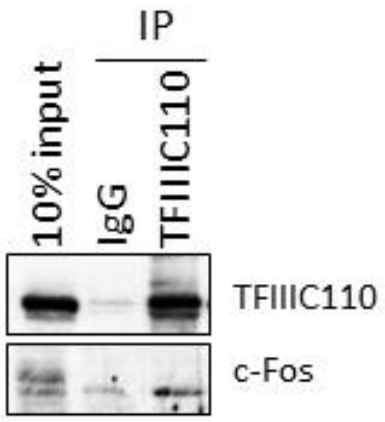

Figure 4.8 c-Fos may associate with TFIIIC

Anti-TFIIIC110 antibody used to immunoprecipitate TFIIIC110 from U2OS whole cell protein extract. Western blot analysis carried out on precipitate with antibodies against TFIIIC110 and c-Fos. IgG used as control for non-specific binding.

4.2.8 Phosphorylation of c-Jun at Serine 63/73 does not alter its association with TFIIIC

c-Jun is transcriptionally activated when phosphorylated at Serine 63 and Serine 73 in its transactivation domain. It is reasonable that this modification may also play a role in any function of c-Jun at tRNA genes. To test whether phosphorylation of c-Jun at Serine 63/73 alters interaction with TFIIIC, I repeated the co-immunoprecipitation experiments in lysates from cells that had been harvested 8 hours after treatment with UVC ($50J/m^2$). UVC treatment resulted in increased phosphorylation of c-Jun at Serine 63/73 (compare lanes 1 and 2 in Figure 4.9). However, under these conditions c-Jun association with TFIIIC110 did not alter (compare lanes 5 and 6).

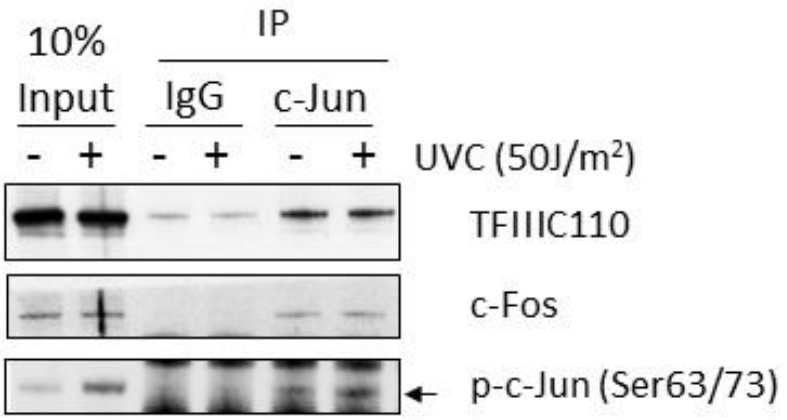

Figure 4.9 Phosphorylation of c-Jun at Serine 63/73 following UVC treatment does not alter c-Jun-TFIIIC association

Anti-c-Jun antibody used to immunoprecipitate c-Jun from protein lysate from U2OS exposed to UVC (50J/m^2) and harvested 8 hours later. Western blot analysis carried out on precipitate with antibodies against TFIIIC110, c-Fos and phospho-c-Jun (Ser63/73). IgG used as control for non-specific binding.

An alternative procedure to alter phosphorylation of c-Jun at Serines 63 and 73 was also used. Treatment with 20μM SP600125 (JNK inhibitor) for 1 hour resulted in reduced levels of phospho-c-Jun (Ser63/73) (compare lanes 1 and 2 in Figure 4.10). As was seen with UVC treatment, the altered levels of p-c-Jun (Ser63/73) had no effect on the association between c-Jun and TFIIIC110 (compare lanes 5 and 6). Technical difficulties prevented blotting of c-Jun directly following immunoprecipitation. It is therefore possible that the amount of c-Jun immunoprecipitated by the anti-c-Jun antibody alters with treatment, most likely due to changes in total protein level. This is unlikely following SP600125 treatment as the total c-Jun protein level does not change (Figure 5.1). However, whether c-Jun protein levels change following the UVC treatment remains to be determined. The amount of c-Fos co-immunoprecipitated with c-Jun does not appear to change with UVC treatment suggesting the amount of c-Jun immunoprecipitated may possibly also remain unchanged. These experiments suggest the association between c-Jun and TFIIIC does not alter with c-Jun phosphorylation at Serine 63/73, however further studies are required to confirm this.

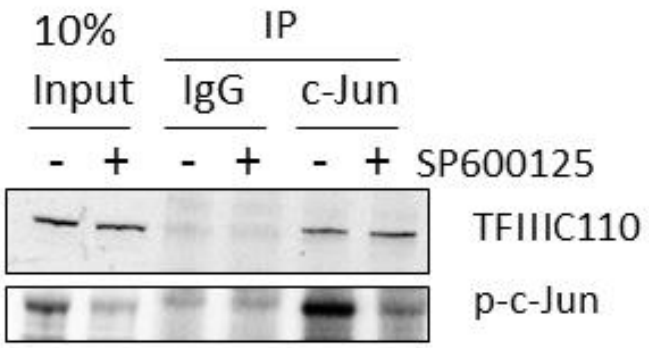

Figure 4.10 Dephosphorylation of c-Jun at Serine 63/73 following treatment with a JNK inhibitor does not alter c-Jun - TFIIIC association

Anti-c-Jun antibody used to immunoprecipitate c-Jun from protein lysate from U2OS following 1 hour treatment with JNK inhibitor SP600125 (20μM). Western blot analysis carried out on precipitate with antibodies against TFIIIC110 and phospho-c-Jun (Ser63/73). IgG used as control for non-specific binding.

4.2.9 Altered expression of TFIIIB protein components in c-Jun null cells stably expressing c-Jun or mutant c-Jun

In order to explore the functional role of c-Jun at tRNA genes, I first analysed the Pol III machinery and Pol III-mediated transcription in c-Jun null mouse embryonic fibroblasts (MEFs) stably transfected with plasmids encoding wild type c-Jun and c-Jun mutants (MacLaren et al., 2004). The mutants included: c-Jun S63/73A, in which the serine residues at positions 63 and 73 were replaced with alanine so they could not be phosphorylated – thus preventing transactivation by JNK; c-Jun Δ284-286 which is lacking amino acids 284-286 resulting in a non-functional DNA binding domain; TAM67-GFP, the N-terminal deletion mutant as described previously, and v-Jun, the oncogenic c-Jun mutant isolated from avian sarcoma virus - this does not require JNK mediated phosphorylation for it to be transcriptionally active. Levels of the TFIIIB subunits, Brf1 and TBP, were analysed by western blot as their expression is known to be regulated by c-Jun (Fromm et al., 2008, Zhong et al., 2011). Preliminary findings show slightly increased levels of Brf1 but not TBP in MEFs expressing wild type c-Jun compared to those expressing the vector alone (Figure 4.11, compare lanes 1 and 2). In MEFs expressing the S63/73A mutant, TBP protein levels were markedly reduced while Brf1 levels appeared similar to the empty vector control (compare lanes 1 and 3). TBP levels were also reduced in the Δ284-286 and TAM67 mutants, however Brf1 levels were higher than in cells expressing wild-

type c-Jun or vector only. In the MEFs expressing v-Jun, TBP were similar to those in the empty vector and wild-type c-Jun cells while Brf1 levels were higher than in empty vector cells and similar to wild-type c-Jun expressing cells. Therefore Brf1 and TBP levels are differentially regulated in response to c-Jun mutants. As TBP and Brf1 are often limiting factors in Pol III-mediated transcription, changes in their protein levels may contribute to differences in tRNA levels that may be seen in these cells, independently of c-Jun presence at tRNA genes.

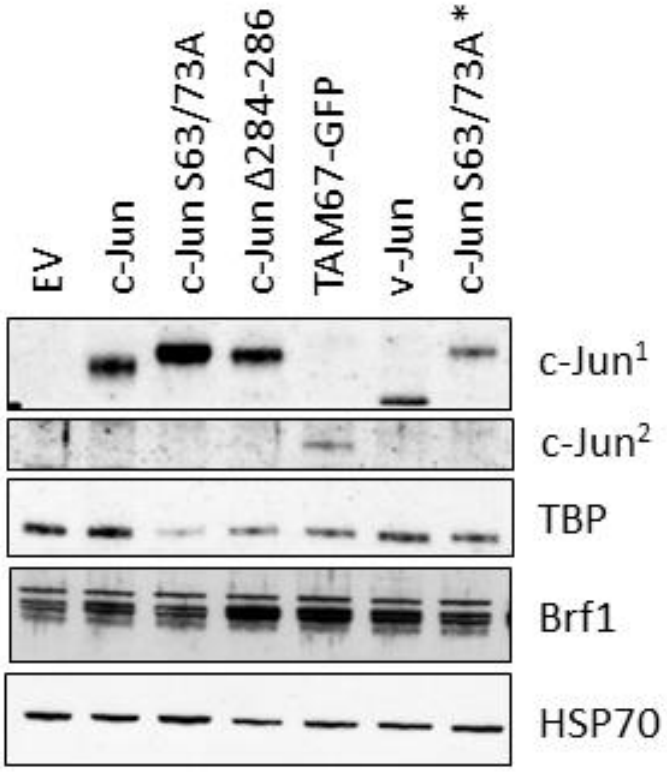

Figure 4.11 Altered expression of TFIIIB protein components in c-Jun null MEFs stably expressing c-Jun or mutant c-Jun

Analysis by western blot of c-Jun, TBP and Brf1 protein levels in c-Jun null MEFs stably expressing empty vector (EV), c-Jun, c-Jun S63/73A (phosphorylation site mutant), c-Jun Δ284-286 (DNA binding site mutant), TAM67-GFP (lacking N-terminus (including transactivation domain)) and v-Jun. Two antibodies against c-Jun were used: [1] raised against N-terminus, [2] raised against DNA binding domain. HSP70 used as a loading control. *Protein sample from c-Jun S63/73A expressing cells harvested at different time to other samples.

4.2.10 tRNA expression in c-Jun null cells stably expressing c-Jun or mutant c-Jun

tRNA levels in the c-Jun null MEFs were determined by qRT-PCR. Preliminary data shows that tRNA levels that do not correspond entirely with either Brf1 or TBP levels (Figure 4.12). tRNA levels are higher in cells expressing wild-type c-Jun compared to those expressing empty vector alone (and therefore containing no c-Jun). Of particular note, tRNA levels are highest in cells expressing the S63/73A mutant, despite these cells containing the lowest TBP level. tRNA expression in these cells appears to be dependent upon the amount of the mutant expressed. In cells harvested at a different time, when expression of the mutant was lower, tRNA levels were also lower and TBP levels slightly higher. Expression of c-Jun and c-Jun mutants in these cells are having an effect upon tRNA expression, however the mechanism for this is uncertain as other effects, such as the changes in TFIIIB expression described above, may contribute to the tRNA levels observed. Thus, these cells are not the best model for determining the role of c-Jun at tRNA genes.

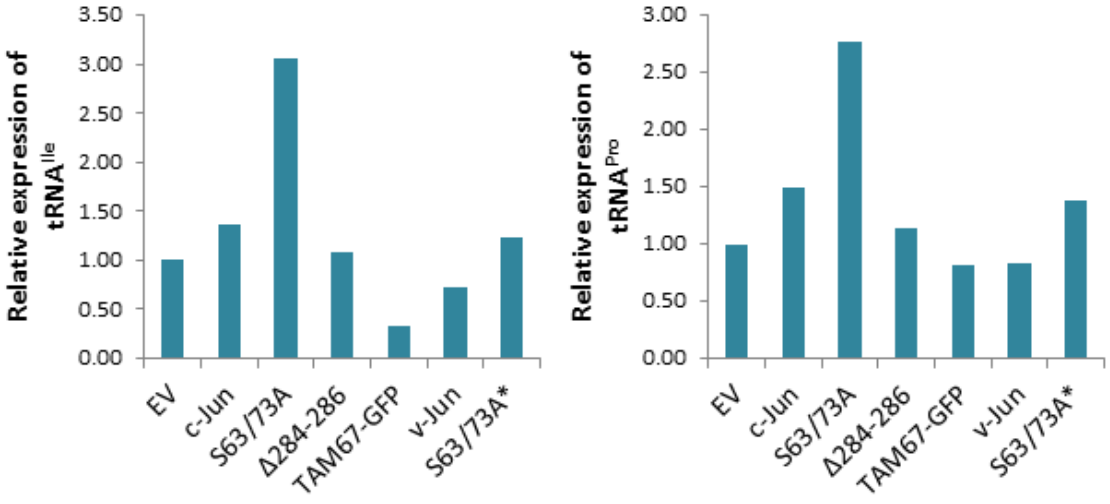

Figure 4.12 tRNA expression differs in c-Jun null MEFs stably expressing c-Jun or mutant c-Jun

Analysis by RT-qPCR of tRNA^{Ile} and tRNA^{Pro} in c-Jun null MEFs stably expressing empty vector (EV), c-Jun, c-Jun S63/73A (phosphorylation site mutant), c-Jun Δ284-286 (DNA binding site mutant), TAM67-GFP (lacking N-terminus (including transactivation domain)) and v-Jun. The expression was normalised to ARPP P0 mRNA levels. n=1 *RNA sample from c-Jun S63/73A expressing cells harvested at different time to other samples.

4.3 Discussion

Several studies have identified AP-1 proteins occupying Pol III target genes (Chesnokov and Schmid, 1996, Felton-Edkins et al., 2006, Raha et al., 2010, Zhong et al., 2011). The data presented by Raha et al. is especially enlightening as, through the employment of ChIP-seq technology, it provides a global view of c-Jun and c-Fos occupancy. Using this data as a basis, in this chapter I have explored AP-1 occupancy at tRNA genes.

c-Jun and c-Fos occupy a large proportion of tRNA genes (Figure 4.1). Nearly all those with c-Jun present also had c-Fos bound, suggesting they may be present as a c-Jun/c-Fos heterodimer. As ChIP-seq does not confirm that c-Jun and c-Fos are on the exact same gene in a single cell (mutually exclusive binding could give the same results), ChIP re-ChIP studies will be required to further support their presence as a dimer. As c-Jun and c-Fos are only present in cells as dimers, the only possibility that would not be excluded by ChIP re-ChIP is that two AP-1 dimers are present, one containing c-Jun and the other c-Fos. While this seems unlikely, it cannot be ruled out.

Interestingly, c-Fos occupies nearly twice as many tRNA genes as c-Jun. This could be due to a more sensitive c-Fos antibody: c-Jun could also be present on these genes but below detectable level. Alternatively, c-Fos could be paired with another protein. c-Fos can form dimers with the other JUN family members and various ATF and MAF family members (Eferl and Wagner, 2003). JunD can be ruled out as a likely partner at Pol III target genes, as it was only found at 15% of Pol III-bound genes (Raha et al., 2010). It will be interesting to examine other potential c-Fos partners in the future.

For overall regulation of tRNA activity, c-Jun and c-Fos would need to ensure changes in the expression of tRNAs that recognise all possible codons. c-Jun and c-Fos together occupy at least one gene for each tRNA isoacceptor, with a few exceptions (Figure 4.2). These exceptions are three isoacceptors with genes at which either only c-Jun or c-Fos is present and five isoacceptors with neither c-Jun nor c-Fos. For the former, it seems likely AP-1 is present as c-Jun and c-Fos cannot function except as part of AP-1. Either the AP-1 dimer present is not c-Jun/c-Fos or, simply, the other subunit was below the level of detection. Of

those isoacceptors with neither c-Jun nor c-Fos, two recognise codons that are usually stop codons. Stop codons do not require recognition by a tRNA, however genes have been identified encoding tRNAs with the corresponding anticodon. These are possible 'suppressor' tRNAs and their role, if they have one, is unknown (Beier and Grimm, 2001). Of the other three isoacceptors with neither c-Jun nor c-Fos, two, $tRNA^{Tyr}$-ATA and $tRNA^{Asn}$-ATT, have only one gene in the human genome (hg18) while $tRNA^{Ile}$-GAT has eight genes. However, in all cases the other isoacceptors for that amino acid can also recognise the corresponding codon according to 'wobble' rules – where a codon may be recognised by an anticodon despite the third base not being complementary according to traditional rules of base pairing. Thus, all codons encoding amino acid incorporation can potentially be recognised by a tRNA encoded by a gene with AP-1 present.

Excluding SINEs, tRNA genes make up the largest proportion of Pol III-transcribed genes in the human genome. Pol III occupancy is associated with gene activity and in *S. cerevisiae* all 286 tRNA genes are occupied by Pol III (Moqtaderi and Struhl, 2004, Harismendy et al., 2003, Roberts et al., 2003). In humans and mice, however, only a subset of tRNA genes is occupied (Moqtaderi et al., 2010, Oler et al., 2010, Barski et al., 2010). Microarray analysis has shown that tRNA levels vary with human tissue type (Dittmar et al., 2006). For example, $tRNA^{Trp}$-CCA is present in spleen tissue at significantly higher levels than brain tissue, while $tRNA^{Glu}$-TTC is higher in brain than spleen tissue. This is supported by ChIP-seq analysis which shows that there are differences in Pol III occupancy at tRNA genes, suggesting different tRNA genes are expressed, in different cell types (Barski et al., 2010, Raha et al., 2010, Oler et al., 2010). In a comparison of HeLa cells and $CD4^+$ T cells, 26% of tRNAs were occupied by Pol III in one cell type but not the other (Barski et al., 2010). How tRNA genes are regulated independently of one another is unclear as promoters are highly similar and regulators often target the Pol III machinery which has a more general effect on Pol III-mediated transcription. It is likely to require the recruitment of a regulator to some genes but not others, such as we see with c-Jun and c-Fos. Determining whether c-Jun and c-Fos may contribute to this will be very interesting.

AP-1 occupancy is highly associated with Pol III occupancy suggesting that c-Jun and c-Fos may activate or support the activation of tRNA genes (Figure 4.3). There are also many Pol III-occupied tRNA genes that do not have AP-1 present. This observation makes it unlikely that active tRNA genes passively recruit c-Jun/c-Fos simply because they are active. Instead, there must be a distinguishing feature between these two groups of Pol III-bound genes that results in AP-1 recruitment. This may be a binding motif or a protein with which AP-1 can interact. Whether AP-1, once recruited, contributes to gene regulation cannot be resolved with this data. An alternative viewpoint is that Pol III occupancy may not be associated directly with active transcription in this cell type. Instead it could indicate that the polymerase is 'poised', ready to transcribe but not yet transcribing. The data for this cell type (K562) is unclear, Moqtaderi et al. conclude that Pol III occupancy is associated directly with tRNA expression while Raha et al. support the poised hypothesis (Moqtaderi et al., 2010, Raha et al., 2010). Whichever is correct, AP-1 has definite association with 'active' genes. Pol III-mediated transcription has many regulators. It is therefore not surprising that 'active' tRNA genes exist that do not have AP-1 present, even if AP-1 does prove to promote transcription.

AP-1 has pro-proliferative activity (Shaulian and Karin, 2001, Eferl and Wagner, 2003). Therefore, it seems most likely that if it does regulate Pol III-mediated transcription it will be towards the outcome of increased protein synthesis (i.e. increased 5S, increased tRNAs) which is required for growth and proliferation. The association of c-Jun and c-Fos occupancy with Pol III occupancy supports this. The presence of c-Jun and c-Fos at many but not all tRNAs also provides an indication that they do indeed have a positive rather than negative effect on tRNA expression. It would theoretically be possible to support increased translation by increasing the transcription of a limited proportion of tRNA genes, as long as tRNAs were produced that could read all possible codons. However, repressing just a proportion of those already active would be unlikely to have a significant effect on translation, as it would simply result in increased availability of Pol III machinery at non-repressed genes. It is certainly not the most efficient method for repression. Well-established repressors of Pol III-mediated transcription, p53 and pRb, act by targeting Pol III machinery, thus

having a general effect on the transcription of Pol III target genes (Cairns and White, 1998, Crighton et al., 2003, Sutcliffe et al., 2000).

So, how are c-Jun and c-Fos recruited to some tRNA genes but not others? Two possible models exist: these genes have either an AP-1 binding motif nearby or a protein present at the gene to which AP-1 can bind. Flanking sequences are known to be important for optimal transcription of Pol III-transcribed genes despite their internal promoters (Dieci et al., 2007). We identified the consensus TRE (TGACTCA) and CRE (TGACGTCA) AP-1 binding motifs one kilobase up or downstream of 73 tRNA genes. However their presence was not associated with c-Jun or c-Fos occupancy at the nearby gene (Figure 4.4). Of those tRNA genes with TRE or CRE nearby, at only 20 genes was the motif within 250bp of the gene (data not shown). As AP-1 binding motifs are often 100-200bp upstream of their target genes (Conkright et al., 2003), the positioning of the motifs near tRNA genes would therefore be unusual if the tRNA gene was the target gene. It is, therefore, not unexpected that these AP-1 binding motifs do not account for AP-1 occupancy at tRNA genes. However, it is possible that these AP-1 motifs may have AP-1 present (ChIP-seq was not analysed for these sites) but this does not correlate with AP-1 occupancy at tRNA genes. Raha et al. found that c-Jun and c-Fos occupancy peaked at the transcription start site of Pol III-transcribed genes, where Pol III occupancy is also at its peak (Raha et al., 2010). This provides further evidence that the feature promoting AP-1 occupancy must be near the promoter site. The TRE motif is the AP-1 binding motif to which c-Jun and c-Fos dimers bind with the highest affinity, followed by the CRE motif (Nakabeppu et al., 1988). However, it remains possible that alternative consensus binding motifs may be responsible for AP-1 occupancy at tRNA genes. Given more time, it would be interesting to take the flanking regions from tRNAs of the three main groups (those with AP-1 and Pol III bound, those with Pol III only and those with neither) and carry out a motif search. This would identify any non-consensus or novel motifs that could be associated with AP-1 occupancy at tRNA genes. However, it is possible that an AP-1 binding motif is not required for recruitment of AP-1 to tRNA genes. There is precedent for this in the regulation of Pol III-mediated transcription. Though E-boxes (Myc binding motifs) are present near many Pol III target genes and c-Myc is also present at a

proportion of Pol III target genes, E-box presence does not correlate with c-Myc occupancy (Raha et al., 2010) (Kirsteen Campbell and R.J. White, unpublished).

Further clues as to how AP-1 may be recruited to tRNA genes come from the association observed between c-Jun and TFIIIC (Figure 4.5). This association was not dependent on DNA (Figure 4.6) and this is consistent with the previous finding that DNA binding sites may not be required for AP-1 occupancy (Figure 4.4). However, one can envision a scenario where AP-1 is recruited through binding to a motif and, once bound, can interact directly with the Pol III machinery. In this way, a motif may be required for complex assembly in the first instance but not required for complex maintenance. Coimmunoprecipitation experiments will detect association between c-Jun and TFIIIC anywhere in the cell, so it is possible that the association detected is not occurring on tRNA genes. However, the presence of c-Jun on tRNA genes suggests that this interaction is most likely to occur at target genes. In addition, preliminary observations suggest Brf1 is also present in complex with c-Jun. ChIP-seq experiments have shown that TFIIIC frequently occupies sites in the human genome independently of TFIIIB (Oler et al., 2010, Moqtaderi et al., 2010). Consequently, a complex containing c-Jun, TFIIIB and TFIIIC is most likely to be found at tRNA genes or other Pol III-transcribed genes rather than elsewhere in the cell.

