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REGULATION OF THE RIBOSOMAL RNA TRANSCRIPTION BY c-MYC ONCOPROTEIN

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ABSTRACT

The transcription factor c-Myc is a key regulator of cell growth and proliferation. c-Myc levels are tightly controlled and deregulated c-Myc is often associated with human cancers. More specifically, disturbances in regulation of c-Myc through proteasome-mediated degradation have been shown to play a critical role in several cancers. Therefore the original focus of this work was to study the mechanisms involved in regulating proteasomal degradation of c-Myc. In our initial studies we observed that upon inhibition of proteasomes, excess c-Myc accumulates primarily in the nucleoli. After further investigation we could show that c-Myc binds to and activates RNA polymerase I-mediated transcription of the ribosomal RNA (rRNA) genes located in the nucleoli and that proteasomes are involved in this process.

We demonstrate that upon an increase in c-Myc levels through either inhibition of the proteasomes or high expression, c-Myc accumulates in the nucleoli. The dynamics of the nucleoplasmic and the nucleolar c-Myc was studied in living cells expressing GFP-fused c-Myc using the Fluorescent loss in photo-bleaching (FLIP) and the Fluorescent recovery after photo-bleaching (FRAP) techniques. We show that c-Myc is relatively stably associated with the nucleoli. In addition, we show that proteasomes accumulate and co-localise with nucleolar c-Myc.

We further investigate the function of c-Myc in the nucleoli. Here we show that c-Myc and Max interact in the nucleoli and are associated with the ribosomal DNA. Upon mitogenic stimulation of quiescent human lymphocytes c-Myc is recruited to the rRNA genes together with pol I and the co-activator complex TRRAP. Association of c-Myc with the rDNA is also accompanied by an increase in rDNA histone acetylation and activation of rRNA transcription. Inhibition of c-Myc by drug treatment or through expression of small RNA interference inhibits rRNA transcription. These results suggest that c-Myc plays a key role in regulating ribosome biogenesis and thus cell growth. We also show that proteasomes are required for activation of rRNA transcription, even though c-Myc levels increase in response to reduced proteasome activity. The role of proteasomes in rDNA transcription remains to be determined.

We also investigate the role of c-Myc in regulation of the nucleolar organisation and induction of nucleolar alterations in cancer cells. Several types of human cancers with nucleolar alterations including cancers of blood, prostate and breast are also associated with deregulated levels of c-Myc. However, it is not known whether c-Myc contributes to the induction of nucleolar changes in these cancers. We show that despite high levels, c-Myc does not accumulate in the nucleoli in lymphoma and breast cancer cell lines. This is intriguing since nucleolar accumulation of excess c-Myc in other cell lines is associated with inhibition of rRNA transcription. In Cos cells, inhibition of c-Myc by drug treatment or through expression of dominant negative c-Myc mutants causes disruption of the nucleolar organization. We also further investigate the function of nucleolar proteasomes and show that the 20S core particles of the proteasome without the 19S regulatory particles are recruited to the nucleoli.

LIST OF PUBLICATIONS

This thesis is based on the following papers and manuscripts that will be referred to by their Roman numerals.

I. **Arabi A**, Rustum C, Hallberg E, Wright AP. (2003) Accumulation of c-Myc and proteasomes at the nucleoli of cells containing elevated c-Myc protein levels. J Cell Sci. 1;116(Pt 9):1707-17

II. **Arabi A,** Wu S, Ridderstråle K, Bierhoff H, Shiue C, Fatyol K, Fahlén S, Hydbring P, Söderberg O, Grummt I, Larsson LG, and Wright AP. (2005) c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. Nat Cell biol. 7(3):303-10

III. **Arabi A**, Berkson R, and Wright AP. (2006) Regulation of the nucleolar structure by the oncoprotein c-Myc and proteasomes. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

bHLHLZ	Basic Helix loop helix Leucine zipper
CP	Core particle
DBD	DNA binding domain
dMyc	Drosophila Myc
E3	Ubiquitin ligase
ER	estrogen receptor
Fbw7	F-box WD-40 domain protein 7
FLIP	Fluorescence loss in photobleaching
FRAP	Fluorescence recovery after photobleaching
GCN5	General control of amino-acid synthesis
GFP	Green fluorescent protein
GSK3K	Glycogen-synthase kinase 3
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HOT	Hydroxytamoxifen
IF	Immunofluorescence
IL2	Interleukin 2
Miz1	Msx-interacting zinc finger protein
mRNA	Messenger RNA
mTOR	Mammalian target of rapamicyn
PA28	Proteasome activator 28
pCAF	p300/CBP-associated factor
PHA	Phytohaemaglutinin
PI3K	phosphatidylinositol-3-kinase
PML	Promyelocytic leukimia
Pol I	RNA polymerase I
Pol II	RNA polymerase II
Pol III	RNA polymerase III
RP	Regulatory particle
rRNA	Ribosomal RNA
SCF	Skp1/Cul1/F box protein
Skp2	S-phase kinase-associated protein 2
TAD	Transactivation domain
tRNA	Transfer RNA
TRRAP	Transcription / transformation domain-associated protein

1 AIMS OF THE STUDY

The general aim of this work was to study the regulation and function of the c-Myc protein and address how deregulation of c-Myc contributes to tumorigenesis.

The specific aims were to:

- Study the mechanisms controlling cellular distribution and dynamics of c-Myc.
- Delineate the mechanisms involved in nucleolar recruitment of c-Myc and the function of c-Myc in nucleoli.
- Study the potential role of c-Myc in induction of the nucleolar alterations in cancer.
- Study the mechanisms regulating c-Myc proteasomal degradation and the significance of this regulation for c-Myc-mediated transcription control.