Further studies are required to determine exactly which part of the Pol III machinery c-Jun is bound to and to determine the residues of c-Jun involved in this interaction. TBP, a component of TFIIIB, is already known to interact with c-Jun and c-Fos (Ransone et al., 1993). However, TBP is also part of the Pol I and II transcriptional apparatus, so this observation is not specific to Pol III. The finding that Brf1 may associate with c-Jun suggests that c-Jun and TBP may interact in the TFIIIB context. The association with TFIIIC could therefore occur via this known interaction with TBP. The N-terminus and bZIP domain of c-Jun interact with TBP (Franklin et al., 1995). It will be interesting to examine whether these regions are required for its association with the Pol III machinery.

Phosphorylation of c-Jun at Serine 63 and Serine 73 promotes its transactivation function (Meng and Xia, 2011). The phosphorylation status of c-Jun at these sites did not appear to alter its interaction with TFIIIC110 (Figure 4.9, Figure 4.10).

This suggests, if c-Jun transactivation function plays a role at tRNA genes, activity and localisation of c-Jun are independent of one another.

Pol II has been found upstream of many Pol III-transcribed genes (Oler et al., 2010, Barski et al., 2010, Raha et al., 2010). This was an unexpected finding and the reasons for its presence there are still not fully understood. It appears to promote Pol III-mediated transcription but is not required for Pol III-mediated transcription. Interestingly, Raha and colleagues found that c-Jun occupancy at tRNA genes resembled Pol III occupancy more than Pol II occupancy (which was further upstream of the TSS) (Raha et al., 2010). However, it is possible that Pol II may promote the recruitment of AP-1 (which is more typically a regulator of Pol II transcription). It will be interesting to further analyse the ChIP-seq data to see if an association exists between Pol II occupancy and c-Jun/c-Fos occupancy at tRNA genes.

In this chapter, it has been demonstrated that c-Jun and c-Fos occupancy is a common but not universal feature of tRNA genes. Therefore, AP-1 has the potential to regulate tRNA expression at two levels, as shown in Figure 4.13. It will be interesting to examine the occupancy of c-Jun and c-Fos at other Pol III target genes. The associated occupancy of these proteins suggests they may be present as a c-Jun/c-Fos AP-1 dimer. However, this dimer is not recruited to tRNA genes by the TRE and CRE binding motifs. Instead, it may occur through direct interaction with Pol III machinery. c-Jun and c-Fos occupancy is associated with active tRNA genes, suggesting it may contribute to the activation of tRNA expression. This is consistent with AP-1's role in the cell.

AP-1 is regulated in response to environmental stresses, growth factors and cytokines and contributes to the cell's response to these stimuli by regulating the expression of genes involved in many processes, most predominantly proliferation and cell death (Shaulian and Karin, 2001). The mitogen-activated kinases, ERK, JNK and p38, are the main mediators of c-Jun and c-Fos activation (Shaulian and Karin, 2001). Interestingly, ERK and JNK have both already been shown to positively regulate Pol III-mediated transcription (Felton-Edkins et al., 2003, Zhong and Johnson, 2009). The regulation of AP-1 activity at Pol III target genes may provide an additional route for the regulation of Pol III-mediated transcription by these kinases. The upregulation of Pol III-mediated transcription

in response to mitogenic factors is required for increased ribosome biogenesis, a prerequisite for cell growth (Ruggero and Pandolfi, 2003). In Chapter Five, I will examine whether JNK may regulate tRNA gene expression via phosphorylation of c-Jun at tRNA genes. The mechanism by which c-Jun could mediate changes in tRNA expression is also examined.

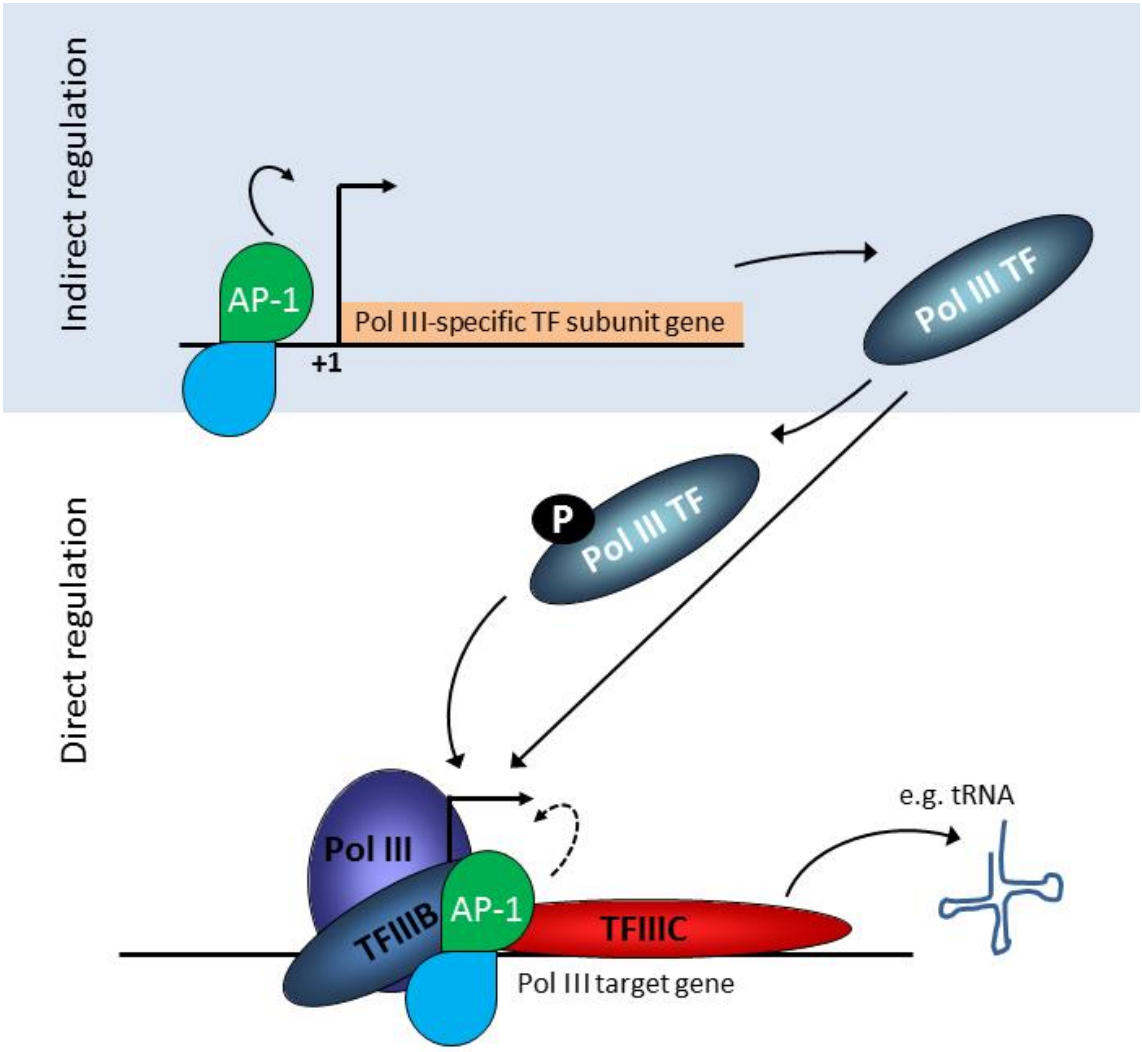

Figure 4.13 Model for regulation of Pol III-mediated transcription by AP-1

The diagram above represents possible routes by which AP-1 may regulate Pol III-mediated transcription. AP-1 subunits c-Jun and ATF2 have been identified bound at genes encoding TFIIIB and TFIIIC subunits (Zhong et al., 2011, Felton-Edkins et al., 2006) providing an indirect route for regulation of Pol III-mediated transcription via regulation of the availability of Pol III-specific transcription factors. AP-1 is also found at Pol III target genes including many tRNA genes, where its recruitment is independent of TRE and CRE AP-1 binding motifs. Instead it may be recruited through its association with the Pol III machinery, as described in this chapter. AP-1 occupancy at tRNA genes correlates with active transcription, suggesting AP-1 may promote Pol III-mediated transcription directly.

5 Regulation of RNA Polymerase III-mediated transcription by c-Jun N-terminal kinases (JNKs)

5.1 Introduction

Protein synthesis is required for nearly all cellular activities. The overall requirement for protein synthesis changes with the state of the cell. In highly proliferating cells, there are higher levels than during quiescence or in cells arrested due to checkpoint activation (Clarke et al., 1996, Johnson et al., 1974, Mauck and Green, 1974). It would be wasteful to produce Pol III products at high levels in the latter cases, but they would be required at high levels in the former case. JNKs are interesting cellular effectors as they can be activated by signals that promote each state (Davis, 2000). They are activated by mitogens and contribute to the highly proliferative state. But they are also activated in response to many stress signals. Interestingly, JNKs have been identified as regulators of Pol III-mediated transcription and could, therefore, contribute to changes in Pol III activity in these conditions (Zhong and Johnson, 2009, Zhong et al., 2011).

Recent work by Zhong and colleagues has shown that JNK can regulate Pol III-mediated transcription by altering the expression of components of the Pol III-specific transcription factor TFIIIB (Zhong and Johnson, 2009). In JNK1-null MEFs, the expression of $tRNA^{Leu}$ and 7SL was half that of wild type MEFs, suggesting JNK1 has a positive effect on Pol III-mediated transcription. JNK2 appears to have an opposing effect as, in JNK2-null MEFs, $tRNA^{Leu}$ and 7SL are significantly higher than in the wild-type cells. These changes in expression corresponded with changes in TBP and Brf1 mRNA and protein levels. Elk1, a JNK-targeted transcription factor, can occupy TBP and Brf1 gene promoters (Zhong et al., 2007, Zhong and Johnson, 2009). This occupancy alters with JNK activity and the authors suggest that Elk1 mediates JNK-dependent changes in TBP and Brf1 expression. Zhong and Johnson also show Bdp1 expression is regulated by TBP protein level and, thus, all three subunits of TFIIIB are coregulated. In converse experiments, the authors activate JNK using anisomycin and examine the Pol III output. Anisomycin is a bacterially-derived compound that can activate JNKs and another MAPK, p38 (Hazzalin et al., 1998). This treatment resulted in a 2-fold increase in expression of a tRNA gene reporter in wild-type and JNK2-null MEFs,

but no change in JNK1-null MEFs was observed. This suggests JNK1 is required for the increased expression. The authors do not further explore the changes in Pol III-mediated transcription that occur following anisomycin treatment. However, it is interesting to note that expression was measured only 30 minutes after treatment with anisomycin. It seems unlikely that this is due to changes in TFIIIB subunit levels, which would be expected to take longer than 30 minutes to achieve and impact Pol III-mediated transcription. A possibility, not excluded by the Zhong study, is that JNK has a direct effect on Pol III-mediated transcription i.e. it regulates Pol III-mediated transcription without the regulation of another gene as an intermediary.

Further observations also support a more direct role for JNK in Pol III-mediated transcription. As described in Chapter 4, the paradigm JNK target, c-Jun, is present at many Pol III-transcribed genes. c-Jun requires phosphorylation at its N-terminus by JNK for full activation so if c-Jun does regulate Pol III transcription initiation it is reasonable to suppose that JNK activity may be required. Like c-Jun, STAT1 has also recently been identified at many Pol III-transcribed genes (Oler et al., 2010). STAT1 is a transcription factor that has increased activity following its phosphorylation by JNK, however its function at Pol III-transcribed genes is so far undetermined. While investigating Elk1's role in regulating TFIIIB expression, Zhong and colleagues also observed Elk1 at a $tRNA^{Leu}$ gene (Zhong et al., 2011). Though it is unclear how widespread Elk1 occupancy is at Pol III target genes, it provides another potential route for JNK regulation of Pol III-mediated transcription. c-Myc binds to and regulates the expression of Pol III target genes (Gomez-Roman et al., 2003). c-Myc is also phosphorylated by JNK, though whether this contributes to its activity at Pol III target genes has not been determined (Noguchi et al., 1999). Further JNK targets may also be identified at Pol III target genes. The phosphorylation of any of these by JNK has the potential to influence Pol III activity. The relevance of the presence of JNK targets at Pol III genes on Pol III-mediated transcription remains to be determined.

Alternatively, JNK could regulate Pol III-mediated transcription through phosphorylation of the Pol III machinery like its fellow MAPK, ERK. ERK can phosphorylate Brf1 following serum stimulation (Felton-Edkins et al., 2003). This phosphorylation promotes TFIIIB interaction with TFIIIC and Pol III and, thus,

activates Pol III-mediated transcription. ERK targets Ser145 on Brf1; however the authors found ERK activity only partially accounts for phosphorylation at this site suggesting another kinase may be involved. Alternatively, Bdp1 and TBP have also been shown to undergo modifications that alter their activity (Hu et al., 2004). JNK has some precedent for phosphorylating polymerase-associated transcription factors. It can phosphorylate TIF-IA, reducing TIF-IA's ability to recruit Pol I to 45S rRNA genes and, therefore, causing reduced rRNA levels (Mayer et al., 2005).

JNK has recently been identified as a histone modifier in a study analysing the role of JNK in neuronal development (Tiwari et al., 2011). It binds at a variety of genes in mouse ES cells, mouse neuronal progenitors and terminally differentiated neurons. Treatment of terminally differentiated neurons with JNK inhibitor SP600125 resulted in reduced expression of JNK-bound genes and reduced phosphorylation at Serine 10 of histone H3 present at these genes. The authors show that JNK can carry out this phosphorylation. Histone H3 Ser10 phosphorylation is associated with active gene transcription at Pol II and Pol III transcribed genes (Sawicka and Seiser, 2012, Zhong et al., 2013a). Therefore, another potential route by which JNK could regulate tRNA expression is through direct modification of the chromatin environment at tRNA genes.

The experiments in this chapter were primarily carried out in the osteosarcoma cell line U2OS. In Chapter 4, I showed that c-Jun and c-Fos are associated with Pol III machinery in these cells (Figure 4.5, Figure 4.8). U2OS have active JNK under normal growth conditions (Figure 5.1). Indeed, bone cancers often have increased JNK activity and this is associated with tumour progression (Eferl and Wagner, 2003). The presence of active JNK in U2OS means that the function of JNK can be investigated through inhibition of basal activity, without prior activation by a JNK stimulant such as anisomycin or UV. This has been done in the literature previously in HCT116 cells which also contain active JNK (Aguilera et al., 2011).

One of the primary difficulties when studying JNK is to differentiate between the function of JNK1, JNK2 and JNK3 (and their different isoforms). JNK3 is not considered here as its expression is limited to the brain, heart and testis. While initially considered to be redundant, it is now well demonstrated that JNK1 and

JNK2 have independent functions (Bode and Dong, 2007). Indeed, as described above, JNK1 and JNK2 appear to have opposing effects in regulating the expression of TFIIIB (Zhong and Johnson, 2009). In the results section below, I refer to JNK as a singular for ease of description and because I have not attempted to distinguish the effects of the different JNK proteins and isoforms. My main tool is the JNK inhibitor SP600125 which has been measured to inhibit both proteins equally *in vitro* (Bennett et al., 2001, Bain et al., 2003). As such, the overall effect of JNKs on Pol III-mediated transcription will be determined. Given further time, this study would be extended to determine the role of the individual JNKs.

The objectives of the experiments carried out in this chapter were to determine if JNK kinases may regulate tRNA expression by phosphorylating a target to be recruited to or already at the tRNA gene. It was necessary to differentiate between these effects and JNK's effect on Pol III machinery levels, as already established by Zhong and Johnson. In particular, I aimed to establish whether the c-Jun present at tRNA genes, as described in Chapter 4, played a functional role at these genes.

5.2 Results

5.2.1 JNK inhibition causes reduction in tRNA levels

Previous studies have shown that JNK is able to regulate Pol III-mediated transcription by modulating the expression of TBP, Brf1 and Bdp1 (Zhong and Johnson, 2009). Influenced by the observation that c-Jun and several other JNK targets can be found at tRNA genes, I decided to explore whether JNK may have a more direct role in regulating tRNA expression. As this effect would be mediated by protein phosphorylation I hypothesise it would occur with much faster kinetics than an effect that requires protein synthesis. A reversible, ATP-competitive JNK inhibitor, SP600125, was employed to observe the effect of JNK inhibition on Pol III products before the change in TFIIIB levels occurred.

U2OS were primarily used for these studies as they have a functional JNK pathway that is active under normal growth conditions. As seen in Figure 5.1, a reduction in phosphorylation of c-Jun at the JNK target site Serine 63 was observed following treatment with 20μM SP600125 for 15 minutes, when compared to the DMSO-treated control. After 60 minutes of treatment, a reduction in c-Jun mRNA was also detected (Figure 5.2). This was as expected following the reduction in transcriptionally active c-Jun (i.e. phosphorylated c-Jun) as c-Jun acts in a positive feedback loop to promote transcription of the c-Jun gene (Angel et al., 1988). There is no detectable difference in c-Jun protein levels after 60 minutes of treatment (Figure 5.1).

Figure 5.1 JNK inhibitor, SP600125, reduces c-Jun phosphorylation (Ser63) in U2OS

Analysis by western blot of c-Jun and phospho-c-Jun(Ser63) protein levels in U2OS following treatment with 20μM SP600125 or DMSO. Cells were harvested at 15, 30 and 60 minutes post-treatment. HSP70 used as a loading control.

A significant reduction in tRNA levels was observed within 15 minutes of addition of the JNK inhibitor, as measured by RT-qPCR (Figure 5.2). Reduced c-Jun phosphorylation by this time point allows for the possibility that c-Jun is the JNK target involved in this effect. Following one hour of treatment, the tRNA levels had decreased further to 50-60% compared to those in the DMSO-treated cells. This rapid effect did not occur through changes in levels of TBP and Brf1 subunits as these remained unchanged during the treatment (Figure 5.3). Changes in TBP and Brf1 mRNA levels were also not observed in this short time course (Figure 5.2).

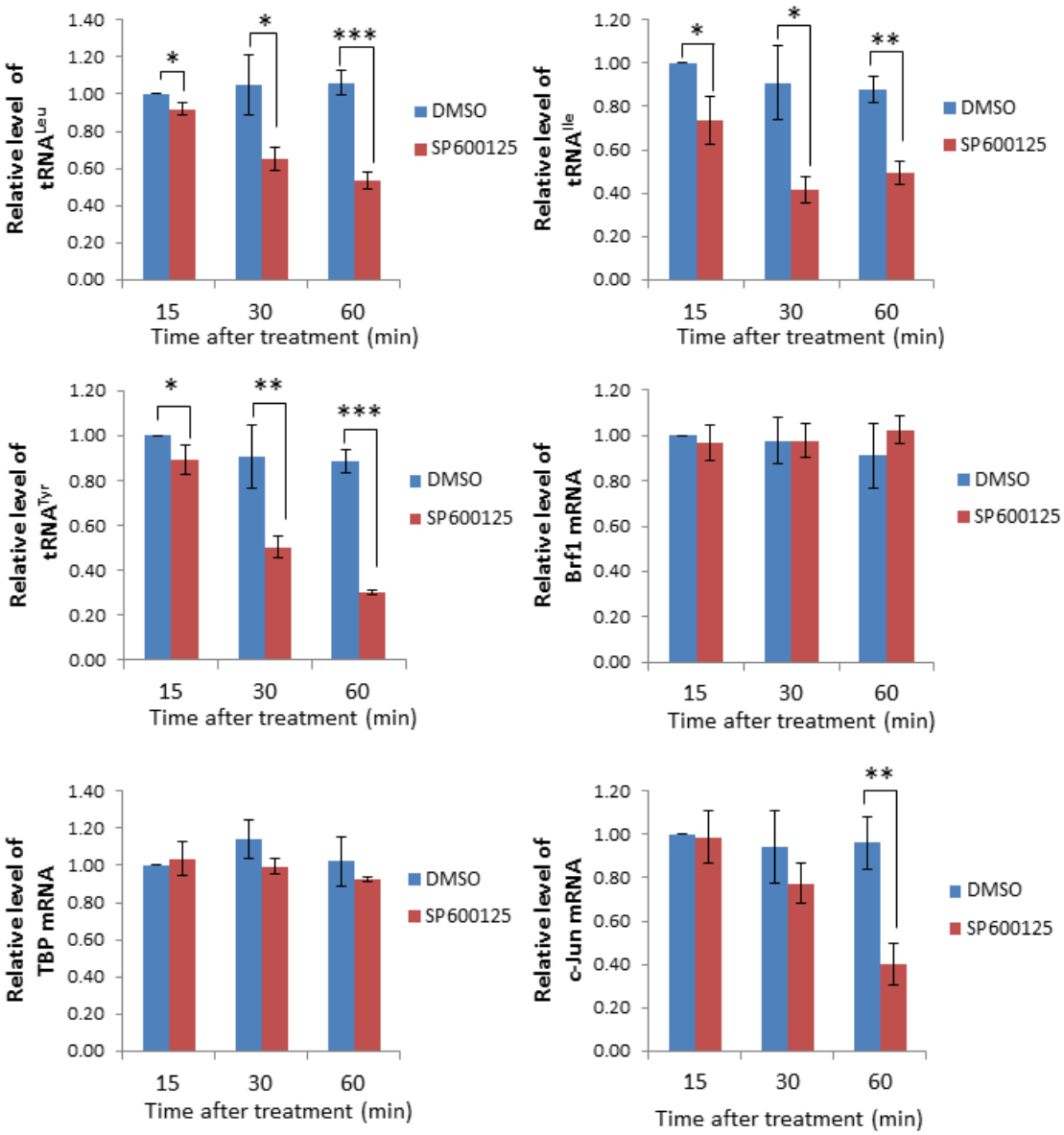

Figure 5.2 Short treatment with SP600125 significantly reduces tRNA levels, while TFIIIB subunit mRNA levels remain unchanged

Analysis by RT-qPCR of tRNA^{Leu}, tRNA^{Tyr}, tRNA^{Ile}, Brf1 mRNA, TBP mRNA and c-Jun mRNA levels in U2OS following treatment with 20μM SP600125 or DMSO. Cells were harvested at 15, 30 and 60 minutes post-treatment. The expression was normalised to ARPP P0 mRNA levels. *p<0.05, **p<0.01, ***p<0.001 n=3

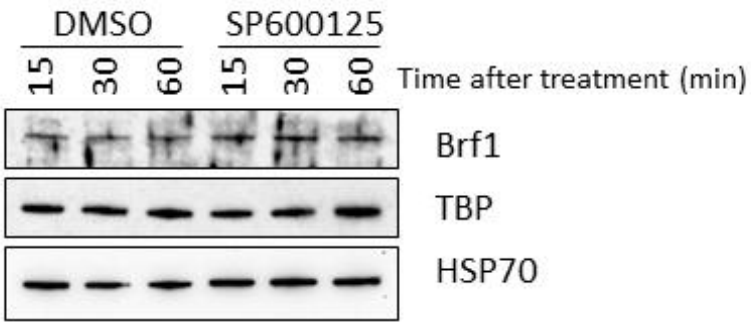

Figure 5.3 TFIIIB subunit protein levels remain unchanged after short JNK inhibitor treatment

Analysis by western blot of Brf1 and TBP protein levels in U2OS following treatment with 20μM SP600125 or DMSO. Cells were harvested 15, 30 and 60 minutes post-treatment. HSP70 used as a loading control.

To determine if JNK can regulate TFIIIB subunit expression levels in U2OS, as demonstrated previously by Zhong and colleagues in MEF and Huh-7 (hepatocellular carcinoma cell line) cells, a longer treatment was carried out. Protein lysates and RNA were harvested after 9 hours treatment with 20μM SP600125. This treatment resulted in a reduction in phospho-c-Jun and total c-Jun protein levels (Figure 5.4). c-Jun mRNA levels were also significantly reduced following treatment (Figure 5.5). tRNA levels were significantly reduced, to a similar level to that seen after 60 minutes treatment (Figure 5.5, Figure 5.2) TBP and Bdp1 mRNA levels were reduced compared to the control samples; however Brf1 mRNA levels did not differ between DMSO and SP600125 treated cells. Preliminary western blot findings suggest both TBP and Brf1 protein levels remain unchanged (Figure 5.4), suggesting the mechanism described by Zhong and Johnson may not occur in U2OS.