2 GENERAL INTRODUCTION TO c-MYC

The discovery of the *c-myc* gene was initiated in the middle of the 1900s by isolation of the *v-myc* gene from the avian myelocytomatosis virus. Subsequently, the *c-myc* gene was isolated from human cells and described as the mammalian homologue of the viral oncogene (Vennstrom et al., 1982). It was soon after discovered that c-Myc was activated in Burkitt's lymphomas, as a result of translocation between chromosome 8 and one of the three chromosomes that contain antibody-coding genes (Hollis et al., 1984). Since then deregulation of c-Myc has been detected in a range of human cancers including breast, colon, cervical and lung cancers (Nesbit et al., 1999).

In humans, the *c-myc* gene encodes a 439 amino acids long transcription factor. It belongs to the Myc protein family which also includes N- and L-Myc. The Myc family members have similar but not entirely redundant functions. c-Myc and N-Myc can substitute each other in vivo (Malynn et al., 2000). c-Myc plays an essential role during mammalian development and disruption of *c-myc* in embryonic stem cells causes early lethality (Davis et al., 1993). Somatic cells survive disruption of c-MYC. However, *c-myc*-null cells have greatly reduced rates of growth and proliferation (Mateyak et al., 1997). Thus, c-Myc is a central promoter of growth and proliferation. Early in vitro studies show that c-Myc expression in quiescent cells is nearly undetectable. After growth stimulation c-Myc mRNA and protein are rapidly induced, concurrent with cell cycle entry. Thereafter c-Myc mRNA and protein decline to low steady-state levels in proliferating cells and to undetectable levels after removal of the growth signals. In contrast to this tight regulation in normal cells, deregulation of c-Myc is often detected in tumours (Spencer and Groudine, 1991).

Besides its essential role in controlling growth and proliferation c-Myc is involved in regulation of a variety of other processes. In-vitro, c-Myc can induce cellular transformation when co-expressed with a cooperating oncogene such as activated Ras (Land et al., 1983). c-Myc expression is down-regulated in cells triggered to undergo differentiation and ectopic expression of c-Myc effectively blocks differentiation (Facchini and Penn, 1998; Larsson et al., 1994). c-Myc also has the ability to induce apoptosis (Evan et al., 1992). In addition, c-Myc has been implicated in inducing genetic instability since over-expression of c-Myc results in chromosomal and extrachromosomal gene rearrangements (Mai et al., 1999).

c-Myc is believed to control these diverse biological processes by both activating and repressing the expression of its numerous target genes. c-Myc activates pol II-mediated transcription by recruitment of basal transcription factors, chromatin modifying and chromatin remodeling complexes to gene promoters. The 150 amino-terminal amino acids in c-Myc have been characterized as the transactivation domain (TAD) (Kato et al., 1990). The TAD contributes to formation of the pre-initiation complex via interaction with the components of the basal transcriptional machinery (McEwan et al., 1996). Additionally, transcription co-activators such as histone acetyltransferases (HATs) and ATP-dependent chromatin remodeling complexes interact with and are recruited by the TAD (McMahon et al., 1998; McMahon et al., 2000). The highly conserved sequences within this region called Myc-boxes (MBs) are also involved in signaling proteasome-mediated degradation of c-Myc (Flinn et al., 1998), and hot spots for mutations that stabilise the c-Myc protein have been found within the MBs in some cancers (Bahram et al., 2000; Gregory and Hann, 2000; Salghetti et al., 1999).

The carboxy-terminal of c-Myc contains the highly conserved basic-helix-loop-helix-leucine zipper (bHLHLZ) motif, required for hetero-dimerisation to Max. Max is a small bHLHLZ-domain protein. In addition to Myc, Max forms heterodimers with the Mnt and the Mad protein-family members (Baudino and Cleveland, 2001). Hetero-dimerisation with Max results in sequence specific DNA-binding and transcription regulation by these proteins. c-Myc-Max heterodimers bind to the DNA sequence CACGTG, belonging to a larger class of sequences (CANNTG) known as E-boxes (Blackwood and Eisenman, 1991). In contrast to c-Myc which activates transcription at E-box containing promoters, Mad-Max and Mnt-Max heterodimers repress transcription at same binding sites **(Fig. 1)**.

In recent years it has become apparent that c-Myc regulates transcription of many genes and that these include RNA polymerase II (pol II)-transcribed protein-coding genes, as well as genes encoding ribosomal RNA and tRNA transcribed by RNA polymerases I (pol I) and III (pol III) (Gomez-Roman et al., 2006). While c-Myc generally has a modest influence on expression of its pol II-transcribed targets, it is a strong activator of pols I and III and as the mechanisms of transcriptional control by c-Myc are deciphered they seem to be somewhat distinct for each Polymerase. Ultimately, delineating the mechanisms that connect transcriptional control by c-Myc to specific cellular events will be essential to understanding the biological behavior of this oncoprotein.

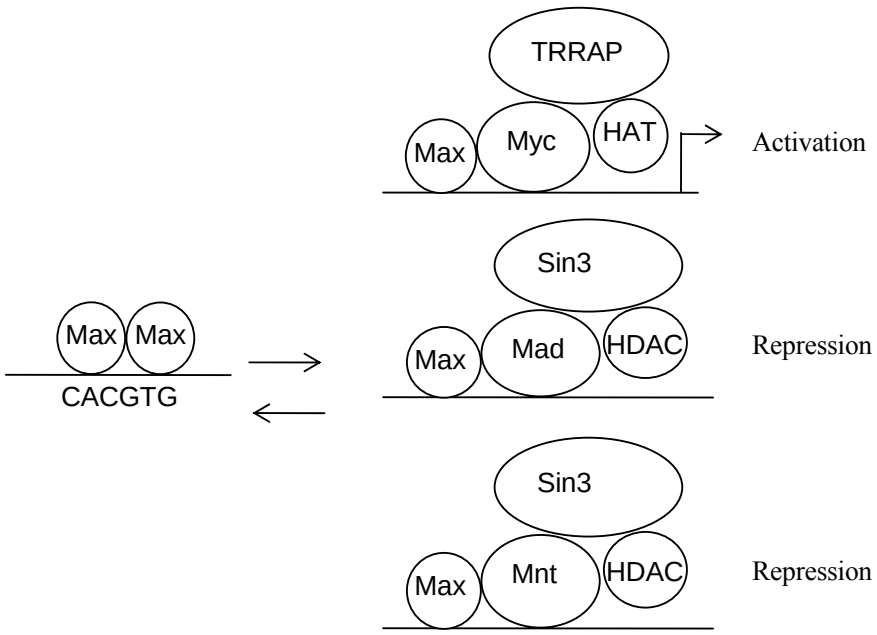

Figure 1. *Transcription regulation by Max-interacting proteins*. Max forms heterodimers with other members of the bHLHLZ protein family. Myc-Max heterodimers bind to the E-boxes in target gene promoters and activate transcription by recruiting histone acetyltransferases (HAT). Mad-Max and Mnt-Max heterodimers bind to the same sites but repress transcription through recruiting histone deacetylases. An equilibrium between these components is believed to regulate the transcription of their targets.

3 REGULATION OF c-MYC ACTIVITY

3.1 EXPRESSION

Expression of the c-MYC gene is tightly regulated and correlates strongly with growth and proliferation. c-Myc expression is high during embryogenesis and targeted disruption of c-Myc in embryonic cells has been shown to be lethal (Davis et al., 1993). In adult organisms high levels of c-Myc is expressed in tissue with high proliferative capacity, whereas low levels are expressed in non-dividing cells (Spencer and Groudine, 1991). Generally, stimulation of cells by external growth signals leads to an increase in c-MYC expression as an immediate early response gene (Henriksson and Luscher, 1996). The Wingless-type MMTV integration site family protein (Wnt) and the Src signaling pathways are involved in upregulation of c-MYC gene expression (Barone and Courtneidge, 1995; van de Wetering et al., 2002). In contrast, signals that inhibit proliferation cause rapid down-regulation of c-Myc expression (Pietenpol et al., 1990). Also, control of mRNA stability plays a role in adjusting c-Myc levels (Jones and Cole, 1987; Lemm and Ross, 2002).

In addition to transcription, translation of c-Myc is responsive to extracellular growth signals, mediated by the phosphotidylinositol 3-kinase (PI3K)/ mammalian target of Rapamycin (mTOR) and the ERK signaling pathways **(Fig. 2)**. Growth signal activation of the PI3K/mTOR and ERK results in an increase in S6 kinase (S6K) activity, which in turn leads to phosphorylation of the ribosomal protein S6. S6 phosphorylation causes a selective increase in translation of mRNAs containing the terminal oligoprymidine (TOP) sequence in the 5'-untranslated region also present in c-Myc (Jefferies et al., 1997; Sekulic et al., 2000; West et al., 1998).

3.2 DEGRADATION BY 26S PROTEASOMES

c-Myc is an unstable protein with a typical half-life of 30 minutes (Hann and Eisenman, 1984). In 1991 it was shown that c-Myc is degraded by the ubiquitin/proteasome pathway (Ciechanover et al., 1991). In this pathway a chain of ubiquitin molecules is covalently attached to the target protein through an enzymatic cascade. In the first step the ubiquitin becomes activated and covalently attached to the active site cystein residue of an E1 ubiquitin activating enzyme by formation of a thiol ester bound. In the second step, the activated ubiquitin is transferred to an E2 ubiquitin conjugating-enzyme and in the final step the ubiquitin is transferred and attached by an isopeptide

bound to a lysine residue in the target protein by an E3 ubiquitin ligase. Additional ubiquitins are then attached to this ubiquitin to form a polyubiquitin chain on the substrate protein. The poly-ubiquitinated target protein is then recognised by the proteolytic enzyme proteasome (Thrower et al., 2000).

The proteasome is a multi-subunit protease, assembled from the 20S proteolytic and the 19S regulatory complexes. The 20S core particle (CP) of the proteasome has a barrel-like structure that is formed by a stack of four oligomeric rings, named α and β. The two inner β rings have glutamyl peptide hydrolyzing, trypsin-like and chymotrypsin-like activities (Chen and Hochstrasser, 1996). The active sites can be accessed only through axial pore at the top and bottom of the stack. The 19S regulatory particle (RP) caps one or both ends of the 20S CP to form a 26S proteasome. The 19S RP is assembled from two main sub-complexes a base with ATPase activity and a lid which is crucial for ubiquitin-dependent proteolysis (Glickman et al., 1998). In isolation, the 20S proteasomes generally show negligible protease activity. The narrow hole of the CP is 10 angstroms in diameter, a size that allows only unfolded proteins to enter (Wenzel and Baumeister, 1995). In addition the N-terminals of the α subunits serve as plugs that keep the CP closed (Groll et al., 2000). Transformation of proteasomes into efficient proteases requires interaction with the 19S RP. The base of the RP contains the AAA- (ATPases Associated with a variety of cellular Activities) ATPAses, which are involved in substrate unfolding and translocation into the 20S proteolytic chamber (Braun et al., 1999; Groll et al., 2000; Kohler et al., 2001). The substrate-linked polyubiquitin chain is recognised by the 19S regulatory subunit 6' (S6') and possibly additional ATPase subunits in the base (Lam et al., 2002).