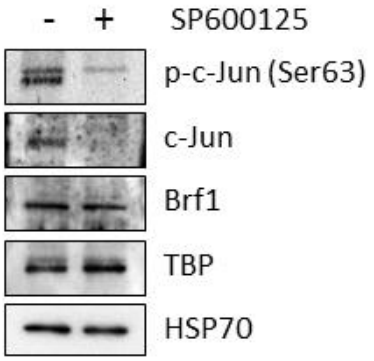

Figure 5.4 Longer treatment with SP600125 reduces total c-Jun levels

Analysis by western blot of c-Jun, phospho-c-Jun(Ser63), TBP and Brf1 protein levels in U2OS following treatment with 20μM SP600125 or DMSO. Cells were harvested 9 hours post-treatment. HSP70 used as a loading control.

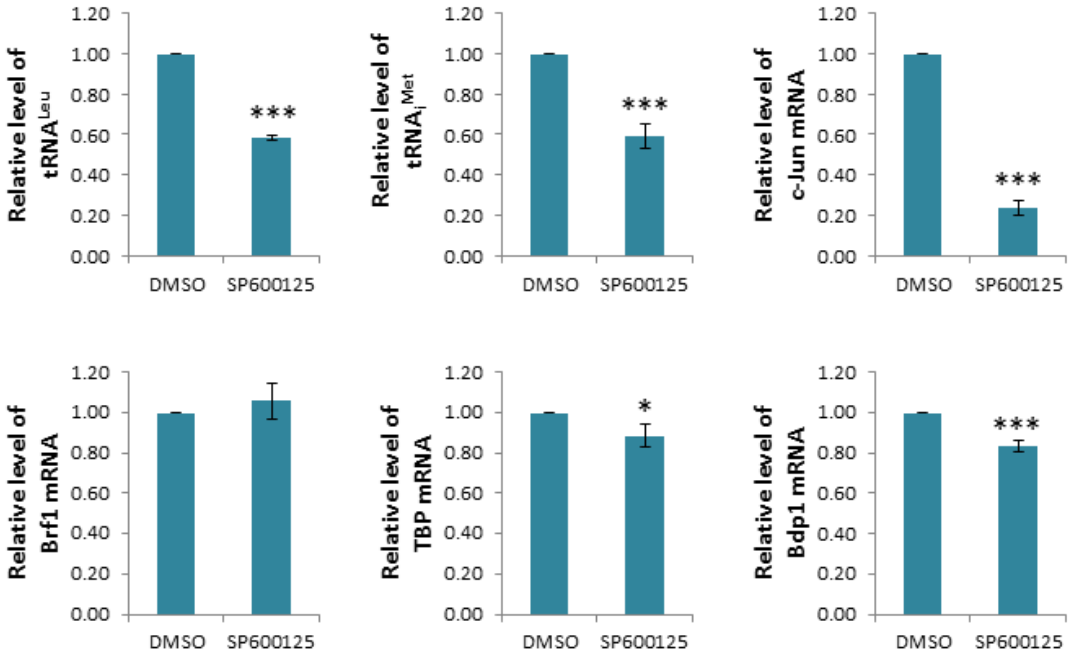

Figure 5.5 Longer treatment with SP600125 significantly reduces tRNA levels and TFIIIB subunit mRNA levels

Analysis by RT-qPCR of $tRNA^{Leu}$, $tRNA_i^{Met,}$ Brf1 mRNA, TBP mRNA and c-Jun mRNA levels in U2OS following treatment with 20μM SP600125 or DMSO. Cells were harvested 9 hours post-treatment. The expression was normalised to ARPP P0 mRNA levels. *p<0.05, ***p<0.001 n=3

5.2.2 Anisomycin treatment results in increased tRNA levels

Based on the above results, we hypothesised that the activation of JNK would cause a rapid increase in tRNA levels. To examine whether this was the case, tRNA levels were measured by RT-qPCR following treatment with anisomycin, a known activator of JNK activity. A treatment curve indicated that 30 minutes of treatment with 100nM anisomycin was sufficient to activate JNK, as phospho-c-Jun(Ser63) and phospho-JNK levels rose above background with this treatment (Figure 5.6). The p-JNK antibody detects the phosphorylated forms of the 54kDa and 46kDa isoforms of JNK. These are the top two bands visible in the blot. A third band also appears to increase following anisomycin treatment; however its identity is unknown (Figure 5.7a).

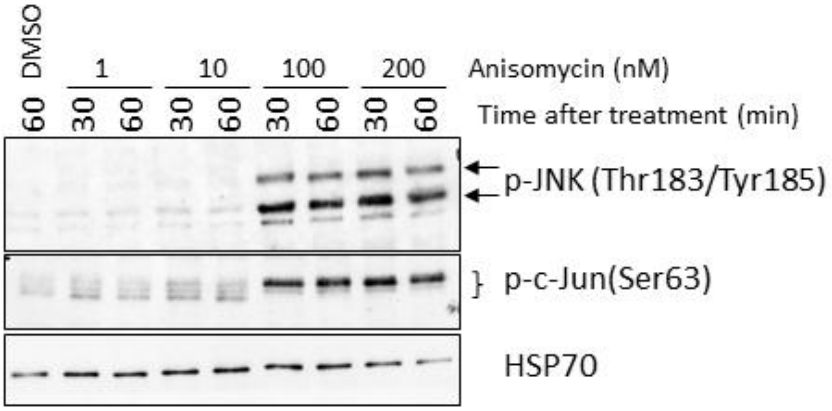

Figure 5.6 Determining the concentration of anisomycin required to induce JNK activity

Analysis by western blot of phospho-JNK(Thr183/Tyr185) and phospho-c-Jun(Ser63) protein levels in U2OS following treatment with anisomycin at 1nM, 10nM, 100nM and 200nM. Cells were harvested 30 and 60 minutes post-treatment. HSP70 used as a loading control.

Preliminary results show that c-Jun mRNA levels increased following 100nM anisomycin treatment for 30 minutes, indicative of increased c-Jun activity (Figure 5.7c). $tRNA^{Tyr}$ levels also increase with 100nM or 200nM of anisomycin (Figure 5.7c). Interestingly, $tRNA^{Leu}$ levels do not appear to change. Brf1 protein levels do not change with anisomycin treatment according to preliminary findings (Figure 5.7b). TBP levels appear to go down, however an upwards shift in the TBP band upon treatment suggests it may be undergoing modification. As the tRNA level changes seen after anisomycin treatment are not concordant with changes in TFIIIB subunit levels, it suggests that tRNA levels may be regulated via a different mechanism to the one described by Zhong and Johnson.

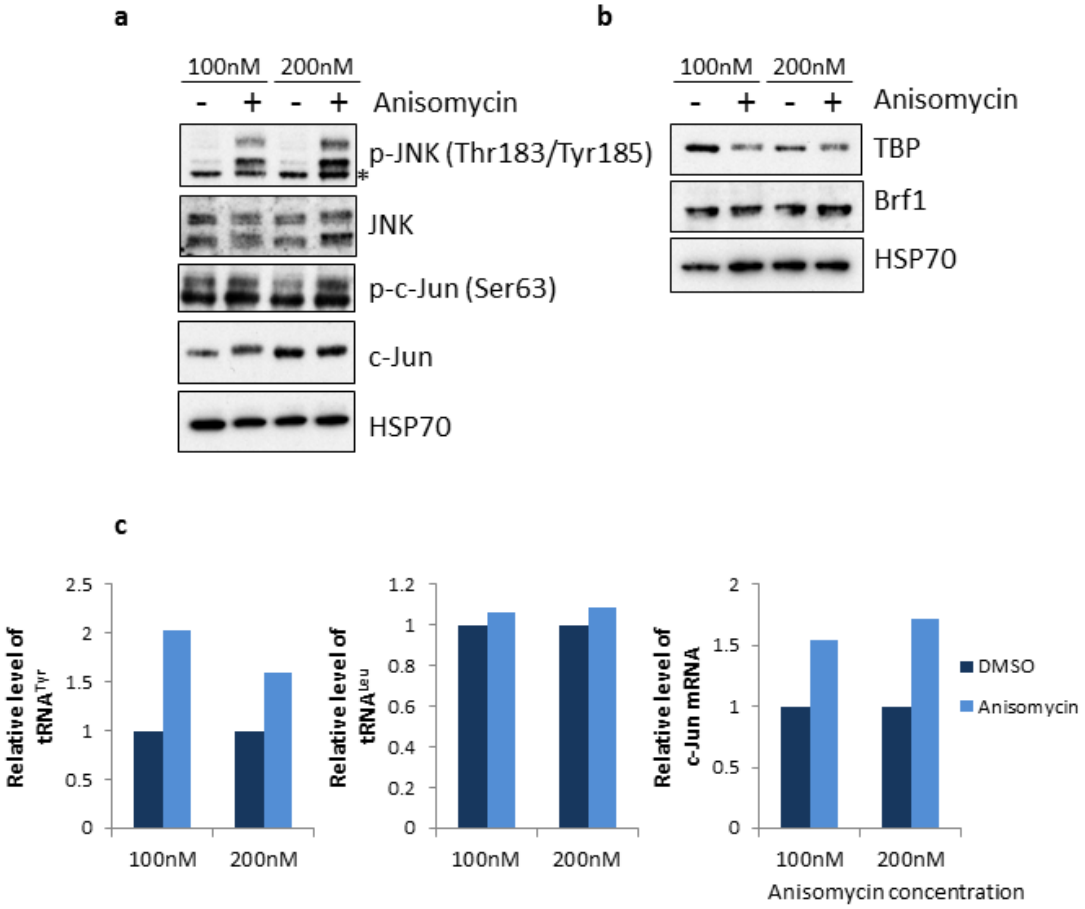

Figure 5.7 Anisomycin treatment may increase tRNA levels

Analysis by western blot of a) JNK, phospho-JNK(Thr183/Tyr185), c-Jun and phospho-c-Jun(Ser63) protein levels and b) Brf1 and TBP protein levels in U2OS following treatment with anisomycin at 100nM and 200nM. Cells were harvested 30 minutes post-treatment. HSP70 used as a loading control. * indicates non-specific band. n=1 c) Analysis by RT-qPCR of $tRNA^{Leu}$, $tRNA^{Tyr}$ and c-Jun mRNA levels in U2OS following treatment with 100nM or 200nM anisomycin or DMSO. Cells were harvested 30 minutes post-treatment. The expression was normalised to ARPP P0 mRNA levels. n=2

5.2.3 Pol III occupancy is reduced at tRNA genes following SP600125 treatment

To further examine what effect the JNK inhibitor SP600125 was having on tRNA genes, the occupancy of Pol III and the associated transcription machinery at tRNA genes were examined by chromatin immunoprecipitation (ChIP). Results from multiple experiments were difficult to combine due to differences in the percentage of input of chromatin associated with each antibody between experiments indicating an interexperimental difference in efficiency. However, Pol III occupancy reproducibly went down at tRNA genes following 60 minutes of treatment with 20μM SP600125 (see representative ChIP in Figure 5.8a). This corresponds with the reduction in tRNA levels that was determined by RT-qPCR (Figure 5.2) and, as Pol III occupancy is associated with gene activity, provides evidence that the reduction in tRNA levels is a transcriptional effect. Preliminary studies show reduced TBP and Brf1 occupancy at tRNA genes following SP600125 treatment (Figure 5.8b). This would explain the reduction in Pol III occupancy, as TFIIIB recruits Pol III to tRNA genes.

5.2.4 Examining histone occupancy and histone modification at tRNA genes following JNK inhibition

As with Pol II-transcribed genes, histone occupancy and histone modification at Pol III-transcribed genes are associated with gene activity. Examination by chromatin immunoprecipitation showed that H3 occupancy increased upon SP600125 treatment (Figure 5.9). This is consistent with the movement of a nucleosome back into the gene from which it had previously been excluded. Preliminary findings also suggest an increase in the association of histone deacetylases 1 and 2 (HDAC1 and HDAC2) with tRNA genes upon JNK inhibition (Figure 5.9). This is also consistent with reduced gene activity. Histone acetylation itself appears to go down, but only slightly (Figure 5.9). Titration of the antibody should give us information as to whether this is a true reflection of the amount of acetylated histone at the tRNA genes at this time point.

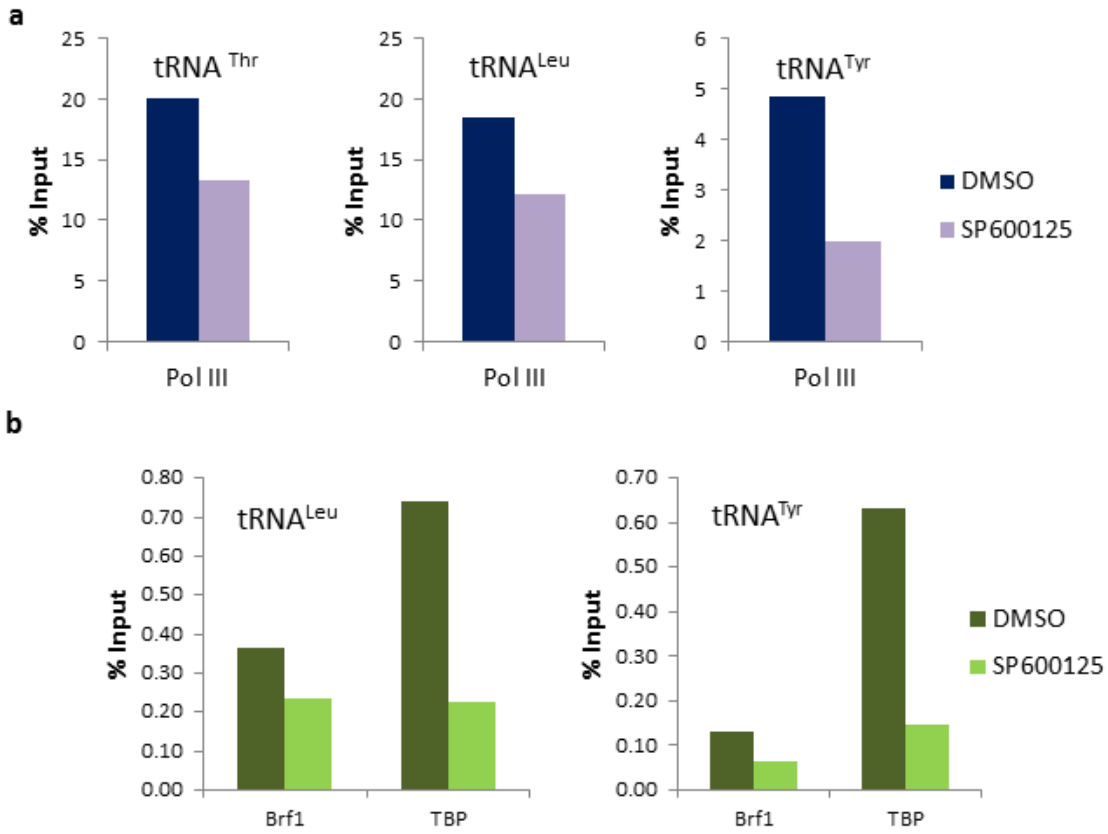

Figure 5.8 SP600125 reduces Pol III and TFIIIB occupancy at tRNA genes

Chromatin immunoprecipitation assays were carried out in U2OS cells with antibodies against a) Pol III subunit, RPC155 (reduced Pol III occupancy at tRNA genes observed upon multiple experiments, representative data shown) and b) TFIIIB subunits TBP and Brf1 (n=1) at tRNA loci following treatment with 20μM SP600125 for 1 hour. ChIP with TAF_I48 used as a negative control. % input calculated: [(IP/input)x100] – [(TAF_I48 IP/input)x100]

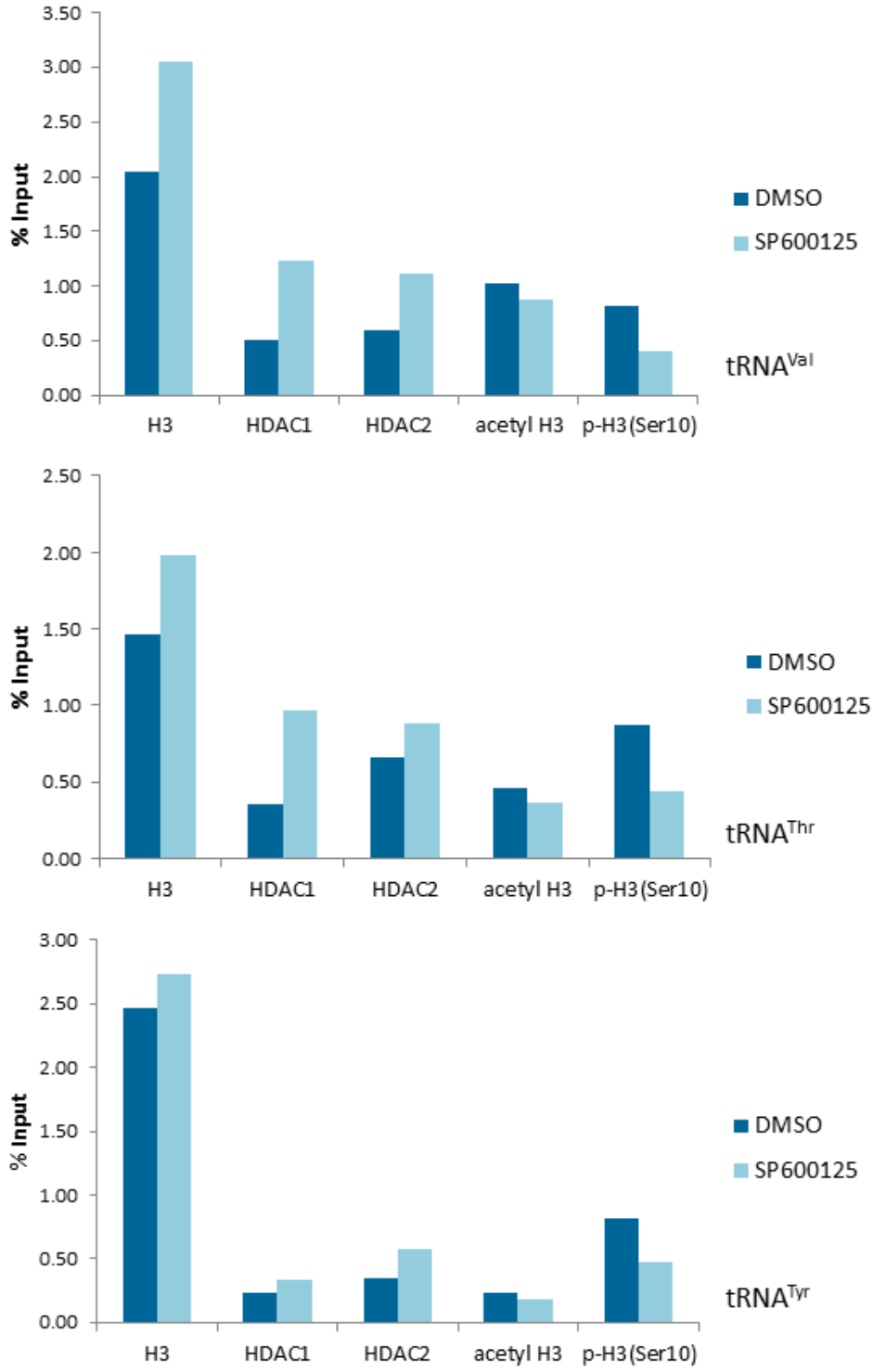

Figure 5.9 SP600125 alters chromatin environment at tRNA genes

Chromatin immunoprecipitation assay in U2OS cells with antibodies against histone H3, HDAC1, HDAC2, pan-acetylated histone H3 and phospho-histone H3(Ser10) at $tRNA^{Tyr}$, $tRNA^{Thr}$ and $tRNA^{Val}$ loci following treatment with 20μM SP600125 for 1 hour. ChIP with TAF_I48 used as a negative control. Acetyl Histone H3 and p-Histone H3 were normalised to H3 % input. % input calculated: [(IP/input)x100] – [(TAF_I48 IP/input)x100] n=1

5.2.5 Reduction in tRNA levels may require c-Jun

One possible mechanism by which JNK could be regulating tRNA transcription is through c-Jun, which is present at many tRNA genes (Raha et al., 2010). While c-Jun was found to occupy tRNA genes in U2OS, the change in occupancy following SP600125 treatment was not reproducible. In some experiments it stayed the same, while in others c-Jun occupancy was reduced (data not shown). Levels of p-c-Jun did go down reproducibly, however the immunoprecipitation efficiency was very low with the anti-p-c-Jun antibody (data not shown). An alternative route to address whether c-Jun has a role in the rapid reduction in tRNA levels observed following JNK inhibitor treatment was, therefore, required.

The effect of SP600125 treatment on c-Jun null mouse embryonic fibroblasts (MEFs) stably expressing c-Jun or an empty vector was examined. Like U2OS, these MEFs have some active JNK during normal growth as p-c-Jun levels are reduced 1 hour after the addition of 20μM SP600125 in c-Jun null MEFs that were stably expressing exogenous c-Jun (Figure 5.10a). Preliminary results show reduction in $tRNA^{Ile}$ level is greater in c-Jun-expressing c-Jun null MEF than in c-Jun null MEFs expressing the empty vector (Figure 5.10b). This suggests c-Jun may be required for reduced tRNA levels following JNK inhibition. Zhong and Johnson showed that JNK regulates TFIIIB levels in MEFs, however the finding above suggests the presence of an alternative JNK-dependent regulatory mechanism is not confined to U2OS (Zhong and Johnson, 2009).