The proteasome activator 28 (PA28), also named 11S, is another complex that can activate the 20S proteasome. The PA28 contains two subunits α and β which form a hexameric ring structure which upon binding to the 20S opens the substrate entrance pore. However, the PA28 lacks ATPase and ubiquitin recognition activities (Li and Rechsteiner, 2001). The PA28 is suggested to be involved in antigen presentation (Wilk et al., 2000). A related nuclear protein PA28γ, which forms homo-hexameric rings has been shown to be involved in growth and cell cycle regulation (Murata et al., 1999).

The E3 ubiquitin ligases have the key role in substrate selection by the proteasome since they interact specifically with degradation signals in target proteins.

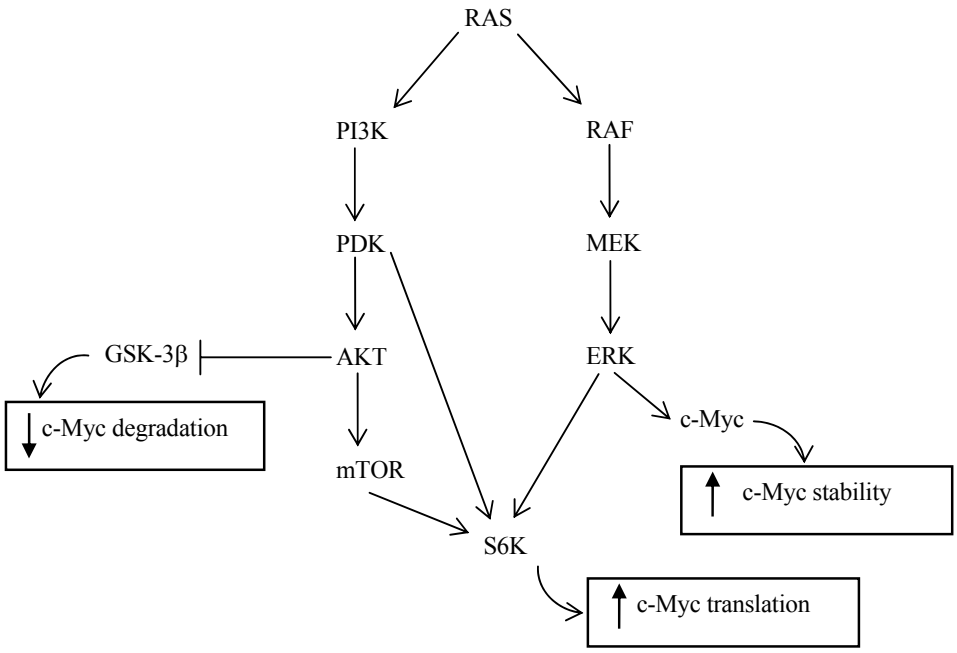

Figure 2. *Regulation of c-Myc levels by extracellular growth signals.* Growth signal stimulation of the signal-transduction pathways downstream of RAS leads to increase in c-Myc levels through multiple mechanisms. Activation of the RAS/MEK/ERK pathway leads to phosphorylation of c-Myc on serine 62 and increases c-Myc protein stability. Activation of the PI3K leads to subsequent phosphorylation and activation of the S6K. Phosphorylation of the S6 subunit of the ribosomes by activated S6K results in an increase in translation via the TOP sequence in the 5'-untranslated region in c-Myc mRNA. In addition, activation of AKT downstream of the PI3K inhibits GSK-3β and thereby prevents phosphorylation of Thr58 which is required for c-Myc degradation.

The degradation determinants in c-Myc are the conserved MB sequences which are located within the amino-terminal TAD (Flinn et al., 1998). MB I mediates control of c-Myc levels by extracellular growth signals. Growth signal activation of the Ras/Raf/Erk pathway results in phosphorylation on Ser62 in MB I which leads to an increase in stability and transcriptional activity of c-Myc. This phosphorylation is a prerequisite for subsequent phosphorylation of Thr58 by Glycogen-synthase kinase-3 (GSK-3), which in turn signals c-Myc's proteasomal degradation (Gregory and Hann, 2000; Sears et al., 2000). Activation of Ras is also upstream of the phosphatidylinositol-3-OH-kinase (PI3K)/AKT-pathway which during early G1 phase of the cell cycle results in inhibition of the GSK-3 and promotes c-Myc stability by

preventing Thr58 phosphorylation. It has also been shown that dephosphorylation of Ser62 by prolyl isomerase/protein phosphatase 2 (PIN1/PP2A) prior to recognition of phosphorylated Thr58 by the ubiquitination machinery is required for c-Myc proteolysis (Yeh et al., 2004). This series of ordered phosphorylations and dephosphorylations are suggested to ensure only a transient increase in c-Myc levels (Dominguez-Sola and Dalla-Favera, 2004).

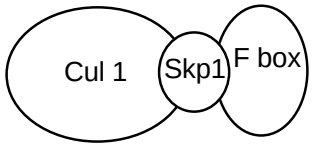

Figure 3. *The SCF ubiquitin ligase complexes ubiquitinate c-Myc.* The SCF is a multimeric protein complex. The substrate specificity of the SCF is determined by the F box proteins. The F box proteins Skp2 and Fbw7 interact specifically with c-Myc.

So far two c-Myc interacting F-box proteins of the Skp1/Cul1/F-box (SCF) ubiquitin ligase have been identified **(Fig. 3)**. The oncoprotein S-phase associated kinase associated protein 2 (Skp2), which interacts with the MB II and the bHLHLZ domain of c-Myc (Kim et al., 2003; von der Lehr et al., 2003) and the F-box WD-40 domain protein 7 (Fbw7) tumor suppressor, which promotes c-Myc ubiquitination via interaction with MB I (Welcker et al., 2004b; Yada et al., 2004). Skp2 can transform cells in cooperation with Ras (Gstaiger et al., 2001; Latres et al., 2001). Defects in Skp2 lead to a decrease in c-Myc-dependent gene activation. Therefore, it is suggested that ubiquitination of c-Myc by Skp2 might be necessary to activate c-Myc, concurrent with targeting its destruction (Kim et al., 2003). The Fbw7-mediated degradation of c-Myc is regulated by phosphorylation of the Thr58 and Ser62 residues in MB I which are hot-spots for cancer mutations. Increased c-Myc levels caused by defects in Fbw7-mediated degradation lead to increased c-Myc-dependent gene activation (Yada et al., 2004). The human Fbw7 locus encodes three protein isoforms α, β and γ, each occupying a distinct cellular location. The Fbw7γ isoform which is nucleolar has been suggested to regulate c-Myc function in the nucleoli (Welcker et al., 2004a). Inhibition of Fbw7γ leads to nucleolar accumulation of c-Myc and increase cell size.

3.3 LOCALIZATION AND DYNAMICS

As first shown in blood cells c-Myc is a predominantly nuclear transcription factor (Abrams et al., 1982; Hann et al., 1983). The nuclear localisation signal in c-Myc is located C-terminally between the amino acids 320 and 328. In most cells c-Myc is evenly distributed in the nucleoplasm under normal growth conditions. Nucleoplasmic c-Myc is highly dynamic and can shuttle between the nucleus and cytoplasm **(paper I)**.

c-Myc has also been detected in a number of distinct nuclear bodies under specific conditions. For example, c-Myc has been reported to associate with ribonucleoprotein (RNP)-containing speckles in proliferating cells and in nuclear bodies described as "large amorphous globules" colocalised with the Heat shock protein 70, in cells over-expressing c-Myc (Henriksson et al., 1988; Koskinen et al., 1991).

c-Myc distribution has also been associated with the Promyelocytic leukemia (PML) nuclear bodies (Smith et al., 2004). PML Bodies are spherical structures that contain the PML protein (Dyck et al., 1994; Weis et al., 1994). The PML protein interacts with many of the proteins generally resident in the PML-nuclear bodies, as well as non-PML body proteins, including c-Myc (Cairo et al., 2005). Some of the proteins found in the PML bodies are present under all conditions, whereas others reside only transiently. Based on the dynamic nature of these bodies it has been suggested that the PML bodies are "nuclear depots" to which numerous proteins are recruited in response to stimuli such as cell stress. Additionally, PML bodies are suggested to be sites for degradation of some nuclear proteins, or play a role in transcription by sequestration and/or modification of transcription factors (Best et al., 2002; Salomoni and Pandolfi, 2002; Zhong et al., 2000a; Zhong et al., 2000b).

c-Myc localisation has been shown to overlap with the PML bodies and this overlap seem to increase during proteasome inhibition, although the mechanism and the consequence of c-Myc recruitment to the PML bodies are not clear. It has been suggested that PML-bodies might provide degradation sites for c-Myc and that break down of PML bodies in cancers might cause c-Myc stabilisation (Smith et al., 2004). c-Myc can also localise in nucleoli. The nucleolar distribution of c-Myc is further described in the following section.

3.3.1 Nucleolar c-Myc

The nucleolus is a non-membranous nuclear organelle. It was initially described as early as in 1890s, but first after half a century was it discovered to be the site of ribosomal RNA transcription and ribosome biogenesis (Jones, 1965). The dynamic processes of ribosomal gene transcription, rRNA processing and ribosomes assembly establish the organisation of the nucleoli (Scheer and Hock, 1999; Schwarzacher and Mosgoeller, 2000). A typical active nucleolus consists of distinct ultrastructural components including the focal center (FC), dense fibrillar component (DFC) and granular component (GC) (Jordan, 1979). The precise site of the rRNA gene transcription in nucleoli which has been debated for decades is most likely to be at the border between the FC and the DFC (Dundr and Raska, 1993; Raska et al., 2004).

Proteins associated with the nucleoli have been shown to move about in a variety of ways. One way is the vectorial movement which originates at the sites of transcription and moves outward to the GC where ribosome assembly and maturation takes place. In a second kind of movement, nucleolar components such as the pre-rRNA processing enzyme fibrillarin, exchange rapidly with the nucleoplasm. Additionally, many proteins seem to pass through or be retained in the nucleoli under specific conditions (Olson and Dundr, 2005). Among the latter are the numerous proteins not related to traditional nucleolar activities (Andersen et al., 2002; Scherl et al., 2002). It is suggested that proteins can enter and leave the nucleolus by free diffusion, but only those with affinity for nucleolar components would be retained (Dundr et al., 2002; Dundr et al., 2000).

c-Myc localises in nucleoli under a variety of conditions. Association of c-Myc with nucleoli under physiological conditions has been shown in human fibroblasts. In these cells during the G0/G1 transition of the cell cycle when both c-Myc and rRNA expression peaks, c-Myc foci can be detected throughout the nucleus including the nucleolus. The nucleolar c-Myc foci coincide with the sites of active rRNA transcription (Grandori et al., 2005). In the human HeLa cell-line which express high levels of c-Myc, phosphorylated c-Myc localises in nucleoli in a fraction of cells (Soldani et al., 2002).