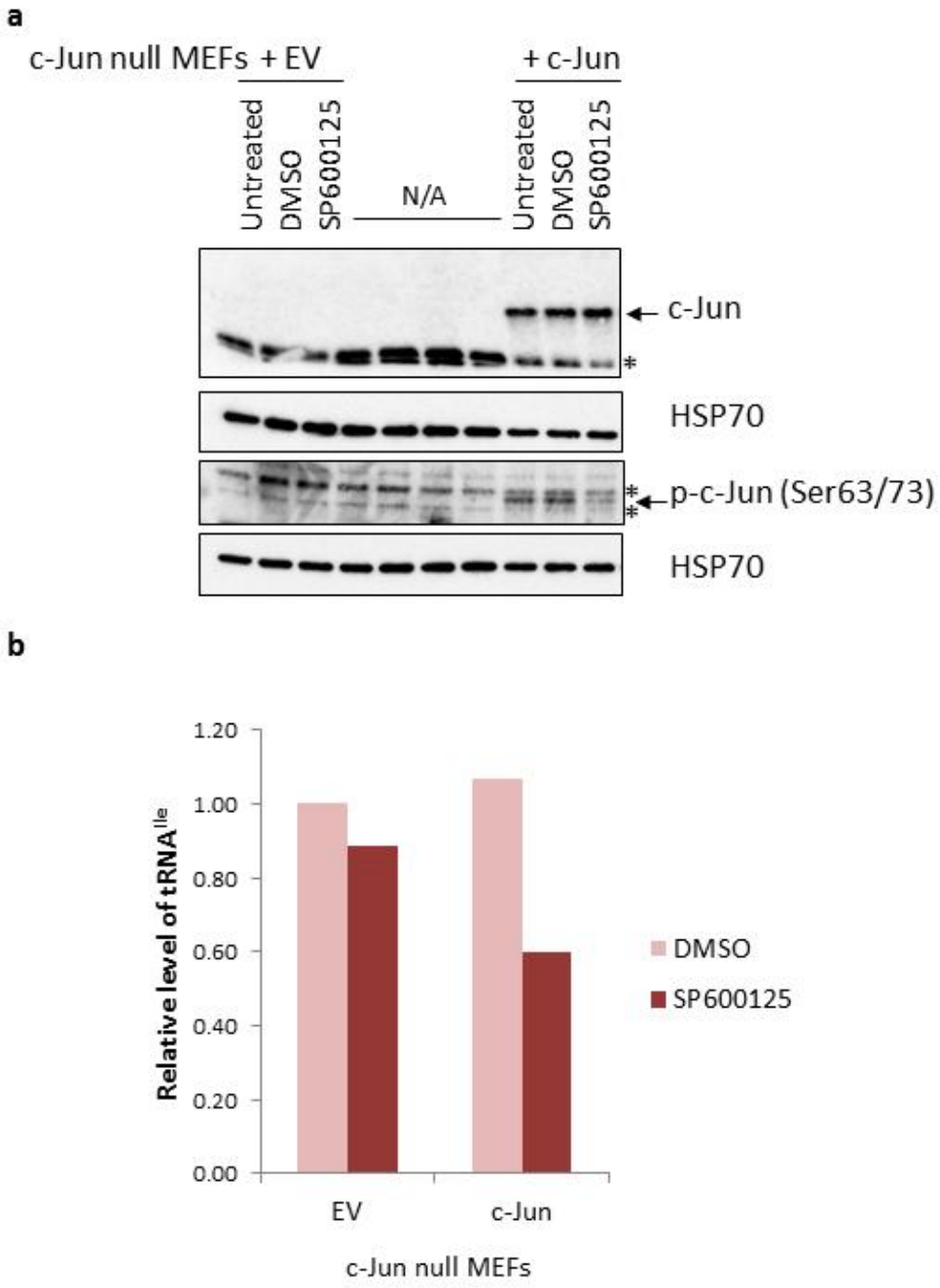

Figure 5.10 SP600125 treatment of cells lacking c-Jun

a) Analysis by western blot of c-Jun and phospho-c-Jun(Ser63/73) protein levels in c-Jun null MEFs stably expressing empty vector (EV) or c-Jun, following treatment with 20μM SP600125. Cells were harvested 1 hour post-treatment. HSP70 used as a loading control. * indicates non-specific band. Arrow indicates specific band. N/A indicates samples that are not described in main text. b) Analysis by RT-qPCR of $tRNA^{Ile}$ level in c-Jun null MEFs stably expressing empty vector (EV) or c-Jun, following treatment with 20μM SP600125. Cells were harvested 1 hour post-treatment. The expression was normalised to ARPP P0 mRNA levels. n=1

5.2.6 JNK inhibition does not reduce the phosphorylation of Brf1 at Threonine 145

In addition to regulating tRNA transcription through one of its known transcription factor targets, it is also possible that the regulation could occur through an unknown target of JNK. While in the process of carrying out the above experiments, I began to investigate this possibility. ERK phosphorylates Brf1 at Thr145 increasing its ability to occupy tRNA genes (Felton-Edkins et al., 2003). However, this site is also phosphorylated by an unknown kinase. Given that JNK and ERK have several shared substrates, JNK is a possible candidate for this unknown kinase. Protein lysates from SP600125-treated and control-treated U2OS were subjected to western blot analysis using an antibody specific to Brf1 phosphorylated at the Thr145 site. A band was identified at the correct size for Brf1 in the phospho-Brf1 blot, however the intensity of this band did not decrease following JNK inhibitor treatment (Figure 5.11). This suggests JNK does not phosphorylate this site in U2OS under normal growth conditions. It remains possible that JNK can directly target a component of the Pol III machinery.

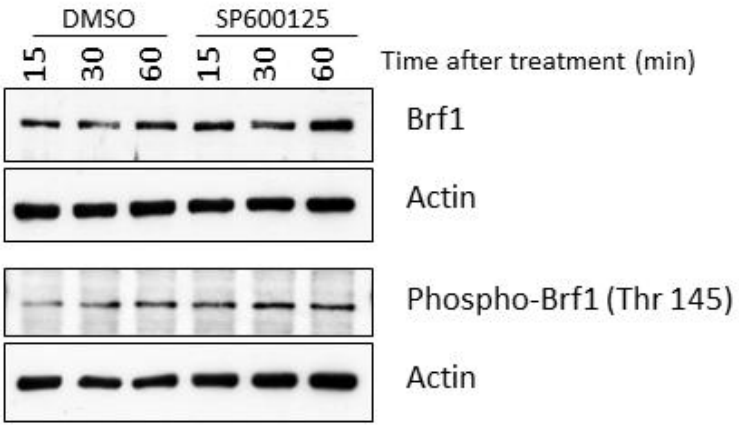

Figure 5.11 SP600125 does not decrease phosphorylation of Brf1 at Thr145

Analysis by western blot of Brf1 and phospho-Brf1(Thr145) protein levels in U2OS following treatment with 20μM SP600125 or DMSO. Cells were harvested at 15, 30 and 60 minutes post-treatment. Actin used as a loading control.

To investigate if JNK could interact with a component of the Pol III machinery, I carried out some preliminary co-immunoprecipitation experiments. I found that TBP and TFIIIC110 were both detectable following JNK immunoprecipitation from HeLa nuclear extract (Figure 5.12). In addition to suggesting the possibility of a phosphorylation target for JNK in the Pol III machinery, it also suggests that JNK may be present at tRNA genes.

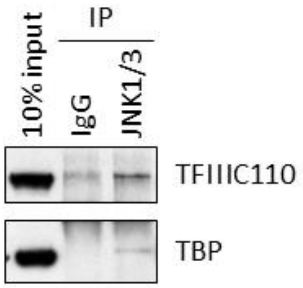

Figure 5.12 JNK interacts with TFIIIC and TFIIIB subunits

Analysis by western blot for TFIIIC110 and TBP in precipitated material from immunoprecipitation with an anti-JNK1/3 antibody in HeLa nuclear extract. Immunoprecipitation with IgG serves as a negative control.

5.2.7 JNK is present at tRNA genes

A recent study identified the presence of JNK at a wide range of genes in differentiating mouse neurons where it plays a functional role regulating their transcription (Tiwari et al., 2011). This study did not describe whether JNK was found at tRNA genes; however we used the publically available dataset from this study to examine this and found JNK present at tRNA genes (Figure 5.13a). The number of genes occupied by JNK reduces during neuronal differentiation. JNK was found at 188 of 433 tRNA genes in embryonic stem cells, 140 in neural progenitors and 138 in terminally differentiated neurons. The tRNA genes occupied also alters between cell types (Figure 5.13b).

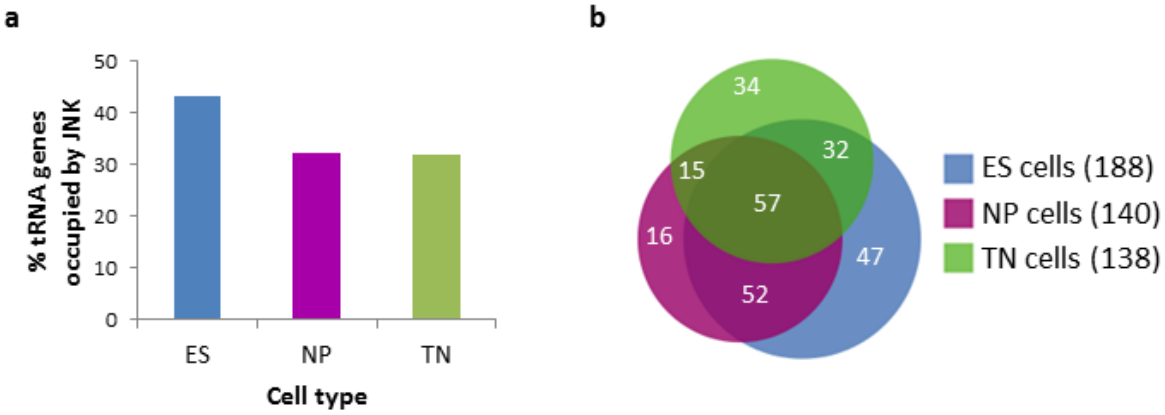

Figure 5.13 JNK occupies tRNA genes in mice

a) Graph showing JNK occupancy at tRNA genes in mouse embryonic stem cells (ES), neuronal progenitors (NP) and terminally differentiated neurons (TN). ChIP-seq data mined from Tiwari et al. 2011 using tRNAs annotated in mm7 mouse genome. b) Venn diagram to show overlap in JNK occupancy at tRNA genes in the three cell types. Total number of tRNAs occupied by JNK in each cell line is displayed in brackets.

Following these findings, we examined whether JNK was present at tRNA genes in U2OS. Preliminary findings suggest that JNK is indeed present here, which, once it can be confirmed, will be especially interesting as it will be, as far as we are aware, the first observation of JNK on the chromatin of human cells (Figure 5.14a). Interestingly, JNK appears to be present at lower levels at an inactive $tRNA^{Leu}$ gene. For Pol II target genes, JNK occupancy is associated with NF-Y binding and the presence of NF-Y binding motifs (CCAAT) nearby (Tiwari et al., 2011). Analysis of motif incidence was carried out for CCAAT near tRNA genes in the human genome and 477 CCAAT motifs were identified within 1kb up- and downstream of tRNAs (analysis carried out by Ann Hedley). To determine if CCAAT sites were enriched at tRNA genes compared to elsewhere in the genome, the number of CCAAT motifs in randomly selected genome stretches of the same length as the tRNA windows was analysed. CCAAT was not enriched at tRNA genes (Figure 5.14b). JNK may be recruited to tRNA genes by an alternative method.

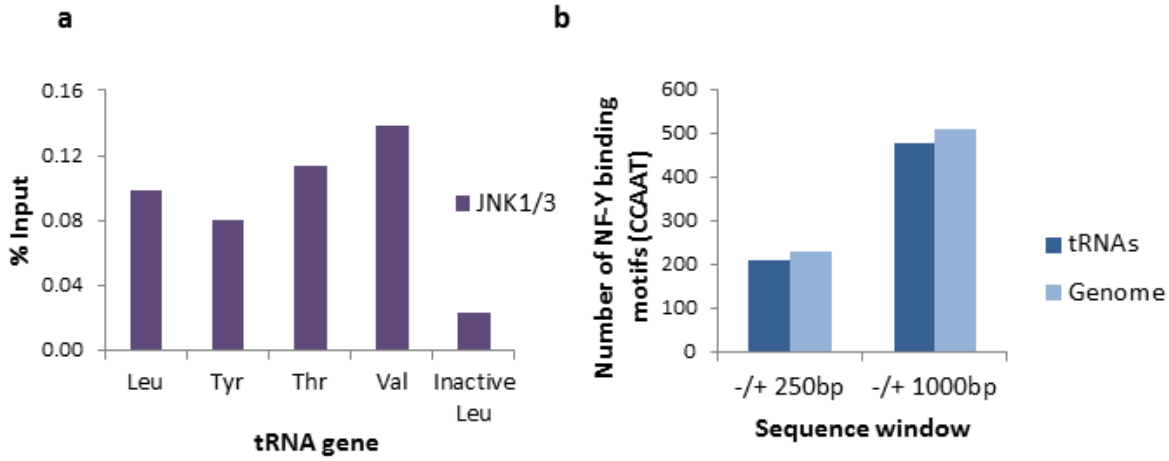

Figure 5.14 JNK appears to occupy tRNA genes in U2OS

a) Chromatin immunoprecipitation in U2OS cells with antibody against JNK1 and JNK3 at tRNA loci. ChIP with TAF_I48 used as a negative control. % input calculated: [(IP/input)x100] – [(TAF_I48 IP/input)x100] JNK observed at tRNA genes in multiple ChIP experiments. Representative data shown. b) The incidence of the NF-Y binding motif CCAAT was analysed 250bp and 1000bp up and downstream of tRNAs in the human genome (labelled 'tRNAs'). The incidence of this motif was also examined in 10 batches of 623 sequences the same length as the tRNAs (tRNA length -/+ 250bp or 1000bp) and from the same chromosomes but selected at random within the chromosome. The mean number of CCAAT motifs within these sequences is displayed (labelled 'genome').

Tiwari and colleagues found that JNK could phosphorylate histone H3 at Ser10. My preliminary results indicate that phospho-histone H3 (Ser10) reduces at tRNA genes following 60 minutes of JNK inhibition (Figure 5.9). Phosphorylation of histone H3 at serine 10, though recently identified as a chromatin mark associated with active genes, is typically a marker of mitosis. Cell cycle analysis by flow cytometry showed no difference in the cell cycle profile between treatments (Figure 5.15) suggesting the reduced H3Ser10 phosphorylation is not due to a reduced number of mitotic cells.

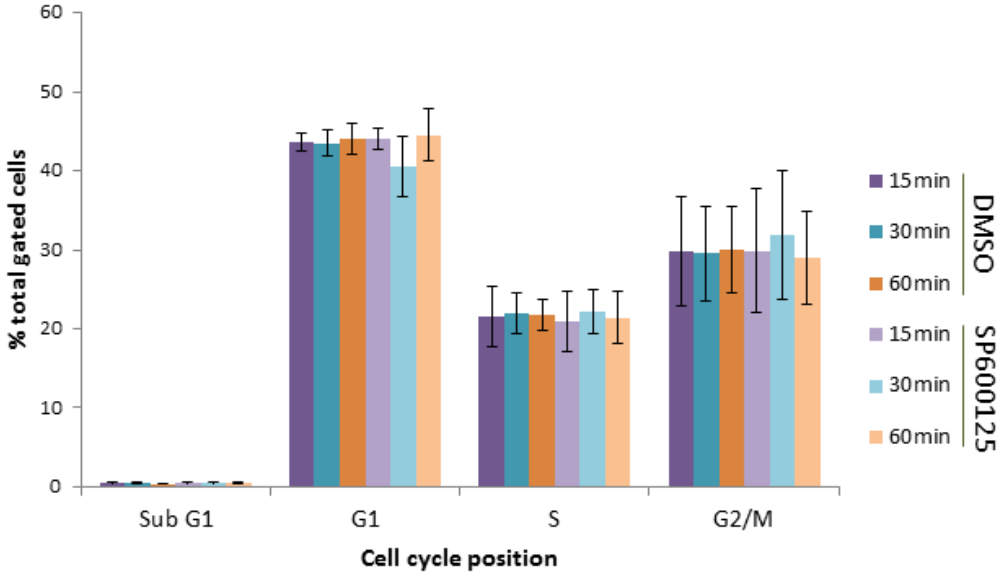

Figure 5.15 SP600125 does not alter cell cycle profile

Following 20μM SP600125 treatment, U2OS cells were fixed and DNA stained with propidium iodide. Cell cycle distribution was determined by flow cytometry. n=3

5.2.8 Alternative JNK inhibitor, JNK-IN-8, reduces tRNA levels

Though widely used, SP600125 has been shown to have off-target effects at the concentrations required for efficient JNK inhibition in a cellular context (Bain et al., 2003). To test whether SP600125's effect on tRNA levels was a JNK-specific effect, an alternative JNK inhibitor, JNK-IN-8, was used (Zhang et al., 2012). The recommended dosage for JNK inhibition using this inhibitor is 1μM for 3 hours in HEK293-ILR cells (human embryonic kidney cells stably expressing Interleukin Receptor 1) (Zhang et al., 2012). A time course in U2OS cells showed that the inhibitor binds to JNK after only 15 minutes, visible as an upward shift in the JNK bands (Figure 5.16).

c-Jun mRNA levels were reduced after 1 hour with 1μM JNK-IN-8 (Figure 5.17b). However, $tRNA^{Leu}$ levels remained unchanged with 1μM JNK-IN-8, even after 8 hours, despite clear effects on c-Jun phosphorylation by JNK-IN-8 (Figure 5.17a). This suggests that c-Jun dephosphorylation at Serine 63 cannot account for the reduced tRNA expression seen following SP600125 treatment. Interestingly, reduced p-JNK and p-c-Jun levels were only detectable after 2 hours of JNK-IN-8 treatment (Figure 5.16). With 20μM SP600125, reduced phosphorylation of c-Jun at Serine 63/73 is detectable after 15 minutes of treatment (Figure 5.1). The concentration of JNK-IN-8 was, therefore, increased to see if tRNA levels were susceptible to JNK-IN-8 treatment at higher levels, despite the possibility that this might increase chances of non-specific effects. 1 hour of treatment with 20μM JNK-IN-8 resulted in $tRNA^{Leu}$ repression (Figure 5.17b). JNK-IN-8 treatment does not appear to repress tRNA expression with the same kinetics as SP600125 treatment, however further examination of tRNA genes is required.

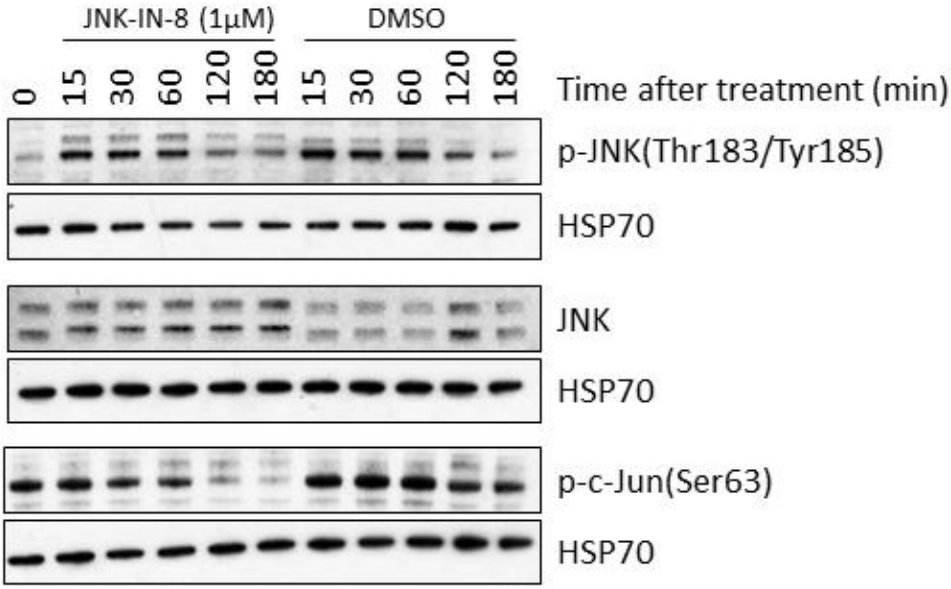

Figure 5.16 JNK inhibitor, JNK-IN-8, reduces phospho-c-Jun (Ser63) levels in U2OS

Analysis by western blot of JNK, phospho-JNK(Thr183/Tyr185) and phospho-c-Jun(Ser63) protein levels in U2OS following treatment with 1μM JNK-IN-8 or DMSO. Cells were harvested 15, 30, 60, 120 and 180 minutes post-treatment. HSP70 used as a loading control.

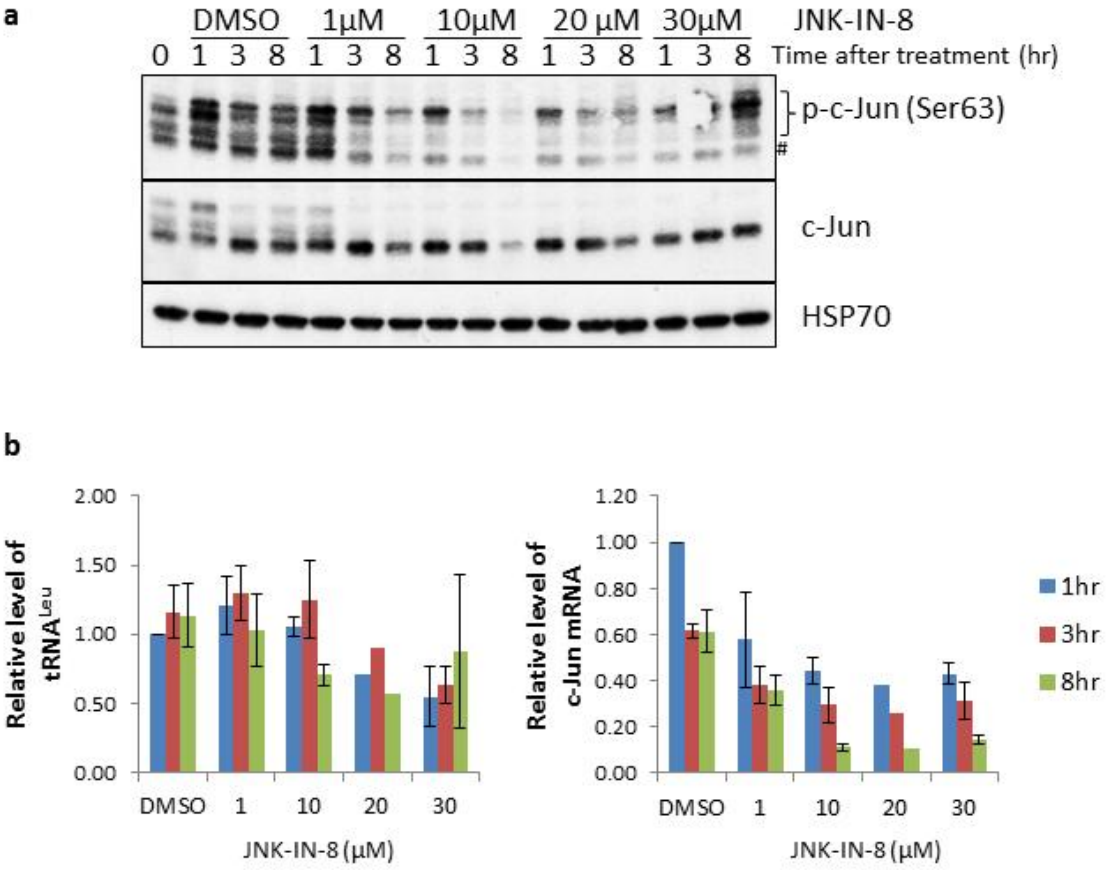

Figure 5.17 Treatment with alternative JNK inhibitor, JNK-IN-8, decreases in tRNA levels

a) Analysis by western blot of c-Jun and phospho-c-Jun(Ser63) protein levels in U2OS following treatment with JNK-IN-8 at 1µM, 10µM, 20µM 30µM or DMSO. Cells were harvested 1, 3 and 8 hours post-treatment. HSP70 used as a loading control. # indicates c-Jun band visible from previous antibody probe. b) Analysis by RT-qPCR of $tRNA^{Leu}$ and c-Jun mRNA levels in U2OS following treatment with JNK-IN-8 at 1µM, 10µM, 20µM 30µM or DMSO. Cells were harvested 1, 3 and 8 hours post-treatment. The expression was normalised to ARPP P0 mRNA levels. n=3 with exception of 20µM treatment where n=2

5.3 Discussion

JNK has been shown to regulate Pol III-mediated transcription through regulation of the expression of TFIIIB, the basal transcription factor that recruits Pol III to its target genes (Zhong and Johnson, 2009). However, additional evidence hinted that this may not be the sole mechanism by which JNK modulates transcription by this polymerase. This is supported by findings described in this chapter. It is not unprecedented for a single factor to regulate Pol III-mediated transcription at multiple levels. For example, ERK has been shown to induce Brf1 activity by both phosphorylating it and inducing its transcription (Felton-Edkins et al., 2003, Goodfellow et al., 2006). c-Myc induces Brf1 expression and promotes Pol III-mediated transcription through binding to TFIIIB (Sansom et al., 2007, Gomez-Roman et al., 2003).