Moreover, following an increase in c-Myc levels, e.g. after inhibition of proteasomes or ectopic expression, it accumulates primarily in the nucleoli as shown in various cell types (**paper I** and (Grandori et al., 2000; Smith et al., 2004; Welcker et al., 2004a). It is therefore possible that nucleoli take part in regulation of c-Myc by providing a sequestration or degradation site.

It has not been studied at ultra-structural level whether a specific sub-nucleolar component is the site of c-Myc accumulation. During proteasome inhibition c-Myc accumulates in nucleolar regions that are distinct from the fibrillarin containing parts **(paper I)**. Fibrillarin is an early pre-rRNA processing factor which is recruited to the nascent rRNAs on active rDNA in the DFC (Dundr et al., 2000). However, it can not be concluded that c-Myc distribution is distinct from the DFC since proteasome inhibition disturbs the nucleolar organisation. For example, it has been shown that the GC and the DFC can no longer be distinguished after proteasome inhibition (Stavreva et al., 2006). In addition, following perturbations in nucleolar function, like several other nucleolar proteins, fibrillarin redistributes (Olson and Dundr, 2005). We show in **paper II** that c-Myc interacts with the rDNA in the nucleoli during both normal and proteasome inhibition conditions. Consistently, the dynamics of the nucleolar c-Myc in living cells show that c-Myc is engaged in a biological interaction in nucleoli **(paper I)**.

4 FUNCTION OF c-MYC

4.1 BIOLOGICAL FUNCTIONS OF c-MYC

c-Myc is involved in regulation of a number of cellular processes including metabolism, growth, division and apoptosis. c-Myc is believed to regulate these processes through its ability to control transcription of specific genes. Thus, target gene identification has been the emphasis in the study of c-Myc's biological functions. c-Myc targets have been identified by both DNA-binding and expression assays. Genome-wide analysis of c-Myc targets has led to identification of more than 1600 potential targets (Coller et al., 2000; Kim et al., 2000; Menssen and Hermeking, 2002; O'Connell et al., 2003). Although the functions of many of these putative targets correspond well with c-Myc's known activities, the molecular mechanisms that connect c-Myc targets to its biological behavior are not yet fully delineated.

One function of c-Myc is controlling the cell cycle. c-Myc is an immediate early response gene and inhibition or loss of c-Myc has been shown to block G0 to S and G1 to S transition upon serum induction of resting cells (de Alboran et al., 2001; Heikkila et al., 1987). Moreover, in *c-myc*-null fibroblasts both the G1 and G2 phases of the cell cycle are prolonged (Mateyak et al., 1997).

c-Myc is believed to control cell division partly through its targets which are direct regulators of the cell-cycle. For example cyclins, cyclin-dependent kinases (Cdks) and Cdk inhibitors are both up and down-regulated by c-Myc (Beier et al., 2000; Bouchard et al., 2001). G1 to S progression is mainly controlled by cyclin D-CDK4 and cyclin E-CDK2 which are inhibited by p15 and p21, respectively. c-Myc has the capacity to promote G1/S transition by repressing the transcription of these CDK-inhibitors through binding to and inhibiting the transcription factor Miz1 (Herold et al., 2002; Staller et al., 2001). The mechanisms of cell cycle control by c-Myc are summarised in **Fig. 4**.

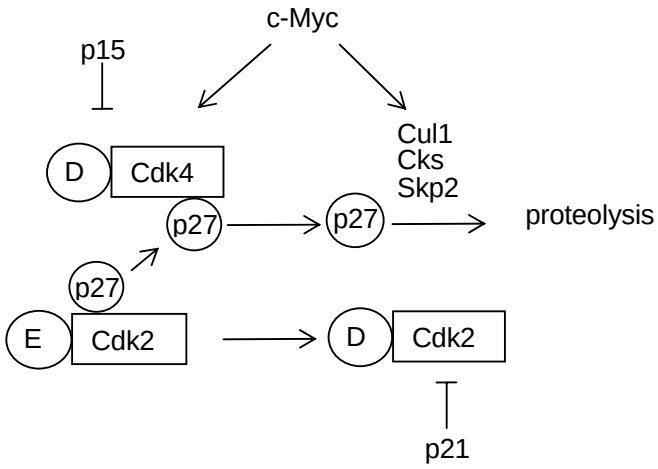

Figure 4. *Mechanisms of cell cycle regulation by c-Myc.* c-Myc activates the transcription of cyclin D/Cdk4. Cyclin D/Cdk4 complexes activate cyclin E/Cdk2 by sequestration of the cyclin E/Cdk2-inhibitor p27. Subsequent ubiquitination and proteasomal degradation of p27 is controlled by transcriptional targets of c-Myc. In addition, transcription of Cdk-inhibitors p15 and p21 is repressed by c-Myc.