Regulation of transcription at multiple levels may ensure both fast and sustained response to cellular conditions. The positive feedback loop of c-Jun provides an example of this (Angel et al., 1988). Phosphorylation of c-Jun increases its ability to activate its target genes. These genes include the gene encoding c-Jun, thus c-Jun protein level will increase and so the response is sustained beyond the initial stimulus. The kinetics of this feedback loop are illustrated in Figure 5.1 and Figure 5.2, where reduced phosphorylation of c-Jun is evident 15 minutes after addition of JNK inhibitor SP600125, yet a significant reduction in c-Jun mRNA level is not detected until 45 minutes later. At this timepoint, c-Jun protein level has not yet reduced.

Treatment of U2OS cells with SP600125 resulted in a dramatic and rapid reduction in levels of all of the tRNAs examined. It would be interesting to look at whether it has a similar effect on other Pol III target genes, such as 7SL and U6. Determining if it is a specific effect on some genes, or a global effect on Pol III-mediated transcription as a whole, would provide mechanistic insight. A specific effect would occur if JNK is acting through its targets at tRNA genes, for example c-Jun is only at a proportion of genes being transcribed by Pol III (Raha et al., 2010). However, if the Pol III machinery is targeted a more general effect would be expected.

Alternatively, reduced tRNA levels may be due to increased tRNA degradation. A specific tRNA degradation pathway has not been defined in humans, however, in yeast the rapid tRNA degradation (RTD) pathway contributes to tRNA levels (Wichtowska et al., 2013). However, the preliminary ChIP assays carried out indicate reduced Pol III at tRNA genes, suggesting that reduced tRNA levels are due to reduced expression (Figure 5.8). Pol III occupancy is considered to be a readout of active transcription. This coincides with reduced TFIIIB occupancy and increased histone H3 occupancy (Figure 5.8b, Figure 5.9). As nucleosomes are excluded from actively transcribed tRNA genes (White, 2002, Wang et al., 2012), an increase in histone H3 is indicative of reduced tRNA gene activity. Given more time, nuclear run-on assays could be carried out in order to confirm that reduced tRNA levels are indeed the result of reduced transcription.

During the short course of JNK inhibitor treatment reduced TFIIIB levels were not detected (see Figure 5.3). This suggests that JNK is regulating Pol III-mediated transcription by a mechanism independent of that published by Zhong and Johnson in 2009. However, it cannot be ruled out that JNK is regulating Pol III activity via alteration of the transcription and/or translation of another Pol II-transcribed gene. Secondary effects such as this can usually be ruled out if the same treatment outcome occurs in the presence of a protein synthesis inhibitor. However, as Pol III products are central to protein synthesis, inhibiting protein synthesis could alter Pol III-mediated transcription by other routes. Given the high speed with which tRNA levels were reduced (15min), faster than target genes that are known to be directly regulated by JNK (c-Jun, TBP and Brf1), it is more likely that the reduction in tRNA transcription is a primary effect of JNK inhibition rather than a secondary effect resulting from changes in expression of other target genes.

Anisomycin (188nM) results in increased expression of a tRNA reporter in mouse embryonic fibroblasts after 30 minutes of treatment (Zhong and Johnson, 2009). Preliminary experiments suggest 30 minutes treatment with anisomycin can also increase endogenous tRNA levels in U2OS (Figure 5.7c). This was not due to increased TFIIIB levels as TBP and Brf1 levels did not increase (Figure 5.7b). Zhong and Johnson did not examine TFIIIB levels following anisomycin treatment. However, they did find the increase in tRNA level was JNK dependent. It will be interesting to determine if this is also true in U2OS.

To investigate if JNK can mediate changes in TFIIIB levels in U2OS cells, a longer treatment with JNK inhibitor was carried out (Figure 5.4 and Figure 5.5). After 9 hours, tRNA levels were repressed to a similar degree as that seen after one hour. However, while Bdp1 and TBP mRNA levels were reduced, Brf1 mRNA and TBP and Brf1 protein levels appeared to be the same as in the untreated cells. This suggests JNK may not be able to regulate their expression in U2OS cells. It would be interesting to investigate if Elk1 is found at TBP and Brf1 genes in U2OS, as Zhong et al. attribute JNK's ability to regulate their expression to Elk1 (Zhong and Johnson, 2009).

Multiple JNK-targeted transcription factors have been identified at Pol III-transcribed genes, yet, for the majority of them, their function there remains an enigma (Raha et al., 2010, Oler et al., 2010). These targets are all transcriptionally activated by JNK, thus JNK inhibition should result in the reduced expression of their target genes. They, therefore, have the potential to cause the change seen in tRNA gene expression. As described in Chapter 4, c-Jun is one of these transcription factors. The preliminary data in Figure 5.10 suggests c-Jun may contribute to JNK-dependent regulation of tRNA expression. It is unclear whether c-Jun occupancy changes at tRNA genes in U2OS following SP600125 treatment, as ChIP assays did not give reproducible results (data not shown). However, p-c-Jun(Ser63/73) occupancy was reproducibly reduced following treatment (data not shown). Preliminary co-immunoprecipitation assays described in Chapter 4 suggest that c-Jun's association with TFIIIC does not change following JNK inhibitor treatment (Figure 4.10). This suggests that c-Jun may remain at tRNA genes and, if it regulates gene expression, phosphorylation of c-Jun rather than its occupancy may mediate this regulation.

Though c-Jun has long been known to regulate the expression of the genes it binds at, the first detailed evidence for the mechanism by which this occurs was published by Aguilera et al. in 2011. They showed that, when bound at target genes, unphosphorylated c-Jun interacts with Mbd3, a component of the nucleosome remodelling and histone deacetylation (NuRD) complex (Aguilera et al., 2011). Mbd3 is required for the recruitment of the histone deacetylase, HDAC1, which can then deacetylate histones at the target gene resulting in reduced transcription. The preliminary ChIP in Figure 5.9 shows reduced HDAC1 and HDAC2 occupancy at tRNA genes following JNK inhibition. This coincides

with a possible increase in unphosphorylated c-Jun occupancy, as suggested by the reduced phospho-c-Jun(Ser63/73) occupancy (data not shown) and unchanged association between c-Jun and TFIIIC (Figure 4.10). These observations support the possibility that tRNA genes might be regulated by the c-Jun-dependent model described by Aguilera and colleagues. Further analysis, including examining the occupancy of Mbd3 at tRNA genes, should be carried out. Certainly, it has been shown that reduced histone acetylation is associated with reduced activity of Pol III target genes (Barski et al., 2010, Oler et al., 2010). However, it is currently unclear if this is a cause of the reduced transcription or a consequence (White, 2011).

An alternative route by which JNK may directly regulate tRNA expression is through the phosphorylation of the Pol III machinery. Phosphorylation of TFIIIB has been shown to alter its recruitment to Pol III target genes (Hu et al., 2004, Fairley et al., 2003, Felton-Edkins et al., 2003, Fairley et al., 2012). While JNK does not appear to contribute to phosphorylation of Brf1 at Thr145, this does not rule out the possibility that JNK phosphorylates another site on the Pol III machinery. This could be examined by a mass spectrometry approach.

The identification of JNK at many protein-coding genes in ES cells highlighted a further route by which JNK can regulate gene expression (Tiwari et al., 2011). The majority of genes bound by JNK were characteristic of the JNK signalling response, encoding proteins involved, for example, in gene expression and the cell cycle. So JNK may potentially regulate many pathways via its activity on the chromatin. Using published ChIP-seq data from this study, we identified JNK at many tRNA genes in mouse embryonic stem (ES) cells, neural progenitor (NP) cells and terminally differentiated neurons (Figure 5.13). While Pol III-mediated transcription has not been followed through neuron differentiation, it has been examined in mouse ES cells (Carrière et al., 2012). Pol III occupancy was found at 284 of the 433 tRNA genes shown in the mouse genome (Genomic tRNA database). This suggests 66% of tRNA genes in ES cells are expressed. Preliminary ChIP data suggests JNK may be present at tRNA genes in U2OS (Figure 5.14a). JNK occupancy of tRNA genes in human cells is further supported by co-immunoprecipitation experiments showing association of JNK with TFIIIC and TBP in HeLa nuclear extract (Figure 5.12). Given more time, it would be interesting to examine whether the Pol II genes bound by JNK in murine cells are also bound

by JNK in U2OS. Given the speed of sequencing, this may be most easily approached by further ChIP-seq experiments which would also provide a more global view of JNK at tRNA genes and other Pol III target genes in U2OS.

How JNK is recruited to tRNA genes is unclear. In mouse ES cells, JNK occupancy at protein-coding genes was associated with occupancy of the transcription factor NF-Y at these genes (Tiwari et al., 2011). Tiwari and colleagues found that JNK-bound genes were enriched for the NF-Y binding motif CCAAT. We found no enrichment of CCAAT sites near tRNA genes in the human genome (Figure 5.14b). Though not enriched, 477 were identified 1kb up- and downstream of tRNAs in the human genome. Therefore, JNK may still be recruited to tRNA genes via this route. Alternatively, JNK could be recruited by another factor occupying Pol III target genes.

As JNK appears to bind at tRNA genes, this is a potential mechanism through which the reduction in tRNA expression following JNK inhibitor treatment could be mediated. SP600125 resulted in reduced expression of some JNK-occupied genes (Tiwari et al., 2011). This was associated with reduced phosphorylation of histone H3 at serine 10, a modification that can be mediated by JNK *in vitro* (Tiwari et al., 2011). Interestingly, preliminary data suggests phospho-histone H3Ser10 occupancy goes down at tRNA genes in U2OS following JNK inhibitor treatment (unlike H3 itself, which goes up) (Figure 5.9). In addition to its association with active gene expression, H3Ser10 phosphorylation is a mitotic marker (Sawicka and Seiser, 2012). SP600125 does not cause a visible change in cell cycle profile according to cell cycle analysis (Figure 5.15). However, given that the experiment was only carried out over 1 hour, further analysis is required to conclude whether this change in H3Ser10 phosphorylation is associated with reduced gene expression rather than a reduced number of cells in mitosis. Since carrying out these experiments, a study has been published showing phospho-H3Ser10 occupancy at Brf1, $tRNA^{Leu}$ and 5S genes in mouse hepatocytes (Zhong et al., 2013a). This occupancy increases following treatment with the chemical carcinogen diethylnitrosamine (DEN) and contributes to the increase in Pol III-mediated transcription that is observed. The mechanism of this contribution remains undetermined. However, based on the data above and the observation that JNK activity contributes to DEN-induced tumour

carcinogenesis (Hui et al., 2008), JNK is a good candidate for the kinase involved in mediating the phosphorylation of Histone H3 that is observed.

I have not examined if JNK can directly regulate tRNA transcription via phosphorylation of its other targets. JNK phosphorylation stabilises p53, thus JNK inhibition should result in reduced p53 levels (Buschmann et al., 2001). As p53 is a negative regulator of Pol III activity, this would be expected to result in increased tRNA levels (Chesnokov et al., 1996, Cairns and White, 1998). As this does not occur, p53 is unlikely to contribute to the reduced tRNA levels observed. However, STAT1, ATF2 and c-Myc all bind Pol III target genes and all exhibit increased transactivational activity upon JNK-mediated phosphorylation (Oler et al., 2010, Felton-Edkins et al., 2006, Raha et al., 2010). Interestingly, STAT1 occupies a large proportion of active tRNA genes in HeLa cells. Of 278 Pol III-occupied sites (of which 227 are tRNA genes) in these cells, 161 have STAT1 bound in interferon-γ –induced HeLa cells (Oler et al., 2010, Rozowsky et al., 2009). However, It remains to be determined if the increased transactivational activity of these JNK targets following JNK activation alters Pol III target gene expression.

Small molecule kinase inhibitors are useful tools, however they can cause off-target effects (Bain et al., 2003). The use of inhibitors was necessary for this study in order to examine tRNA levels independently of JNK-dependent changes in TFIIIB levels. SP600125 is the only JNK inhibitor used widely in the literature. Concentrations used range from 5-50μM (Mayer et al., 2005, Aguilera et al., 2011), however non-specific inhibitory activity has been described at these concentrations (Bain et al., 2003, Fabian et al., 2005). An alternative JNK inhibitor, JNK-IN-8, became available during the course of this project (Zhang et al., 2012). Treatment with the recommended dose (for HEK293 cells), 1μM, did not result in the rapid reduction of tRNA levels seen with SP600125 treatment in U2OS cells (Figure 5.17). This was despite the fact that JNK appears to be inhibited, as determined by reduced p-JNK levels and a mobility shift in JNK. At higher JNK-IN-8 concentrations (20 μM), tRNA levels were reduced after 1 hour treatment. There are several possible reasons why 1μM JNK-IN-8 treatment does not result in same effect on tRNA levels as SP600125 and higher concentrations of JNK-IN-8. Firstly, a certain level of inhibition of JNK may be required to reduce tRNA levels and this is not met with 1μM JNK-IN-8. It would be interesting

to examine whether lower concentrations of SP600125 result in similar kinetics to 1μM JNK-IN-8 treatment, just as higher concentrations of JNK-IN-8 (20-30μM) result in a similar effect to 20μM SP600125. Secondly, only one set of tRNA primers has been used to examine tRNA levels following JNK-IN-8 treatment so far and this tRNA is unlikely to be representative of all tRNAs. Finally, SP600125 and JNK-IN-8 may both be having off-target effects at 20μM and it is this unknown target that is reducing tRNA levels.

The off-target effects of JNK inhibitors have been examined to some extent by kinase assays. Two studies of JNK-IN-8 suggest that this inhibitor inhibits JNKs specifically at 1μM when compared to all other kinases examined (Zhang et al., 2012, International Centre for Kinase Profiling). The possible off-target effects of JNK-IN-8 at higher concentrations have not been examined. The inhibitory effect of SP600125 has been examined by kinase assay at 1μM and 10μM (International Centre for Kinase Profiling, Bain et al., 2003). At both concentrations, the activity of multiple kinases (in addition to JNKs) was inhibited by 50% or more. I examined the published literature on these kinases to see if they might account for the changes in tRNA levels and other effects I had observed upon SP600125 treatment. While no particular kinase stood out as the most likely candidate, several possible candidates were present. In both studies, CK1 was inhibited more than JNK itself. While CK1 has not been shown to regulate Pol III-mediated transcription, a putative phosphorylation target site for CK1 has been identified on Maf1 using prediction software (Rollins et al., 2007). Maf1 is a repressor of Pol III-mediated transcription and phosphorylation of Maf1 prevents this repressive activity (Moir and Willis, 2013). If CK1 could phosphorylate Maf1, inhibition of CK1 by SP600125 would result in reduced Maf1 phosphorylation and could account to the decrease in tRNA levels seen. Another kinase inhibited to a greater degree than JNK was Aurora B (International Centre for Kinase Profiling). While a role for Aurora B in regulating Pol III-mediated transcription is undetermined, inhibition of this kinase could account for the decrease in phospho-Histone H3(Ser10) that was observed upon SP600125 treatment (Figure 5.9), as Aurora B can phosphorylate this site (Goto et al., 2002). AMPK and DYRK2 were also inhibited to a greater degree than JNK according to kinase assays (Bain et al., 2003, International Centre for Kinase Profiling). These kinases both have the potential to regulate Pol III-mediated

transcription indirectly, AMPK through the upregulation of Maf1 activity by inhibiting mTORC1 and DYRK2 through the negative regulation of c-Jun and c-Myc (Gwinn et al., 2008, Taira et al., 2012). These kinases can therefore be described as negative regulators of Pol III-mediated transcription and their inhibition by SP600125 would likely result in an increase rather than a decrease of tRNA levels. Thus, they are unlikely to account for the outcome of SP600125 treatment observed. CK2 and ERK can both positively regulate Pol III-mediated transcription through the phosphorylation of TFIIIB (Johnston et al., 2002, Felton-Edkins et al., 2003). These kinases are not inhibited to the same degree as JNK upon SP600125 treatment, however 10μM SP600125 does reduce their activity to 55-65% of the control treatment (Bain et al., 2003). It is therefore possible that inhibition of either of these kinases could account for the reduced tRNA levels and Pol III recruitment at tRNA genes that was observed upon treatment with 20μM SP600125 in U2OS (Figure 5.1, Figure 5.8). Clearly further examination is required to determine the kinase responsible for reduced Pol III-mediated transcription observed upon SP600125 treatment. Carrying out the treatment in JNK1/JNK2 double null cells would confirm whether JNK inhibition was required.

A reductionist approach using *in vitro* experiments may prove very useful for examining the various possibilities described in this chapter. Such techniques were not originally considered as they have been overshadowed by new technologies and have been criticised for their 'non-physiological' conditions. For example, in *in vitro* transcription (IVT) assays genes are not in their normal chromosome environment and it has become apparent that the regulation of a gene is often influenced by the surrounding chromatin. However, benefits include the ability to control the environment. For example, using IVT, the ability of JNK targets to regulate Pol III-mediated transcription could be examined without possible JNK-induced changes in TFIIIB levels. JNK occupancy at tRNA genes and the potential phosphorylation of the Pol III machinery could also be examined using *in vitro* approaches.

As described above, several observations point to the possibility that JNK can regulate Pol III-mediated transcription by a mechanism independent of its ability to modulate TFIIIB subunit expression. In this chapter, I have demonstrated that JNK inhibitor treatment can result in a dramatic reduction in tRNA levels and

this appears to be mediated by reduced transcription of the tRNA genes. It will be interesting to examine whether this observation is also applicable at other Pol III target genes. Multiple routes by which JNK may modulate tRNA expression have been identified and examined (Figure 5.18). JNK-mediated upregulation of Pol III-mediated transcription could contribute to the cell's response to mitogenic stimuli, as Pol III products are required for growth and proliferation. As JNK also has an important role in mediating the response of a cell to stress, it may also be able to contribute to repression of Pol III-mediated transcription that is frequently observed following stress. These contrasting effects could arise from differences between JNK1 and JNK2 activity. Indeed, it has been demonstrated that JNK1 and JNK2 have opposing effects on the regulation of TFIIIB subunit expression (Zhong et al., 2007, Zhong and Johnson, 2009). Undoubtedly, the role of the JNK family in the regulation of Pol III-mediated transcription warrants further examination.

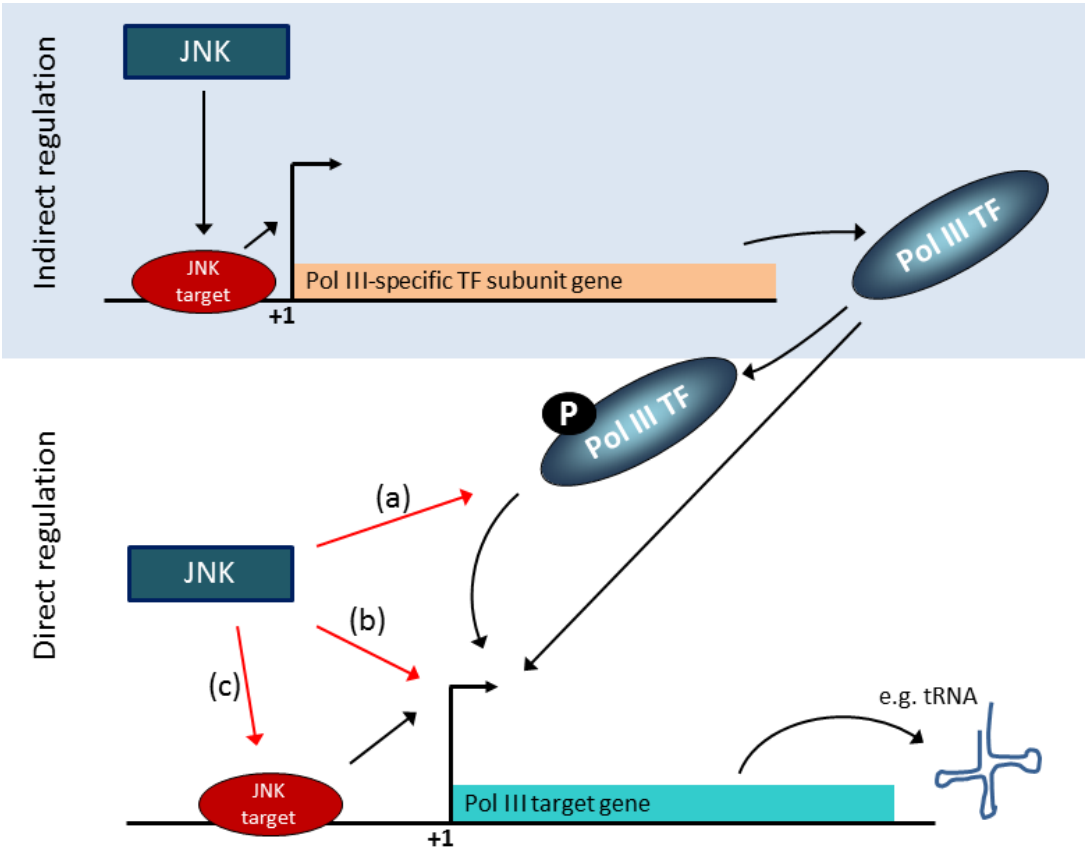

Figure 5.18 Model for regulation of Pol III-mediated transcription by JNK

JNK has been shown to regulate Pol III-mediated transcription indirectly by modulating the expression of TFIIIB subunits, TBP and Brf1, in (Zhong and Johnson, 2009) (on blue background). Possible routes of direct regulation by JNK include (a) the phosphorylation of a component of the Pol III machinery, altering its recruitment to/activity at Pol III target gene, (b) direct binding of JNK to Pol III target genes and modification of chromatin, altering gene environment and (c) phosphorylation of a JNK substrate that has been identified to bind at Pol III target genes, such as c-Jun or Elk1.

6 Final Discussion

Transcription by RNA Polymerase III in humans is subject to regulation by many factors including p53, RB, c-Myc, ERK, Maf1, CK2 and JNKs (Chesnokov et al., 1996, Cairns and White, 1998, White et al., 1996, Gomez-Roman et al., 2003, Felton-Edkins et al., 2003, Reina et al., 2006, Ghavidel and Schultz, 2001, Johnston et al., 2002, Zhong and Johnson, 2009). These factors share the common feature of being regulated in response to stress and nutrient availability, times when the cell undergoes changes in proliferation rate. Having multiple regulators advocates the importance of the regulation of Pol III-mediated transcription in response to the cell's environment. Pol III produces 5S rRNA and tRNAs, essential components of the translation machinery. Other Pol III products essential for cellular function include U6 snRNA, which is required for mRNA splicing, H1, which facilitates the processing of the 5' end of pre-tRNAs, and MRP RNA, which is essential for rRNA processing. These RNAs share a common purpose: they are all essential for protein synthesis and, as a consequence, cell growth. In order to maintain cellular size and use energy efficiently, cell growth is tightly regulated in response to the proliferation state of the cell. Tight regulation of Pol III-mediated transcription is part of this cell growth regulation.

In this thesis, the contribution of p53, AP-1 and JNKs to the regulation of Pol III-mediated transcription has been examined. In Chapter Three, it is demonstrated that repression of Pol III activity is not a universal response to p53 induction. Indeed, doxorubicin treatment results in a robust induction of p53 and, independently of p53, a significant repression of Pol III-mediated transcription. In light of the published literature, this suggests that p53 may only repress Pol III-mediated transcription in response to oncogenic stress, when p53 is induced by ARF (Morton et al., 2007). In Chapter Four, the occupancy of c-Jun and c-Fos, which together constitute a form of the transcription factor AP-1, at tRNA genes is shown to be associated with occupancy of Pol III, suggesting they may positively regulate Pol III-mediated transcription. c-Jun N-terminal kinases (JNKs) are key regulators of c-Jun, inducing c-Jun transactivational activity by phosphorylation of its transactivation domain. In Chapter 5, JNK is demonstrated to be able to regulate the transcription of Pol III target genes independently of its previously published ability to regulate TFIIIB expression (Zhong and Johnson,

2009). Various potential routes by which this may occur are identified. JNK occupies multiple tRNA genes and may regulate tRNA expression by the phosphorylation of the Pol III pre-initiation complex or by modifying the chromatin environment, making it more conducive to transcription. In addition, it could regulate tRNA expression through phosphorylation of c-Jun or one of several other JNK targets that occupy tRNA genes.

This study highlights the multiple levels at which Pol III-mediated transcription is regulated. JNKs regulate the expression of TFIIIB subunits, altering the level of Pol III machinery available (Zhong and Johnson, 2009). p53 alters Pol III-specific transcription factor availability by an alternative route; sequestering TFIIIB away from Pol III target genes (Cairns and White, 1998, Crighton et al., 2003). ERK, a MAPK family member like JNK, phosphorylates TFIIIB, promoting the recruitment of TFIIIB to the target promoter (Felton-Edkins et al., 2003). The binding of regulators at Pol III target genes, where they can repress or promote Pol III activity, is a further mechanism of regulation. The association of c-Jun and c-Fos with active tRNA genes suggests they may contribute to regulation at this level. These different levels of regulation are represented diagrammatically in Figure 6.1. Further routes of regulation that may also influence the quantity of Pol III products in the cell include regulating the stability and degradation of Pol III machinery at the mRNA and protein level and of the Pol III products themselves. The regulation of Pol III-mediated transcription will also include feedback loops. For example, 5S rRNA can regulate p53 activity (Donati et al., 2013). Interestingly, a single regulator can act at multiple levels of regulation. For example, c-Myc regulates Brf1 expression and also recruits histone acetyltransferase activity to enhance Pol III recruitment to target genes (Gomez-Roman et al., 2003, Kenneth et al., 2007). In Chapter Five, it is demonstrated that JNK, also, may act at multiple levels. Multiple levels of regulation ensure that the levels of Pol III products in the cell can be regulated rapidly and then maintained over a longer period of time if required. They also ensure that the cell makes efficient use of resources and they allow the integration of signals from multiple signalling pathways. The amount of tRNA, 5S rRNA and the other Pol III products in the cell is the outcome of the combination of these multiple regulatory routes.

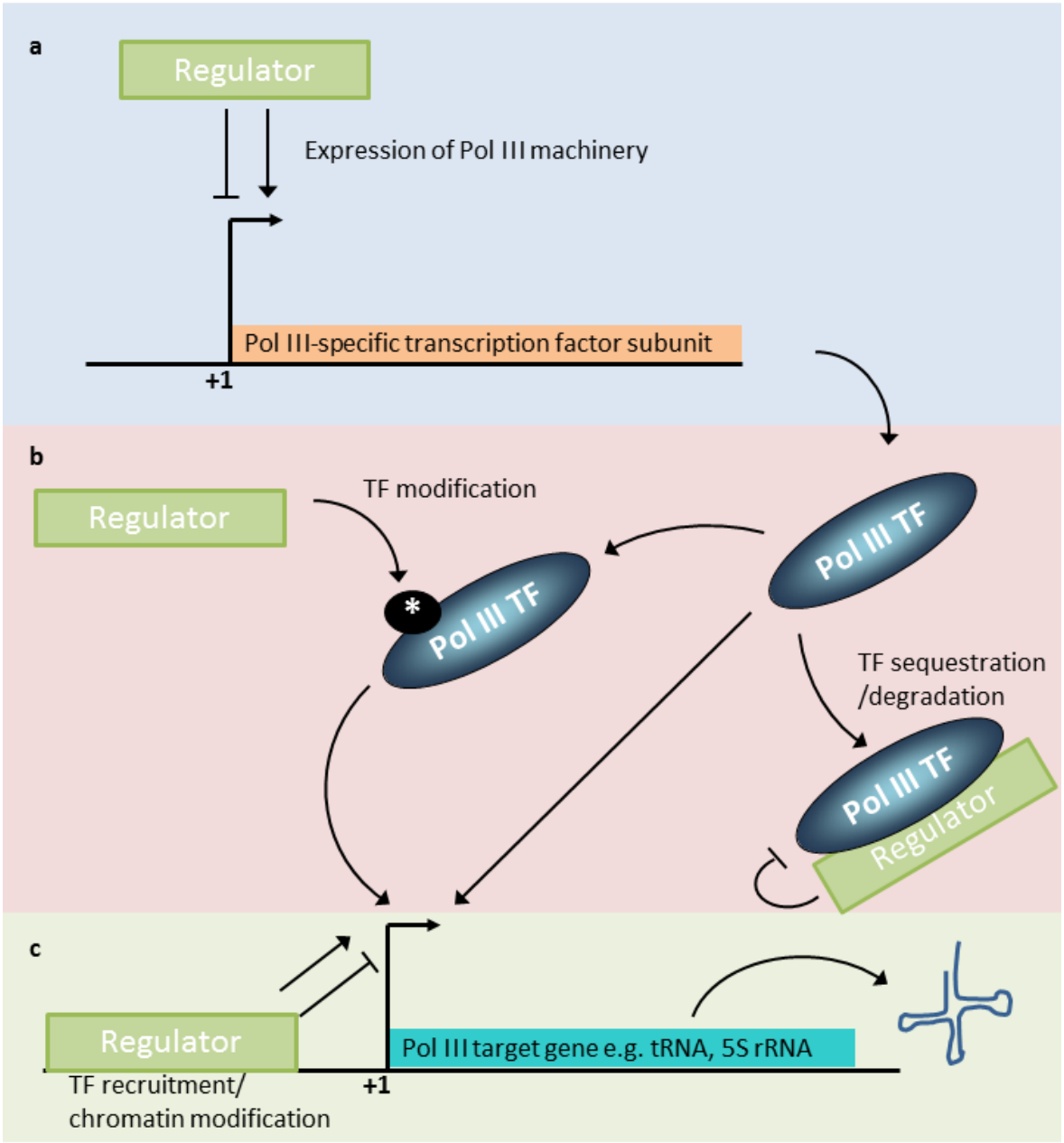

Figure 6.1 The multiple levels of regulation of Pol III-mediated transcription

The diagram above represents some of the routes by which Pol III-mediated transcription can be regulated. These are divided into three groups: a) the regulation of expression of the Pol III-specific transcription factors TFIIIB and TFIIIC (encoded by Pol II-transcribed genes), b) modification, sequestration and degradation of Pol III-specific transcription factors and c) regulator binding at Pol III target genes and altering expression by promoting/inhibiting recruitment of Pol III-specific transcription factors or modification of chromatin environment. TF = transcription factor. Feedback loops and the regulation of mRNA for Pol III-TF subunits may also contribute to Pol III-mediated transcription.

The regulation of tRNA expression has been the main focus of this thesis, primarily because tRNAs are the subject of most studies exploring the role of p53, AP-1 and JNK on Pol III-mediated transcription in human cells (Crighton et al., 2003, Morton et al., 2007, Zhong and Johnson, 2009, Zhong et al., 2011, Zhong et al., 2013b). However, as noted above, Pol III-mediated transcription produces many other non-coding RNAs that are equally important for cellular function (Dieci et al., 2007). A degree of coordination is expected between the regulation of all genes transcribed by Pol III as many Pol III regulators target the Pol III-specific transcription factors (Marshall and White, 2008). This will have a global impact on the formation of pre-initiation complexes at Pol III target genes (White, 2008). However, recent genome-wide studies of transcription factor binding have identified Pol II-associated transcription factors, including c-Jun, c-Fos, STAT1, c-Myc and Ets1, at Pol III target genes in human cells (Raha et al., 2010, Oler et al., 2010). These have the potential to contribute to the expression of the genes they occupy and, as they are only present at a proportion of Pol III-bound genes, may contribute to differential expression of Pol III target genes (Dittmar et al., 2006, White, 2011). By employing RNA-seq analysis, future studies will gain a more global view of Pol III product levels and how these change in different cellular contexts. In combination with ChIP-seq analysis, this will contribute significantly to our understanding of the regulation of Pol III-mediated transcription.

Several known regulators of Pol III-mediated transcription can also regulate Pol I-mediated transcription, including RB, c-Myc and p53 [reviewed in (White, 2008)]. Coordinated regulation is beneficial to the cell as 45S rRNA and 5S rRNA are required in equimolar quantities for ribosome biogenesis (Phillips and McConkey, 1976). p53 represses Pol III-mediated transcription by binding to TFIIIB and sequestering it away from Pol III target genes (Chesnokov et al., 1996, Cairns and White, 1998, Crighton et al., 2003). Similarly, p53 represses Pol I-mediated transcription by binding to SL1 (like TFIIIB, the TBP-containing transcription factor) and preventing its recruitment to the promoter of the rRNA gene (Zhai and Comai, 2000). JNK2 can phosphorylate TIF-IA, impairing TIF-IA's recruitment to rRNA genes where it is required to promote Pol I recruitment (Mayer et al., 2005). JNKs may also directly regulate Pol III-mediated transcription, as demonstrated in Chapter Five, and indirect regulation has

already been shown (Zhong and Johnson, 2009). JNK2 has a negative effect on TFIIIB subunit expression (Zhong and Johnson, 2009). Conversely, JNK1 can positively regulate TFIIIB expression (Zhong and Johnson, 2009). As JNK2 has a negative effect on both Pol I and Pol III activity, it will be interesting to examine if JNK1 has a positive effect on Pol I-mediated transcription. JNKs are activated by both mitogens and stress stimuli and the differences in the activity of JNK1 and JNK2 could contribute to the altered levels of Pol III and Pol I activity that occur following these stimuli (increased following mitogenic stimuli and decreased following stress). As demonstrated in Chapter Four, the binding of c-Jun and c-Fos at active Pol III target genes indicates that they may have a positive influence of Pol III-mediated transcription. Whether they can regulate Pol I-mediated transcription has not been shown. However, c-Jun contributes directly to rRNA processing by binding to the RNA helicase DDX21 and promoting its translocation to the nucleolus, the site of Pol I-mediated transcription and rRNA processing (Holmström et al., 2008). Exploring the coordinated regulation of Pol I- and Pol III-mediated transcription will provide further insight into the control of ribosome biogenesis and cell growth.

The regulation of Pol III-mediated transcription by oncogenes and tumour-suppressor proteins has been the subject of intense study over the past decade. Pol III products and Pol III-specific transcription factors have been found upregulated in many tumours compared to untransformed tissue (Winter et al., 2000, Chen et al., 1997, Zhao et al., 2004, Hong et al., 2010). Higher levels of protein synthesis are required to sustain the increased growth and proliferation of a cancer cell. However, it remains to be determined if Pol III product overexpression has transformation potential. p53, AP-1 and JNK all have the potential to contribute to the increased Pol III-mediated transcription observed in tumours. Wild type tumour suppressor function of p53 is estimated to be lost in over 80% of human tumours (Lozano and Elledge, 2000). Several common cancer-derived mutants of p53 can no longer repress Pol III-mediated transcription *in vitro* (Stein et al., 2002a). On the other hand, AP-1 and JNK both have pro-tumorigenic activity, contributing to tumour development in a variety of tissues (see Sections 1.6.3 and 1.7.3). It will be interesting to examine whether their association with active Pol III-mediated transcription may be a factor of this contribution.

In conclusion, it is evident that many pathways converge to regulate Pol III-mediated transcription in mammalian cells. This ensures that cellular levels of Pol III products meet the cell's requirements, while efficiency is maintained. In this thesis, I have demonstrated that p53 contributes to the regulation of Pol III-mediated transcription in a stress-specific manner. I also show that AP-1 and JNK both have the potential to directly activate Pol III-mediated transcription. This is in accord with their known pro-proliferative functions, as cell growth is a prerequisite for successful proliferation. In particular, this thesis demonstrates the complexity of the regulation of Pol III-mediated transcription and highlights many directions for future study.

References

Aguilera, C., Nakagawa, K., Sancho, R., Chakraborty, A., Hendrich, B. & Behrens, A. 2011. c-Jun N-terminal phosphorylation antagonises recruitment of the Mbd3/NuRD repressor complex. Nature, 469, 231-235.

Angel, P., Hattori, K., Smeal, T. & Karin, M. 1988. The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell, 55, 875-85.

Arias, J., Alberts, A. S., Brindle, P., Claret, F. X., Smeal, T., Karin, M., Feramisco, J. & Montminy, M. 1994. Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. Nature, 370, 226-9.

Arnold, J. J., Smidansky, E. D., Moustafa, I. M. & Cameron, C. E. 2012. Human mitochondrial RNA polymerase: Structure–function, mechanism and inhibition. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, 1819, 948-960.

Arrand, J. R. & Rymo, L. 1982. Characterization of the major Epstein-Barr virus-specific RNA in Burkitt lymphoma-derived cells. J Virol, 41, 376-89.

Bain, J., Mclauchlan, H., Elliott, M. & Cohen, P. 2003. The specificities of protein kinase inhibitors: an update. Biochem. J., 371, 199-204.

Barski, A., Chepelev, I., Liko, D., Cuddapah, S., Fleming, A. B., Birch, J., Cui, K., White, R. J. & Zhao, K. 2010. Pol II and its associated epigenetic marks are present at Pol III-transcribed noncoding RNA genes. Nat Struct Mol Biol, 17, 629-634.

Bartkiewicz, M., Gold, H. & Altman, S. 1989. Identification and characterization of an RNA molecule that copurifies with RNase P activity from HeLa cells. Genes Dev, 3, 488-99.

Baumann, M., Pontiller, J. & Ernst, W. 2010. Structure and basal transcription complex of RNA polymerase II core promoters in the mammalian genome: an overview. Mol Biotechnol, 45, 241-7.

Baxter, G. C. & Stanners, C. P. 1978. The effect of protein degradation on cellular growth characteristics. J Cell Physiol, 96, 139-45.

Beckerman, R. & Prives, C. 2010. Transcriptional Regulation by P53. Cold Spring Harbor Perspectives in Biology, 2, 1-18.

Behrens, A., Jochum, W., Sibilia, M. & Wagner, E. F. 2000. Oncogenic transformation by ras and fos is mediated by c-Jun N-terminal phosphorylation. Oncogene, 19, 2657-63.

Beier, H. & Grimm, M. 2001. Misreading of termination codons in eukaryotes by natural nonsense suppressor tRNAs. Nucleic Acids Research, 29, 4767-4782.

Bennett, B. L., Sasaki, D. T., Murray, B. W., O'leary, E. C., Sakata, S. T., Xu, W., Leisten, J. C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S. S., Manning, A. M. & Anderson, D. W. 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proceedings of the National Academy of Sciences of the United States of America, 98, 13681-13686.

Bode, A. M. & Dong, Z. 2007. The functional contrariety of JNK. Molecular Carcinogenesis, 46, 591-598.

Bogoyevitch, M. A. & Kobe, B. 2006. Uses for JNK: the Many and Varied Substrates of the c-Jun N-Terminal Kinases. Microbiol. Mol. Biol. Rev., 70, 1061-1095.

Boguta, M. 2013. Maf1, a general negative regulator of RNA polymerase III in yeast. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, 1829, 376-384.

Boyle, W. J., Smeal, T., Defize, L. H., Angel, P., Woodgett, J. R., Karin, M. & Hunter, T. 1991. Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. Cell, 64, 573-84.

Bredow, S., Surig, D., Muller, J., Kleinert, H. & Benecke, B. J. 1990. Activating-transcription-factor (ATF) regulates human 7S L RNA transcription by RNA polymerase III in vivo and in vitro. Nucleic Acids Res, 18, 6779-84.

Brown, J. P., Wei, W. & Sedivy, J. M. 1997. Bypass of Senescence After Disruption of p21CIP1/WAF1 Gene in Normal Diploid Human Fibroblasts. Science, 277, 831-834.

Brown, J. R., Nigh, E., Lee, R. J., Ye, H., Thompson, M. A., Saudou, F., Pestell, R. G. & Greenberg, M. E. 1998. Fos family members induce cell cycle entry by activating cyclin D1. Mol Cell Biol, 18, 5609-19.

Brown, P. H., Alani, R., Preis, L. H., Szabo, E. & Birrer, M. J. 1993. Suppression of oncogene-induced transformation by a deletion mutant of c-jun. Oncogene, 8, 877-86.

Brusselbach, S., Mohle-Steinlein, U., Wang, Z. Q., Schreiber, M., Lucibello, F. C., Muller, R. & Wagner, E. F. 1995. Cell proliferation and cell cycle progression are not impaired in fibroblasts and ES cells lacking c-Fos. Oncogene, 10, 79-86.

Bryant, G. O., Martel, L. S., Burley, S. K. & Berk, A. J. 1996. Radical mutations reveal TATA-box binding protein surfaces required for activated transcription in vivo. Genes & Development, 10, 2491-2504.

Budde, A. & Grummt, I. 1999. p53 represses ribosomal gene transcription. Oncogene, 18, 1119-24.

Buschmann, T., Potapova, O., Bar-Shira, A., Ivanov, V. N., Fuchs, S. Y., Henderson, S., Fried, V. A., Minamoto, T., Alarcon-Vargas, D., Pincus, M. R., Gaarde, W. A., Holbrook, N. J., Shiloh, Y. & Ronai, Z. E. 2001. Jun NH2-Terminal Kinase Phosphorylation of p53 on Thr-81 Is Important for p53 Stabilization and Transcriptional Activities in Response to Stress. Mol. Cell. Biol., 21, 2743-2754.

Cairns, C. A. & White, R. J. 1998. p53 is a general repressor of RNA polymerase III transcription. EMBO J, 17, 3112-23.

Calo, V., Migliavacca, M., Bazan, V., Macaluso, M., Buscemi, M., Gebbia, N. & Russo, A. 2003. STAT proteins: from normal control of cellular events to tumorigenesis. J Cell Physiol, 197, 157-68.

Camacho, J. A., Peterson, C. J., White, G. J. & Morgan, H. E. 1990. Accelerated ribosome formation and growth in neonatal pig hearts. Am J Physiol, 258, C86-91.

Canella, D., Bernasconi, D., Gilardi, F., Lemartelot, G., Migliavacca, E., Praz, V., Cousin, P., Delorenzi, M., Hernandez, N. & Consortium, T. C. 2012. A multiplicity of factors contributes to selective RNA polymerase III occupancy of a subset of RNA polymerase III genes in mouse liver. Genome Research.

Carrière, L., Graziani, S., Alibert, O., Ghavi-Helm, Y., Boussouar, F., Humbertclaude, H., Jounier, S., Aude, J.-C., Keime, C., Murvai, J., Foglio, M., Gut, M., Gut, I., Lathrop, M., Soutourina, J., Gérard, M. & Werner, M. 2012. Genomic binding of Pol III transcription machinery and relationship with TFIIS transcription factor distribution in mouse embryonic stem cells. Nucleic Acids Research, 40, 270-283.

Chakraborty, A., Uechi, T. & Kenmochi, N. 2011. Guarding the 'translation apparatus': defective ribosome biogenesis and the p53 signaling pathway. Wiley Interdisciplinary Reviews-Rna, 2, 507-522.

Chan, P. P. & Lowe, T. M. 2009. GtRNAdb: a database of transfer RNA genes detected in genomic sequence. Nucleic Acids Research, 37, D93-D97.

Chang, Q., Zhang, Y., Beezhold, K. J., Bhatia, D., Zhao, H., Chen, J., Castranova, V., Shi, X. & Chen, F. 2009. Sustained JNK1 activation is associated with altered histone H3 methylations in human liver cancer. J Hepatol, 50, 323-33.

Chen, F., Beezhold, K. & Castranova, V. 2009. JNK1, a potential therapeutic target for hepatocellular carcinoma. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 1796, 242-251.

Chen, R. H., Abate, C. & Blenis, J. 1993. Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. Proc Natl Acad Sci U S A, 90, 10952-6.

Chen, R. H., Juo, P. C., Curran, T. & Blenis, J. 1996. Phosphorylation of c-Fos at the C-terminus enhances its transforming activity. Oncogene, 12, 1493-502.

Chen, W., Bocker, W., Brosius, J. & Tiedge, H. 1997. Expression of neural BC200 RNA in human tumours. J Pathol, 183, 345-51.

Chesnokov, I., Chu, W. M., Botchan, M. R. & Schmid, C. W. 1996. p53 inhibits RNA polymerase III-directed transcription in a promoter-dependent manner. Mol Cell Biol, 16, 7084-8.

Chesnokov, I. & Schmid, C. W. 1996. Flanking sequences of an Alu source stimulate transcription in vitro by interacting with sequence-specific transcription factors. J Mol Evol, 42, 30-6.

Chinenov, Y. & Kerppola, T. K. 2001. Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. Oncogene, 20, 2438-52.

Christophorou, M. A., Ringshausen, I., Finch, A. J., Swigart, L. B. & Evan, G. I. 2006. The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. Nature, 443, 214-7.

Chu, W. M., Ballard, R., Carpick, B. W., Williams, B. R. & Schmid, C. W. 1998. Potential Alu function: regulation of the activity of double-stranded RNA-activated kinase PKR. Mol Cell Biol, 18, 58-68.

Chu, W. M., Wang, Z., Roeder, R. G. & Schmid, C. W. 1997. RNA polymerase III transcription repressed by Rb through its interactions with TFIIIB and TFIIIC2. J Biol Chem, 272, 14755-61.

Clarke, E. M., Peterson, C. L., Brainard, A. V. & Riggs, D. L. 1996. Regulation of the RNA polymerase I and III transcription systems in response to growth conditions. J Biol Chem, 271, 22189-95.

Conkright, M. D., Guzmán, E., Flechner, L., Su, A. I., Hogenesch, J. B. & Montminy, M. 2003. Genome-Wide Analysis of CREB Target Genes Reveals A Core Promoter Requirement for cAMP Responsiveness. Molecular Cell, 11, 1101-1108.

Conlon, I. & Raff, M. 1999. Size Control in Animal Development. Cell, 96, 235-244.

Cox, M. L. & Meek, D. W. 2010. Phosphorylation of serine 392 in p53 is a common and integral event during p53 induction by diverse stimuli. Cellular Signalling, 22, 564-571.

Crighton, D., O'prey, J., Bell, H. S. & Ryan, K. M. 2007. p73 regulates DRAM-independent autophagy that does not contribute to programmed cell death. Cell Death Differ, 14, 1071-9.

Crighton, D., Wilkinson, S., O'prey, J., Syed, N., Smith, P., Harrison, P. R., Gasco, M., Garrone, O., Crook, T. & Ryan, K. M. 2006. DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell, 126, 121-34.

Crighton, D., Woiwode, A., Zhang, C., Mandavia, N., Morton, J. P., Warnock, L. J., Milner, J., White, R. J. & Johnson, D. L. 2003. p53 represses RNA polymerase III transcription by targeting TBP and inhibiting promoter occupancy by TFIIIB. EMBO J, 22, 2810-20.

Crook, T., Marston, N. J., Sara, E. A. & Vousden, K. H. 1994. Transcriptional activation by p53 correlates with suppression of growth but not transformation. Cell, 79, 817-827.

Dai, C. & Gu, W. 2010. p53 post-translational modification: deregulated in tumorigenesis. Trends in Molecular Medicine, 16, 528-536.

Daniels, G. R. & Deininger, P. L. 1985. Repeat sequence families derived from mammalian tRNA genes. Nature, 317, 819-22.

Davis, R. J. 2000. Signal transduction by the JNK group of MAP kinases. Cell, 103, 239-52.

Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M. & Davis, R. J. 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell, 76, 1025-37.

Dieci, G., Bosio, M. C., Fermi, B. & Ferrari, R. 2013. Transcription reinitiation by RNA polymerase III. Biochimica et Biophysica Acta - Gene Regulatory Mechanisms, 1829, 331-341.

Dieci, G., Fiorino, G., Castelnuovo, M., Teichmann, M. & Pagano, A. 2007. The expanding RNA polymerase III transcriptome. Trends Genet, 23, 614-22.