Similar to some other oncoproteins like adenovirus E1A and E2F, c-Myc is a potent inducer of both proliferation and apoptosis. Expression of c-Myc in the absence of survival factors such as growth signals induces apoptosis (Evan et al., 1992). It is suggested that the oncoprotein-induced apoptotic pathway is suppressed as long as the cells receive survival signals. This regulation would sensitise cell-cycle entry to apoptosis in absence of appropriate growth stimulation. This will potentially inhibit the expansion of cells with deregulated oncogenes, which would be unable to survive in the absence of growth stimulatory signals unless apoptosis is inhibited (Pelengaris et al., 2002).

The precise molecular mechanisms by which c-Myc activates apoptosis are not known, however activation of the pro-apoptotic proteins such as p53 and Bax and inhibition of anti-apoptotic proteins such as Bcl2 has been implicated (Eischen et al., 2001a; Eischen et al., 2001b; Juin et al., 2002; Lindstrom and Wiman, 2003) . The release of cytochrome c from mitochondria is downstream of the c-Myc-activated apoptotic pathway (Juin et al., 1999). Upon release to the cytoplasm, cytochrome c activates caspases which ultimately break up the cell components and cause death.

The largest functional group of c-Myc targets are genes implicated in metabolism. These include genes involved in nucleotide and DNA metabolism like the CAD (carbamoyl phosphate synthase), ODC (ornithine decarboxylase), and thymidine kinase genes (Bello-Fernandez et al., 1993; Miltenberger et al., 1995). The genes involved in amino acid, lipid and sugar transport and metabolism also belong to this group of c-Myc targets. Thus, by regulating metabolism c-Myc provides the cells with the building blocks for growth.

Another large functional group of c-Myc targets is the pol II-transcribed genes encoding components of the protein synthesis machinery (Coller et al., 2000; Kim et al., 2000; Menssen and Hermeking, 2002) **(Table 1)**. One class of these targets consists of genes that encode the ribosomal proteins (r-proteins), which together with rRNAs are assembled in nucleoli into ribosomes. It has been shown in both yeast and higher eukaryotes that the precise equimolar amounts of r-proteins are required to ensure effective ribosome biogenesis and that deletion of a single r-protein has direct inhibitory effect on cell growth (Ruggero and Pandolfi, 2003). The second class is the nucleolar proteins. These proteins are involved in rRNA transcription and processing, and ribosome assembly. The third class includes genes encoding translation-initiation and elongation factors. The elF4E and elF2α protein which are rate limiting for translation belong to this class of c-Myc targets (Rosenwald et al., 1993).

Table 1. *c-Myc regulated proteins involved in ribosome-genesis and translation control*. c-Myc targets are genes encoding the large (L) and small (S) ribosome subunit proteins. Among the nucleolar proteins which are regulated by c-Myc are proteins involved in rRNA transcription and processing. Also, some translation elongation factors (elF), translation initiation factor (IF) are regulated by c-Myc.

Ribosomal proteins	L3, L15, L37, L39, S2, S3a, S6
Nucleolar proteins	Nucleophosmin, Nucleolin, Fibrillain, Nopp140, UBF
Translation factors	elF-4A, elF-4E, elF-3, EEF1a1, IF3X

Protein synthesis is strongly connected to growth (Rudra and Warner, 2004). Therefore c-Myc targets involved in protein synthesis are likely mediators of its growth promoting function. The requirement of c-Myc for promoting growth has been demonstrated in c-MYC-deficient fibroblasts that have reduced rates of rRNA

synthesis, protein synthesis and growth, defined by accumulation of mass (Mateyak et al., 1997). The connection of c-Myc to growth has also been reported in c-Myc over-expressing B lymphocytes of mice and in human hepatocytes (Iritani and Eisenman, 1999; Kim et al., 2000). In addition to pol II-transcribed c-Myc targets coupled to protein synthesis, the Pol III and I-mediated transcription of tRNAs and rRNAs is activated by c-Myc. Thus, c-Myc has the potential to coordinate growth through the regulation of genes involved in translation, through activation of three distinct transcriptional systems **(Fig. 6)**.

As described earlier c-Myc is a down stream target of mTOR **(Fig. 2)**. mTOR is a phosphatidylinositol kinase-related kinase that controls many processes related to growth. c-Myc seems to cooperate closely with mTOR in growth regulation (Schmelzle and Hall, 2000) **(Fig. 5)**.

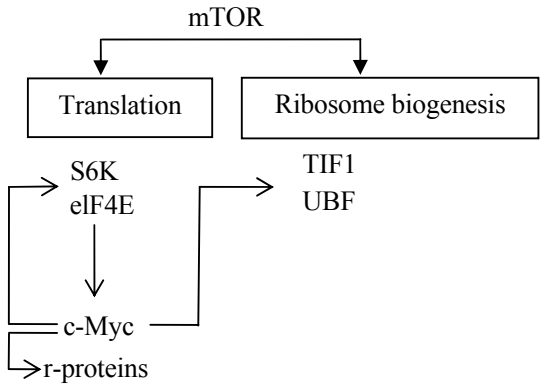

Figure 5. *mTOR and c-Myc control common growth-related components.* mTOR regulates translation in part by activating the S6K and elF4E proteins. Activation of these proteins induces the 5'TOP and 5'cap sequence dependent translation, respectively. Translation of c-Myc and r-proteins is induced following S6K activation. c-Myc translation is also induced by elF4E activation. The TIF1A and UBF components of the rRNA transcription machinery are phosphorylated and activated by mTOR. elF4E, r-proteins and UBF and possibly S6K are transcriptional targets of c-Myc.