Dittmar, K. A., Goodenbour, J. M. & Pan, T. 2006. Tissue-Specific Differences in Human Transfer RNA Expression. PLoS Genet, 2, e221.

Domitrovich, A. M. & Kunkel, G. R. 2003. Multiple, dispersed human U6 small nuclear RNA genes with varied transcriptional efficiencies. Nucleic Acids Research, 31, 2344-2352.

Donati, G., Peddigari, S., Mercer, C. A. & Thomas, G. 2013. 5S ribosomal RNA is an essential component of a nascent ribosomal precursor complex that regulates the Hdm2-p53 checkpoint. Cell Rep, 4, 87-98.

Eferl, R., Ricci, R., Kenner, L., Zenz, R., David, J. P., Rath, M. & Wagner, E. F. 2003. Liver tumor development. c-Jun antagonizes the proapoptotic activity of p53. Cell, 112, 181-92.

Eferl, R. & Wagner, E. F. 2003. AP-1: a double-edged sword in tumorigenesis. Nat Rev Cancer, 3, 859-868.

Eichhorn, K. & Jackson, S. P. 2001. A Role for TAF3B2 in the Repression of Human RNA Polymerase III Transcription in Nonproliferating Cells. Journal of Biological Chemistry, 276, 21158-21165.

El-Deiry, W. S. 1998. Regulation of p53 downstream genes. Seminars in Cancer Biology, 8, 345-357.

El-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W. & Vogelstein, B. 1992. Definition of a consensus binding site for p53. Nat Genet, 1, 45-9.

Ellenberger, T. E., Brandl, C. J., Struhl, K. & Harrison, S. C. 1992. The GCN4 basic region leucine zipper binds DNA as a dimer of uninterrupted alpha helices: crystal structure of the protein-DNA complex. Cell, 71, 1223-37.

Ernens, I., Goodfellow, S. J., Innes, F., Kenneth, N. S., Derblay, L. E., White, R. J. & Scott, P. H. 2006. Hypoxic stress suppresses RNA polymerase III recruitment and tRNA gene transcription in cardiomyocytes. Nucleic Acids Res, 34, 286-94.

Espinosa, J. M. 2008. Mechanisms of regulatory diversity within the p53 transcriptional network. Oncogene, 27, 4013-23.

Fabian, M. A., Biggs, W. H., 3rd, Treiber, D. K., Atteridge, C. E., Azimioara, M. D., Benedetti, M. G., Carter, T. A., Ciceri, P., Edeen, P. T., Floyd, M., Ford, J. M., Galvin, M., Gerlach, J. L., Grotzfeld, R. M., Herrgard, S., Insko, D. E., Insko, M. A., Lai, A. G., Lelias, J. M., Mehta, S. A., Milanov, Z. V., Velasco, A. M., Wodicka, L. M., Patel, H. K., Zarrinkar, P. P. & Lockhart, D. J. 2005. A small molecule-kinase interaction map for clinical kinase inhibitors. Nat Biotechnol, 23, 329-36.

Fairley, Jennifer a., Mitchell, Louise e., Berg, T., Kenneth, Niall s., Von schubert, C., Silljé, Herman h. W., Medema, René h., Nigg, Erich a. & White, Robert j. 2012. Direct Regulation of tRNA and 5S rRNA Gene Transcription by Polo-like Kinase 1. Molecular Cell.

Fairley, J. A., Scott, P. H. & White, R. J. 2003. TFIIIB is phosphorylated, disrupted and selectively released from tRNA promoters during mitosis in vivo. EMBO J, 22, 5841-50.

Felton-Edkins, Z. A., Fairley, J. A., Graham, E. L., Johnston, I. M., White, R. J. & Scott, P. H. 2003. The mitogen-activated protein (MAP) kinase ERK induces tRNA synthesis by phosphorylating TFIIIB. EMBO J, 22, 2422-32.

Felton-Edkins, Z. A., Kondrashov, A., Karali, D., Fairley, J. A., Dawson, C. W., Arrand, J. R., Young, L. S. & White, R. J. 2006. Epstein-Barr Virus Induces Cellular Transcription Factors to Allow Active Expression of EBER Genes by RNA Polymerase III. Journal of Biological Chemistry, 281, 33871-33880.

Fleischmann, A., Jochum, W., Eferl, R., Witowsky, J. & Wagner, E. F. 2003. Rhabdomyosarcoma development in mice lacking Trp53 and Fos: Tumor suppression by the Fos protooncogene. Cancer Cell, 4, 477-482.

Franklin, C. C., Mcculloch, A. V. & Kraft, A. S. 1995. In vitro association between the Jun protein family and the general transcription factors, TBP and TFIIB. Biochem J, 305 (Pt 3), 967-74.

Fridman, J. S. & Lowe, S. W. 2003. Control of apoptosis by p53. Oncogene, 22, 9030-9040.

Fromm, J. A., Johnson, S. A. & Johnson, D. L. 2008. Epidermal growth factor receptor 1 (EGFR1) and its variant EGFRvIII regulate TATA-binding protein expression through distinct pathways. Mol Cell Biol, 28, 6483-95.

Ghavidel, A. & Schultz, M. C. 2001. TATA Binding Protein-Associated CK2 Transduces DNA Damage Signals to the RNA Polymerase III Transcriptional Machinery. Cell, 106, 575-584.

Gil, J. & Peters, G. 2006. Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. Nat Rev Mol Cell Biol, 7, 667-677.

Gjidoda, A. & Henry, R. W. 2013. RNA polymerase III repression by the retinoblastoma tumor suppressor protein. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, 1829, 385-392.

Gold, H. A., Topper, J. N., Clayton, D. A. & Craft, J. 1989. The RNA processing enzyme RNase MRP is identical to the Th RNP and related to RNase P. Science, 245, 1377-80.

Gomez-Roman, N., Grandori, C., Eisenman, R. N. & White, R. J. 2003. Direct activation of RNA polymerase III transcription by c-Myc. Nature, 421, 290-4.

Goodfellow, S. J., Graham, E. L., Kantidakis, T., Marshall, L., Coppins, B. A., Oficjalska-Pham, D., Gerard, M., Lefebvre, O. & White, R. J. 2008. Regulation of RNA polymerase III transcription by Maf1 in mammalian cells. J Mol Biol, 378, 481-91.

Goodfellow, S. J., Innes, F., Derblay, L. E., Maclellan, W. R., Scott, P. H. & White, R. J. 2006. Regulation of RNA polymerase III transcription during hypertrophic growth. EMBO J, 25, 1522-33.

Goodfellow, S. J. & White, R. J. 2007. Regulation of RNA polymerase III transcription during mammalian cell growth. Cell Cycle, 6, 2323-6.

Goto, H., Yasui, Y., Nigg, E. A. & Inagaki, M. 2002. Aurora-B phosphorylates Histone H3 at serine28 with regard to the mitotic chromosome condensation. Genes to Cells, 7, 11-17.

Gottesfeld, J. M., Wolf, V. J., Dang, T., Forbes, D. J. & Hartl, P. 1994. Mitotic repression of RNA polymerase III transcription in vitro mediated by phosphorylation of a TFIIIB component. Science, 263, 81-4.

Grandori, C., Cowley, S. M., James, L. P. & Eisenman, R. N. 2000. The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu Rev Cell Dev Biol, 16, 653-99.

Grasso, C. S., Wu, Y. M., Robinson, D. R., Cao, X., Dhanasekaran, S. M., Khan, A. P., Quist, M. J., Jing, X., Lonigro, R. J., Brenner, J. C., Asangani, I. A., Ateeq, B., Chun, S. Y., Siddiqui, J., Sam, L., Anstett, M., Mehra, R., Prensner, J. R., Palanisamy, N., Ryslik, G. A., Vandin, F., Raphael, B. J., Kunju, L. P., Rhodes, D. R., Pienta, K. J., Chinnaiyan, A. M. & Tomlins, S. A. 2012. The mutational landscape of lethal castration-resistant prostate cancer. Nature, 487, 239-43.

Gridasova, A. A. & Henry, R. W. 2005. The p53 tumor suppressor protein represses human snRNA gene transcription by RNA polymerases II and III independently of sequence-specific DNA binding. Mol Cell Biol, 25, 3247-60.

Grigoriadis, A. E., Schellander, K., Wang, Z. Q. & Wagner, E. F. 1993. Osteoblasts are target cells for transformation in c-fos transgenic mice. J Cell Biol, 122, 685-701.

Gruda, M. C., Van Amsterdam, J., Rizzo, C. A., Durham, S. K., Lira, S. & Bravo, R. 1996. Expression of FosB during mouse development: normal development of FosB knockout mice. Oncogene, 12, 2177-85.

Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B. & Davis, R. J. 1996. Selective interaction of JNK protein kinase isoforms with transcription factors. EMBO J, 15, 2760-70.

Gupta, S., Campbell, D., Derijard, B. & Davis, R. J. 1995. Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science, 267, 389-93.

Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A., Vasquez, D. S., Turk, B. E. & Shaw, R. J. 2008. AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint. Molecular Cell, 30, 214-226.

Haag, J. R. & Pikaard, C. S. 2011. Multisubunit RNA polymerases IV and V: purveyors of non-coding RNA for plant gene silencing. Nat Rev Mol Cell Biol, 12, 483-492.

Hai, T. & Curran, T. 1991. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. Proc Natl Acad Sci U S A, 88, 3720-4.

Harismendy, O., Gendrel, C. G., Soularue, P., Gidrol, X., Sentenac, A., Werner, M. & Lefebvre, O. 2003. Genome-wide location of yeast RNA polymerase III transcription machinery. EMBO J, 22, 4738-47.

Hazzalin, C. A., Le Panse, R., Cano, E. & Mahadevan, L. C. 1998. Anisomycin Selectively Desensitizes Signalling Components Involved in Stress Kinase Activation and fos andjun Induction. Molecular and Cellular Biology, 18, 1844-1854.

Healy, S., Khan, P. & Davie, J. R. 2013. Immediate early response genes and cell transformation. Pharmacology & Therapeutics, 137, 64-77.

Hibi, M., Lin, A., Smeal, T., Minden, A. & Karin, M. 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev, 7, 2135-48.

Hirsch, H. A., Gu, L. & Henry, R. W. 2000. The retinoblastoma tumor suppressor protein targets distinct general transcription factors to regulate RNA polymerase III gene expression. Mol Cell Biol, 20, 9182-91.

Ho, J. S., Ma, W., Mao, D. Y. & Benchimol, S. 2005. p53-Dependent transcriptional repression of c-myc is required for G1 cell cycle arrest. Mol Cell Biol, 25, 7423-31.

Holmström, T. H., Mialon, A., Kallio, M., Nymalm, Y., Mannermaa, L., Holm, T., Johansson, H., Black, E., Gillespie, D., Salminen, T. A., Langel, Ü., Valdez, B. C. & Westermarck, J. 2008. c-Jun Supports Ribosomal RNA Processing and Nucleolar Localization of RNA Helicase DDX21. Journal of Biological Chemistry, 283, 7046-7053.

Hong, Y., Downey, T., Eu, K. W., Koh, P. K. & Cheah, P. Y. 2010. A 'metastasis-prone' signature for early-stage mismatch-repair proficient sporadic colorectal

cancer patients and its implications for possible therapeutics. Clin Exp Metastasis, 27, 83-90.

Hopper, A. K. & Phizicky, E. M. 2003. tRNA transfers to the limelight. Genes & Development, 17, 162-180.

Horikoshi, N., Usheva, A., Chen, J., Levine, A. J., Weinmann, R. & Shenk, T. 1995. Two domains of p53 interact with the TATA-binding protein, and the adenovirus 13S E1A protein disrupts the association, relieving p53-mediated transcriptional repression. Mol Cell Biol, 15, 227-34.

Horn, H. F. & Vousden, K. H. 2007. Coping with stress: multiple ways to activate p53. Oncogene, 26, 1306-16.

Howe, J. G. & Shu, M. D. 1989. Epstein-Barr virus small RNA (EBER) genes: unique transcription units that combine RNA polymerase II and III promoter elements. Cell, 57, 825-34.

Hsieh, Y. J., Kundu, T. K., Wang, Z., Kovelman, R. & Roeder, R. G. 1999a. The TFIIIC90 subunit of TFIIIC interacts with multiple components of the RNA polymerase III machinery and contains a histone-specific acetyltransferase activity. Mol Cell Biol, 19, 7697-704.

Hsieh, Y. J., Wang, Z., Kovelman, R. & Roeder, R. G. 1999b. Cloning and characterization of two evolutionarily conserved subunits (TFIIIC102 and TFIIIC63) of human TFIIIC and their involvement in functional interactions with TFIIIB and RNA polymerase III. Mol Cell Biol, 19, 4944-52.

Hu, P., Samudre, K., Wu, S., Sun, Y. & Hernandez, N. 2004. CK2 Phosphorylation of Bdp1 Executes Cell Cycle-Specific RNA Polymerase III Transcription Repression. Molecular Cell, 16, 81-92.

Huet, J., Conesa, C., Manaud, N., Chaussivert, N. & Sentenac, A. 1994. Interactions between yeast TFIIIB components. Nucleic Acids Res, 22, 2282-8.

Hui, L., Zatloukal, K., Scheuch, H., Stepniak, E. & Wagner, E. F. 2008. Proliferation of human HCC cells and chemically induced mouse liver cancers requires JNK1-dependent p21 downregulation. J Clin Invest, 118, 3943-53.

Imbriano, C., Gurtner, A., Cocchiarella, F., Di Agostino, S., Basile, V., Gostissa, M., Dobbelstein, M., Del Sal, G., Piaggio, G. & Mantovani, R. 2005. Direct p53 transcriptional repression: in vivo analysis of CCAAT-containing G2/M promoters. Mol Cell Biol, 25, 3737-51.

International Centre for Kinase Profiling, T. Kinase Inhibitor Database, http://www.kinase-screen.mrc.ac.uk/kinase-inhibitors [Online]. [Accessed 2013].

Jelinek, W. R., Toomey, T. P., Leinwand, L., Duncan, C. H., Biro, P. A., Choudary, P. V., Weissman, S. M., Rubin, C. M., Houck, C. M., Deininger, P. L. & Schmid, C. W. 1980. Ubiquitous, interspersed repeated sequences in mammalian genomes. Proc Natl Acad Sci U S A, 77, 1398-402.

Jochum, W., Passegue, E. & Wagner, E. F. 2001. AP-1 in mouse development and tumorigenesis. Oncogene, 20, 2401-12.

Johnson, G. L. & Lapadat, R. 2002. Mitogen-Activated Protein Kinase Pathways Mediated by ERK, JNK, and p38 Protein Kinases. Science, 298, 1911-1912.

Johnson, L. F., Abelson, H. T., Green, H. & Penman, S. 1974. Changes in RNA in relation to growth of the fibroblast. I. Amounts of mRNA, rRNA, and tRNA in resting and growing cells. Cell, 1, 95-100.

Johnson, S. S., Zhang, C., Fromm, J., Willis, I. M. & Johnson, D. L. 2007. Mammalian Maf1 Is a Negative Regulator of Transcription by All Three Nuclear RNA Polymerases. Molecular Cell, 26, 367-379.

Johnston, I. M., Allison, S. J., Morton, J. P., Schramm, L., Scott, P. H. & White, R. J. 2002. CK2 forms a stable complex with TFIIIB and activates RNA polymerase III transcription in human cells. Mol Cell Biol, 22, 3757-68.

Kaneko, H., Dridi, S., Tarallo, V., Gelfand, B. D., Fowler, B. J., Cho, W. G., Kleinman, M. E., Ponicsan, S. L., Hauswirth, W. W., Chiodo, V. A., Kariko, K., Yoo, J. W., Lee, D.-K., Hadziahmetovic, M., Song, Y., Misra, S., Chaudhuri, G., Buaas, F. W., Braun, R. E., Hinton, D. R., Zhang, Q., Grossniklaus, H. E., Provis, J. M., Madigan, M. C., Milam, A. H., Justice, N. L., Albuquerque, R. J. C., Blandford, A. D., Bogdanovich, S., Hirano, Y., Witta, J., Fuchs, E., Littman, D. R., Ambati, B. K., Rudin, C. M., Chong, M. M. W., Provost, P., Kugel, J. F., Goodrich, J. A., Dunaief, J. L., Baffi, J. Z. & Ambati, J. 2011. DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration. Nature, 471, 325-330.

Kantidakis, T., Ramsbottom, B. A., Birch, J. L., Dowding, S. N. & White, R. J. 2010. mTOR associates with TFIIIC, is found at tRNA and 5S rRNA genes, and targets their repressor Maf1. Proc Natl Acad Sci U S A, 107, 11823-8.

Karin, M. & Hawkins, P. T. 1996. The Regulation of AP-1 Activity by Mitogen-Activated Protein Kinases [and Discussion]. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 351, 127-134.

Kasibhatla, S., Brunner, T., Genestier, L., Echeverri, F., Mahboubi, A. & Green, D. R. 1998. DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. Mol Cell, 1, 543-51.

Kassavetis, G. A., Braun, B. R., Nguyen, L. H. & Geiduschek, E. P. 1990. S. cerevisiae TFIIIB is the transcription initiation factor proper of RNA polymerase III, while TFIIIA and TFIIIC are assembly factors. Cell, 60, 235-45.

Kenneth, N. S., Ramsbottom, B. A., Gomez-Roman, N., Marshall, L., Cole, P. A. & White, R. J. 2007. TRRAP and GCN5 are used by c-Myc to activate RNA polymerase III transcription. Proc Natl Acad Sci U S A, 104, 14917-22.

Khan, S. H., Moritsugu, J. & Wahl, G. M. 2000. Differential requirement for p19ARF in the p53-dependent arrest induced by DNA damage, microtubule disruption, and ribonucleotide depletion. Proceedings of the National Academy of Sciences, 97, 3266-3271.

Ko, L. J. & Prives, C. 1996. p53: puzzle and paradigm. Genes & Development, 10, 1054-1072.

Kruse, J. P. & Gu, W. 2009. Modes of p53 regulation. Cell, 137, 609-22.

Kundu, T. K., Wang, Z. & Roeder, R. G. 1999. Human TFIIIC relieves chromatin-mediated repression of RNA polymerase III transcription and contains an intrinsic histone acetyltransferase activity. Mol Cell Biol, 19, 1605-15.

Labuda, D., Sinnett, D., Richer, C., Deragon, J. M. & Striker, G. 1991. Evolution of mouse B1 repeats: 7SL RNA folding pattern conserved. J Mol Evol, 32, 405-14.

Lambert, P. F., Kashanchi, F., Radonovich, M. F., Shiekhattar, R. & Brady, J. N. 1998. Phosphorylation of p53 serine 15 increases interaction with CBP. J Biol Chem, 273, 33048-53.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., Fitzhugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., Levine, R., Mcewan, P., Mckernan, K., Meldrim, J., Mesirov, J. P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., Mcmurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J. C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R. H., Wilson, R. K., Hillier, L. W., Mcpherson, J. D., Marra, M. A., Mardis, E. R., Fulton, L. A., Chinwalla, A. T., Pepin, K. H., Gish, W. R., Chissoe, S. L., Wendl, M. C., Delehaunty, K. D., Miner, T. L., Delehaunty, A., Kramer, J. B., Cook, L. L., Fulton, R. S., Johnson, D. L., Minx, P. J., Clifton, S. W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J. F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., et al. 2001. Initial sequencing and analysis of the human genome. Nature, 409, 860-921.

Landschulz, W. H., Johnson, P. F. & Mcknight, S. L. 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science, 240, 1759-64.

Lane, D. P. 1992. p53, guardian of the genome. Nature, 358, 15-16.

Laptenko, O. & Prives, C. 2006. Transcriptional regulation by p53: one protein, many possibilities. Cell Death Differ, 13, 951-61.

Larminie, C. G., Cairns, C. A., Mital, R., Martin, K., Kouzarides, T., Jackson, S. P. & White, R. J. 1997. Mechanistic analysis of RNA polymerase III regulation by the retinoblastoma protein. EMBO J, 16, 2061-71.

Latonen, L. & Laiho, M. 2005. Cellular UV damage responses--Functions of tumor suppressor p53. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 1755, 71-89.

Lau, E. & Ronai, Z. E. A. 2012. ATF2 – at the crossroad of nuclear and cytosolic functions. Journal of Cell Science, 125, 2815-2824.

Lawler, S., Fleming, Y., Goedert, M. & Cohen, P. 1998. Synergistic activation of SAPK1/JNK1 by two MAP kinase kinases in vitro. Current Biology, 8, 1387-1391.

Levine, A. J. & Oren, M. 2009. The first 30 years of p53: growing ever more complex. Nat Rev Cancer, 9, 749-58.

Li, B., Tournier, C., Davis, R. J. & Flavell, R. A. 1999. Regulation of IL-4 expression by the transcription factor JunB during T helper cell differentiation. EMBO J, 18, 420-32.

Li, M. & Gu, W. 2011. A Critical Role for Noncoding 5S rRNA in Regulating Mdmx Stability. Molecular Cell, 43, 1023-1032.

Lin, T., Chao, C., Saito, S. I., Mazur, S. J., Murphy, M. E., Appella, E. & Xu, Y. 2005. p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. Nat Cell Biol, 7, 165-171.

Liu, W. M., Chu, W. M., Choudary, P. V. & Schmid, C. W. 1995. Cell stress and translational inhibitors transiently increase the abundance of mammalian SINE transcripts. Nucleic Acids Res, 23, 1758-65.

Lowe, S. W. & Sherr, C. J. 2003. Tumor suppression by Ink4a–Arf: progress and puzzles. Current Opinion in Genetics & Development, 13, 77-83.

Lozano, G. & Elledge, S. J. 2000. p53 sends nucleotides to repair DNA. Nature, 404, 24-5.

Maclaren, A., Black, E. J., Clark, W. & Gillespie, D. a. F. 2004. c-Jun-Deficient Cells Undergo Premature Senescence as a Result of Spontaneous DNA Damage Accumulation. Molecular and Cellular Biology, 24, 9006-9018.

Mariner, P. D., Walters, R. D., Espinoza, C. A., Drullinger, L. F., Wagner, S. D., Kugel, J. F. & Goodrich, J. A. 2008. Human Alu RNA is a modular transacting repressor of mRNA transcription during heat shock. Mol Cell, 29, 499-509.

Marshall, L. & White, R. J. 2008. Non-coding RNA production by RNA polymerase III is implicated in cancer. Nat Rev Cancer, 8, 911-4.

Mauck, J. C. & Green, H. 1974. Regulation of pre-transfer RNA synthesis during transition from resting to growing state. Cell, 3, 171-177.

Mayer, C., Bierhoff, H. & Grummt, I. 2005. The nucleolus as a stress sensor: JNK2 inactivates the transcription factor TIF-IA and down-regulates rRNA synthesis. Genes & Development, 19, 933-941.

Mcstay, B. & Grummt, I. 2008. The epigenetics of rRNA genes: from molecular to chromosome biology. Annu Rev Cell Dev Biol, 24, 131-57.

Meek, D. W. 2009. Tumour suppression by p53: a role for the DNA damage response? Nat Rev Cancer, 9, 714-23.

Meek, D. W. & Anderson, C. W. 2009. Posttranslational Modification of p53: Cooperative Integrators of Function. Cold Spring Harbor Perspectives in Biology, 1.

Mellert, H., Sykes, S. M., Murphy, M. E. & Mcmahon, S. B. 2007. The ARF/oncogene pathway activates p53 acetylation within the DNA binding domain. Cell Cycle, 6, 1304-6.

Meng, Q. & Xia, Y. 2011. c-Jun, at the crossroad of the signaling network. Protein Cell, 2, 889-98.

Mohit, A. A., Martin, J. H. & Miller, C. A. 1995. p493F12 kinase: a novel MAP kinase expressed in a subset of neurons in the human nervous system. Neuron, 14, 67-78.

Moir, R. D. & Willis, I. M. 2013. Regulation of pol III transcription by nutrient and stress signaling pathways. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, 1829, 361-375.

Moore, P. B. & Steitz, T. A. 2002. The involvement of RNA in ribosome function. Nature, 418, 229-235.

Moqtaderi, Z. & Struhl, K. 2004. Genome-wide occupancy profile of the RNA polymerase III machinery in Saccharomyces cerevisiae reveals loci with incomplete transcription complexes. Mol Cell Biol, 24, 4118-27.

Moqtaderi, Z., Wang, J., Raha, D., White, R. J., Snyder, M., Weng, Z. & Struhl, K. 2010. Genomic binding profiles of functionally distinct RNA polymerase III transcription complexes in human cells. Nat Struct Mol Biol, 17, 635-640.

Morton, J. P., Kantidakis, T. & White, R. J. 2007. RNA polymerase III transcription is repressed in response to the tumour suppressor ARF. Nucleic Acids Res, 35, 3046-52.

Morton, S., Davis, R. J., Mclaren, A. & Cohen, P. 2003. A reinvestigation of the multisite phosphorylation of the transcription factor c-Jun. EMBO J, 22, 3876-3886.

Mossink, M. H., Van Zon, A., Scheper, R. J., Sonneveld, P. & Wiemer, E. a. C. 2003. Vaults: a ribonucleoprotein particle involved in drug resistance? Oncogene, 22, 7458-7467.

Muller, P. a. J. & Vousden, K. H. 2013. p53 mutations in cancer. Nat Cell Biol, 15, 2-8.

Murray-Zmijewski, F., Slee, E. A. & Lu, X. 2008. A complex barcode underlies the heterogeneous response of p53 to stress. Nat Rev Mol Cell Biol, 9, 702-712.

Nakabeppu, Y., Ryder, K. & Nathans, D. 1988. DNA binding activities of three murine Jun proteins: stimulation by Fos. Cell, 55, 907-15.

Nelson, J. D., Denisenko, O. & Bomsztyk, K. 2006. Protocol for the fast chromatin immunoprecipitation (ChIP) method. Nat. Protocols, 1, 179-185.

Nguyen, V. T., Kiss, T., Michels, A. A. & Bensaude, O. 2001. 7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes. Nature, 414, 322-5.

Nielsen, S., Yuzenkova, Y. & Zenkin, N. 2013. Mechanism of eukaryotic RNA polymerase III transcription termination. Science, 340, 1577-80.

Noguchi, K., Kitanaka, C., Yamana, H., Kokubu, A., Mochizuki, T. & Kuchino, Y. 1999. Regulation of c-Myc through Phosphorylation at Ser-62 and Ser-71 by c-Jun N-Terminal Kinase. Journal of Biological Chemistry, 274, 32580-32587.

Nyathi, Y., Wilkinson, B. M. & Pool, M. R. 2013. Co-translational targeting and translocation of proteins to the endoplasmic reticulum. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1833, 2392-2402.

Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y. & Taya, Y. 2000. p53AIP1, a Potential Mediator of p53-Dependent Apoptosis, and Its Regulation by Ser-46-Phosphorylated p53. Cell, 102, 849-862.

Oler, A. J., Alla, R. K., Roberts, D. N., Wong, A., Hollenhorst, P. C., Chandler, K. J., Cassiday, P. A., Nelson, C. A., Hagedorn, C. H., Graves, B. J. & Cairns, B. R. 2010. Human RNA polymerase III transcriptomes and relationships to Pol II promoter chromatin and enhancer-binding factors. Nat Struct Mol Biol, 17, 620-8.

Ozanne, B. W., Spence, H. J., Mcgarry, L. C. & Hennigan, R. F. 2006. Transcription factors control invasion: AP-1 the first among equals. Oncogene, 26, 1-10.

Papachristou, D. J., Batistatou, A., Sykiotis, G. P., Varakis, I. & Papavassiliou, A. G. 2003. Activation of the JNK–AP-1 signal transduction pathway is associated with pathogenesis and progression of human osteosarcomas. Bone, 32, 364-371.

Paulson, K. E. & Schmid, C. W. 1986. Transcriptional inactivity of Alu repeats in HeLa cells. Nucleic Acids Res, 14, 6145-58.

Phillips, W. F. & Mcconkey, E. H. 1976. Relative stoichiometry in ribosomal proteins in HeLa cell nucleoli. Journal of Biological Chemistry, 251, 2876-2881.

Pluta, K., Lefebvre, O., Martin, N. C., Smagowicz, W. J., Stanford, D. R., Ellis, S. R., Hopper, A. K., Sentenac, A. & Boguta, M. 2001. Maf1p, a negative effector of RNA polymerase III in Saccharomyces cerevisiae. Mol Cell Biol, 21, 5031-40.

Raha, D., Wang, Z., Moqtaderi, Z., Wu, L., Zhong, G., Gerstein, M., Struhl, K. & Snyder, M. 2010. Close association of RNA polymerase II and many transcription factors with Pol III genes. Proceedings of the National Academy of Sciences, 107, 3639-3644.

Ransone, L. J., Kerr, L. D., Schmitt, M. J., Wamsley, P. & Verma, I. M. 1993. The bZIP domains of Fos and Jun mediate a physical association with the TATA box-binding protein. Gene Expr, 3, 37-48.

Reina, J. H., Azzouz, T. N. & Hernandez, N. 2006. Maf1, a new player in the regulation of human RNA polymerase III transcription. PLoS One, 1, e134.

Rinn, J. L. & Huarte, M. 2011. To repress or not to repress: this is the guardian's question. Trends Cell Biol, 21, 344-53.

Roberts, D. N., Stewart, A. J., Huff, J. T. & Cairns, B. R. 2003. The RNA polymerase III transcriptome revealed by genome-wide localization and activity-occupancy relationships. Proc Natl Acad Sci U S A, 100, 14695-700.

Rollins, J., Veras, I., Cabarcas, S., Willis, I. & Schramm, L. 2007. Human Maf1 negatively regulates RNA polymerase III transcription via the TFIIB family members Brf1 and Brf2. Int J Biol Sci, 3, 292-302.

Rosa, M. D., Gottlieb, E., Lerner, M. R. & Steitz, J. A. 1981. Striking similarities are exhibited by two small Epstein-Barr virus-encoded ribonucleic acids and the adenovirus-associated ribonucleic acids VAI and VAII. Mol Cell Biol, 1, 785-96.

Rosette, C. & Karin, M. 1996. Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. Science, 274, 1194-7.

Rozowsky, J., Euskirchen, G., Auerbach, R. K., Zhang, Z. D., Gibson, T., Bjornson, R., Carriero, N., Snyder, M. & Gerstein, M. B. 2009. PeakSeq enables systematic scoring of ChIP-seq experiments relative to controls. Nat Biotech, 27, 66-75.

Rudin, C. M. & Thompson, C. B. 2001. Transcriptional activation of short interspersed elements by DNA-damaging agents. Genes Chromosomes Cancer, 30, 64-71.

Ruggero, D. & Pandolfi, P. P. 2003. Does the ribosome translate cancer? Nat Rev Cancer, 3, 179-92.

Russell, J. & Zomerdijk, J. C. 2005. RNA-polymerase-I-directed rDNA transcription, life and works. Trends Biochem Sci, 30, 87-96.

Sabapathy, K., Hochedlinger, K., Nam, S. Y., Bauer, A., Karin, M. & Wagner, E. F. 2004. Distinct Roles for JNK1 and JNK2 in Regulating JNK Activity and c-Jun-Dependent Cell Proliferation. Molecular Cell, 15, 713-725.

Saez, E., Rutberg, S. E., Mueller, E., Oppenheim, H., Smoluk, J., Yuspa, S. H. & Spiegelman, B. M. 1995. c-fos is required for malignant progression of skin tumors. Cell, 82, 721-732.

Sansom, O. J., Meniel, V. S., Muncan, V., Phesse, T. J., Wilkins, J. A., Reed, K. R., Vass, J. K., Athineos, D., Clevers, H. & Clarke, A. R. 2007. Myc deletion rescues Apc deficiency in the small intestine. Nature, 446, 676-679.

Sawicka, A. & Seiser, C. 2012. Histone H3 phosphorylation – A versatile chromatin modification for different occasions. Biochimie, 94, 2193-2201.

Schramm, L. & Hernandez, N. 2002. Recruitment of RNA polymerase III to its target promoters. Genes Dev, 16, 2593-620.

Schramm, L., Pendergrast, P. S., Sun, Y. & Hernandez, N. 2000. Different human TFIIIB activities direct RNA polymerase III transcription from TATA-containing and TATA-less promoters. Genes Dev, 14, 2650-63.

Schreiber, M., Kolbus, A., Piu, F., Szabowski, A., Mohle-Steinlein, U., Tian, J., Karin, M., Angel, P. & Wagner, E. F. 1999. Control of cell cycle progression by c-Jun is p53 dependent. Genes Dev, 13, 607-19.

Scott, P. H., Cairns, C. A., Sutcliffe, J. E., Alzuherri, H. M., Mclees, A., Winter, A. G. & White, R. J. 2001. Regulation of RNA polymerase III transcription during cell cycle entry. J Biol Chem, 276, 1005-14.

Sharrocks, A. D. 2001. The ETS-domain transcription factor family. Nat Rev Mol Cell Biol, 2, 827-837.

Shaulian, E. & Karin, M. 2001. AP-1 in cell proliferation and survival. Oncogene, 20, 2390-400.

Shaulian, E. & Karin, M. 2002. AP-1 as a regulator of cell life and death. Nat Cell Biol, 4, E131-6.

She, Q. B., Chen, N., Bode, A. M., Flavell, R. A. & Dong, Z. 2002. Deficiency of c-Jun-NH(2)-terminal kinase-1 in mice enhances skin tumor development by 12-O-tetradecanoylphorbol-13-acetate. Cancer Res, 62, 1343-8.

She, X., Jiang, Z., Clark, R. A., Liu, G., Cheng, Z., Tuzun, E., Church, D. M., Sutton, G., Halpern, A. L. & Eichler, E. E. 2004. Shotgun sequence assembly and recent segmental duplications within the human genome. Nature, 431, 927-30.

Sherr, C. J. 2006. Divorcing ARF and p53: an unsettled case. Nat Rev Cancer, 6, 663-73.

Shieh, S.-Y., Ikeda, M., Taya, Y. & Prives, C. 1997. DNA Damage-Induced Phosphorylation of p53 Alleviates Inhibition by MDM2. Cell, 91, 325-334.

Shilatifard, A. 2004. Transcriptional elongation control by RNA polymerase II: a new frontier. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression, 1677, 79-86.

Shor, B., Wu, J., Shakey, Q., Toral-Barza, L., Shi, C., Follettie, M. & Yu, K. 2010. Requirement of the mTOR kinase for the regulation of Maf1 phosphorylation and control of RNA polymerase III-dependent transcription in cancer cells. J Biol Chem, 285, 15380-92.

Smeal, T., Binetruy, B., Mercola, D. A., Birrer, M. & Karin, M. 1991. Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. Nature, 354, 494-6.

Sorek, R., Ast, G. & Graur, D. 2002. Alu-containing exons are alternatively spliced. Genome Res, 12, 1060-7.

Stein, T., Crighton, D., Boyle, J. M., Varley, J. M. & White, R. J. 2002a. RNA polymerase III transcription can be derepressed by oncogenes or mutations that

compromise p53 function in tumours and Li-Fraumeni syndrome. Oncogene, 21, 2961-70.

Stein, T., Crighton, D., Warnock, L. J., Milner, J. & White, R. J. 2002b. Several regions of p53 are involved in repression of RNA polymerase III transcription. Oncogene, 21, 5540-7.

Stott, F. J., Bates, S., James, M. C., Mcconnell, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H. & Peters, G. 1998. The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. EMBO J, 17, 5001-14.

Stults, D. M., Killen, M. W., Pierce, H. H. & Pierce, A. J. 2008. Genomic architecture and inheritance of human ribosomal RNA gene clusters. Genome Res, 18, 13-8.

Sun, X. X., Dai, M. S. & Lu, H. 2008. Mycophenolic acid activation of p53 requires ribosomal proteins L5 and L11. J Biol Chem, 283, 12387-92.

Sutcliffe, J. E., Brown, T. R., Allison, S. J., Scott, P. H. & White, R. J. 2000. Retinoblastoma protein disrupts interactions required for RNA polymerase III transcription. Mol Cell Biol, 20, 9192-202.

Sutcliffe, J. E., Cairns, C. A., Mclees, A., Allison, S. J., Tosh, K. & White, R. J. 1999. RNA polymerase III transcription factor IIIB is a target for repression by pocket proteins p107 and p130. Mol Cell Biol, 19, 4255-61.

Tafolla, E., Wang, S., Wong, B., Leong, J. & Kapila, Y. L. 2005. JNK1 and JNK2 Oppositely Regulate p53 in Signaling Linked to Apoptosis Triggered by an Altered Fibronectin Matrix: JNK LINKS FAK AND p53. Journal of Biological Chemistry, 280, 19992-19999.

Taira, N., Mimoto, R., Kurata, M., Yamaguchi, T., Kitagawa, M., Miki, Y. & Yoshida, K. 2012. DYRK2 priming phosphorylation of c-Jun and c-Myc modulates cell cycle progression in human cancer cells. J Clin Invest, 122, 859-72.

Takahashi, H., Ogata, H., Nishigaki, R., Broide, D. H. & Karin, M. 2010. Tobacco smoke promotes lung tumorigenesis by triggering IKKbeta- and JNK1-dependent inflammation. Cancer Cell, 17, 89-97.

Teichmann, M., Wang, Z. & Roeder, R. G. 2000. A stable complex of a novel transcription factor IIB- related factor, human TFIIIB50, and associated proteins mediate selective transcription by RNA polymerase III of genes with upstream promoter elements. Proc Natl Acad Sci U S A, 97, 14200-5.

Thimmappaya, B., Weinberger, C., Schneider, R. J. & Shenk, T. 1982. Adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times after infection. Cell, 31, 543-51.

Tiwari, V. K., Stadler, M. B., Wirbelauer, C., Paro, R., Schubeler, D. & Beisel, C. 2011. A chromatin-modifying function of JNK during stem cell differentiation. Nat Genet, 44, 94-100.

Upadhya, R., Lee, J. & Willis, I. M. 2002. Maf1 is an essential mediator of diverse signals that repress RNA polymerase III transcription. Mol Cell, 10, 1489-94.

Van Dam, H. & Castellazzi, M. 2001. Distinct roles of Jun : Fos and Jun : ATF dimers in oncogenesis. Oncogene, 20, 2453-64.

Varambally, S., Yu, J., Laxman, B., Rhodes, D. R., Mehra, R., Tomlins, S. A., Shah, R. B., Chandran, U., Monzon, F. A., Becich, M. J., Wei, J. T., Pienta, K. J., Ghosh, D., Rubin, M. A. & Chinnaiyan, A. M. 2005. Integrative genomic and proteomic analysis of prostate cancer reveals signatures of metastatic progression. Cancer Cell, 8, 393-406.

Vogelstein, B., Lane, D. & Levine, A. J. 2000. Surfing the p53 network. Nature, 408, 307-10.

Vogt, P. K. 2002. Fortuitous convergences: the beginnings of JUN. Nat Rev Cancer, 2, 465-9.

Vousden, K. H. & Prives, C. 2009. Blinded by the Light: The Growing Complexity of p53. Cell, 137, 413-31.

Wagner, E. F. & Nebreda, A. R. 2009. Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer, 9, 537-549.

Walter, P. & Blobel, G. 1982. Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. Nature, 299, 691-8.

Wang, H. D., Yuh, C. H., Dang, C. V. & Johnson, D. L. 1995. The hepatitis B virus X protein increases the cellular level of TATA-binding protein, which mediates transactivation of RNA polymerase III genes. Mol Cell Biol, 15, 6720-8.

Wang, H. Y., Cheng, Z. & Malbon, C. C. 2003. Overexpression of mitogen-activated protein kinase phosphatases MKP1, MKP2 in human breast cancer. Cancer Lett, 191, 229-37.

Wang, J., Zhuang, J., Iyer, S., Lin, X., Whitfield, T. W., Greven, M. C., Pierce, B. G., Dong, X., Kundaje, A., Cheng, Y., Rando, O. J., Birney, E., Myers, R. M., Noble, W. S., Snyder, M. & Weng, Z. 2012. Sequence features and chromatin structure around the genomic regions bound by 119 human transcription factors. Genome Research, 22, 1798-1812.

Wang, Z. Q., Grigoriadis, A. E., Mohle-Steinlein, U. & Wagner, E. F. 1991. A novel target cell for c-fos-induced oncogenesis: development of chondrogenic tumours in embryonic stem cell chimeras. EMBO J, 10, 2437-50.

Werner, F. & Grohmann, D. 2011. Evolution of multisubunit RNA polymerases in the three domains of life. Nat Rev Micro, 9, 85-98.

White, R. J. 2001. *Gene Transcription: Mechanisms and Control*, Blackwell Science Ltd.

White, R. J. 2002. *RNA Polymerase III Transcription*, Springer.

White, R. J. 2004. RNA polymerase III transcription and cancer. Oncogene, 23, 3208-16.

White, R. J. 2008. RNA polymerases I and III, non-coding RNAs and cancer. Trends Genet, 24, 622-9.

White, R. J. 2011. Transcription by RNA polymerase III: more complex than we thought. Nat Rev Genet, 12, 459-463.

White, R. J., Gottlieb, T. M., Downes, C. S. & Jackson, S. P. 1995. Cell cycle regulation of RNA polymerase III transcription. Mol Cell Biol, 15, 6653-62.

White, R. J., Trouche, D., Martin, K., Jackson, S. P. & Kouzarides, T. 1996. Repression of RNA polymerase III transcription by the retinoblastoma protein. Nature, 382, 88-90.

Whitfield, J., Neame, S. J., Paquet, L., Bernard, O. & Ham, J. 2001. Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. Neuron, 29, 629-43.

Whitmarsh, A., Shore, P., Sharrocks, A. & Davis, R. 1995. Integration of MAP kinase signal transduction pathways at the serum response element. Science, 269, 403-407.

Wichtowska, D., Turowski, T. W. & Boguta, M. 2013. An interplay between transcription, processing, and degradation determines tRNA levels in yeast. Wiley Interdisciplinary Reviews: RNA, n/a-n/a.

Widmann, C., Gibson, S., Jarpe, M. B. & Johnson, G. L. 1999. Mitogen-Activated Protein Kinase: Conservation of a Three-Kinase Module From Yeast to Human. Physiological Reviews, 79, 143-180.

Will, C. L. & Lührmann, R. 2011. Spliceosome Structure and Function. Cold Spring Harbor Perspectives in Biology, 3.

Winter, A. G., Sourvinos, G., Allison, S. J., Tosh, K., Scott, P. H., Spandidos, D. A. & White, R. J. 2000. RNA polymerase III transcription factor TFIIIC2 is overexpressed in ovarian tumors. Proc Natl Acad Sci U S A, 97, 12619-24.

Wisdom, R., Johnson, R. S. & Moore, C. 1999. c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. EMBO J, 18, 188-197.

Wyatt, M. D. & Pittman, D. L. 2006. Methylating Agents and DNA Repair Responses: Methylated Bases and Sources of Strand Breaks. Chemical Research in Toxicology, 19, 1580-1594.

Yang, S. H., Whitmarsh, A. J., Davis, R. J. & Sharrocks, A. D. 1998. Differential targeting of MAP kinases to the ETS-domain transcription factor Elk-1. EMBO J, 17, 1740-9.

Yang, Z., Zhu, Q., Luo, K. & Zhou, Q. 2001. The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription. Nature, 414, 317-22.

Yazgan, O. & Pfarr, C. M. 2002. Regulation of two JunD isoforms by Jun N-terminal kinases. J Biol Chem, 277, 29710-8.

Yee, K. S. & Vousden, K. H. 2005. Complicating the complexity of p53. Carcinogenesis, 26, 1317-22.

Young, L. S. & Rickinson, A. B. 2004. Epstein-Barr virus: 40 years on. Nat Rev Cancer, 4, 757-68.

Young, M. R., Li, J. J., Rincon, M., Flavell, R. A., Sathyanarayana, B. K., Hunziker, R. & Colburn, N. 1999. Transgenic mice demonstrate AP-1 (activator protein-1) transactivation is required for tumor promotion. Proc Natl Acad Sci U S A, 96, 9827-32.

Young, M. R., Yang, H.-S. & Colburn, N. H. 2003. Promising molecular targets for cancer prevention: AP-1, NF-[kappa]B and Pdcd4. Trends in Molecular Medicine, 9, 36-41.

Zhai, W. & Comai, L. 2000. Repression of RNA polymerase I transcription by the tumor suppressor p53. Mol Cell Biol, 20, 5930-8.

Zhang, T., Inesta-Vaquera, F., Niepel, M., Zhang, J., Ficarro, Scott b., Machleidt, T., Xie, T., Marto, Jarrod a., Kim, N., Sim, T., Laughlin, John d., Park, H., Lograsso, Philip v., Patricelli, M., Nomanbhoy, Tyzoon k., Sorger, Peter k., Alessi, Dario r. & Gray, Nathanael s. 2012. Discovery of Potent and Selective Covalent Inhibitors of JNK. Chemistry & Biology, 19, 140-154.

Zhang, Y., Liu, G. & Dong, Z. 2001. MSK1 and JNKs mediate phosphorylation of STAT3 in UVA-irradiated mouse epidermal JB6 cells. J Biol Chem, 276, 42534-42.

Zhao, H., Langerød, A., Ji, Y., Nowels, K. W., Nesland, J. M., Tibshirani, R., Bukholm, I. K., Kåresen, R., Botstein, D., Børresen-Dale, A.-L. & Jeffrey, S. S. 2004. Different Gene Expression Patterns in Invasive Lobular and Ductal Carcinomas of the Breast. Molecular Biology of the Cell, 15, 2523-2536.

Zhao, K. W., Li, D., Zhao, Q., Huang, Y., Silverman, R. H., Sims, P. J. & Chen, G. Q. 2005. Interferon-alpha-induced expression of phospholipid scramblase 1 through STAT1 requires the sequential activation of protein kinase Cdelta and JNK. J Biol Chem, 280, 42707-14.

Zhong, Q., Shi, G., Zhang, Q., Zhang, Y., Levy, D. & Zhong, S. 2013a. Role of phosphorylated histone H3 serine 10 in DEN-induced deregulation of Pol III genes and cell proliferation and transformation. Carcinogenesis.

Zhong, Q., Shi, G., Zhang, Y., Levy, D. & Zhong, S. 2013b. Elk1 and AP-1 sites in the TBP promoter mediate alcohol-induced deregulation of Pol III-dependent genes. Gene, 526, 54-60.

Zhong, S., Fromm, J. & Johnson, D. L. 2007. TBP is differentially regulated by c-Jun N-terminal kinase 1 (JNK1) and JNK2 through Elk-1, controlling c-Jun expression and cell proliferation. Mol Cell Biol, 27, 54-64.

Zhong, S. & Johnson, D. L. 2009. The JNKs differentially regulate RNA polymerase III transcription by coordinately modulating the expression of all TFIIIB subunits. Proc Natl Acad Sci U S A, 106, 12682-7.

Zhong, S., Machida, K., Tsukamoto, H. & Johnson, D. L. 2011. Alcohol Induces RNA Polymerase III-dependent Transcription through c-Jun by Co-regulating TATA-binding Protein (TBP) and Brf1 Expression. Journal of Biological Chemistry, 286, 2393-2401.