As shown in various cell types proliferation to a large extent depends on the capacity of cells to increase in size and to coordinate this growth with division (Neufeld and Edgar, 1998). Hence, c-Myc has the potential to regulate proliferation by

controlling growth. However, several studies indicate that growth regulation by c-Myc can be uncoupled from cell division. For example, ectopic expression of c-Myc in hepatocytes leads to increase in cell size but does not affect cell division (Kim et al., 2000). Similarly in mouse B cells and embryonic fibroblasts regulation of growth by c-Myc seems to not have an effect on cell division (Beier et al., 2000; Iritani and Eisenman, 1999). Thus, it is still a matter of debate if c-Myc regulates cell division primarily through regulation of cell cycle genes or by controlling growth.

Myc controls the transcription of growth-related genes in invertebrates. In *Drosophila*, dMyc mutant cells are smaller whereas cells over-expressing dMyc are larger than normal cells (de la Cova et al., 2004; Johnston et al., 1999; Orian et al., 2003). Interestingly, the phenotype of the dMyc-mutant fly is similar to the minute class of the fly with mutations in the ribosomal protein genes indicating that dMyc might be connected to ribosome biogenesis. Unlike mammalian c-Myc, dMyc seems to regulate growth only via activation of pol II-transcribed genes. The rDNA in *Drosophila* does not contain E-boxes and dMyc could not be found associated with the rDNA. However, dMyc-activated expression of pol I and TIF-1A precede the increase in rRNA expression (Grewal et al., 2005). Thus, in spite of mechanistical differences, the role of c-Myc in growth regulation is evolutionarily conserved. While dMyc controls cell size, the overall cell number and cycle time seem not to be affected by dMyc, suggesting that Myc–mediated growth control in *Drosophila* might be uncoupled from cell division.

c-Myc is also involved in a process known as cell competition. Cell competition was first described in Drosophila whereby cells with reduced growth rates, typically achieved by reducing the dose of a ribosomal protein, are eliminated via apoptosis (Morata and Ripoll, 1975). It has been shown that such cell competition can also take place by juxtaposing cells with different levels of c-Myc. The slower-growing cells with wild-type levels of c-Myc can be out-competed by nearby cells over-expressing c-Myc, which both grow faster and actively eliminate their slower growing neighbors by inducing apoptosis (de la Cova et al., 2004; Moreno and Basler, 2004). Cell competition is suggested to control normal organ size during development. In disease, cell competition might increase the clonal expansion of c-Myc over-expressing cells, while simultaneously inducing apoptosis in non-tumor cells (Donaldson and Duronio, 2004).

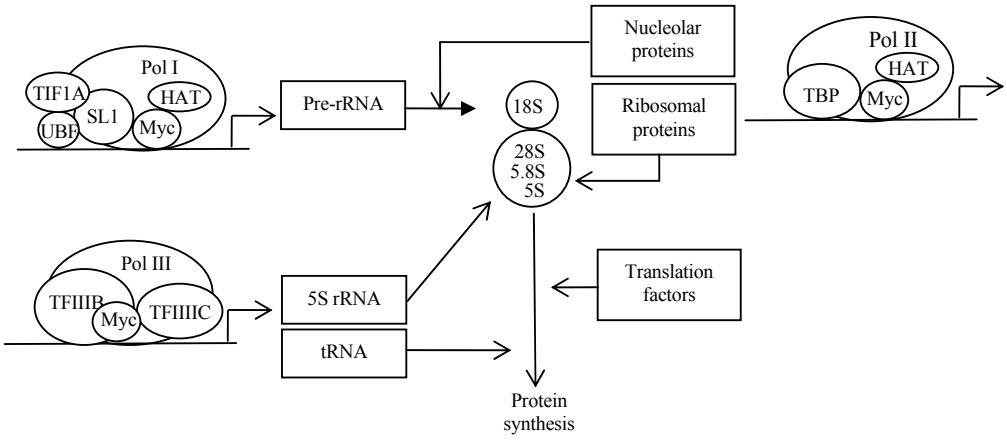

Figure 6. *c-Myc controls protein synthesis through regulation of pol I, II and III.* c-Myc activates pre-rRNA transcription by pol I. Pre-rRNA processing by nucleolar proteins generates the 18S, 5,8S and 18S rRNAs. These rRNAs together with the 5S rRNA transcribed by pol III, and the ribosomal proteins, assemble to form the ribosomes which are the sites of protein synthesis. The tRNA and several nucleolar and ribosomal proteins are transcriptional targets of c-Myc.

4.2 MECHANISMS OF TRANSCRIPTION REGULATION

In eukaryotes nuclear RNA transcription is catalyzed by three RNA polymerases. Transcription of the protein-coding genes is driven by Pol II, whereas Pol I and Pol III catalyse transcription of the rRNA and tRNA genes (Chambon, 1975). The genomic DNA is wrapped around the histones to form nucleosomes and a structure known as the chromatin. The chromatin is further organized into a compact structure. Generally, transcriptionally inactive regions coincide with the highly condense chromatin, named heterochromatin, whereas active genes correspond to chromatin with a loosened conformation called euchromatin (Croston and Kadonaga, 1993; Svaren and Horz, 1993). Opening up the chromatin is necessary to allow transcription to occur. Gene promoters often contain regulatory elements to which transcription factors bind. Transcription factors aid the assembly of the pre-initiation complex at the promoter and

transcription initiation by chromatin remodeling. The pol II-pre-initiation complex contains numerous proteins including TATA-binding protein (TBP) and TBP associated factors (TAFs), and is required by the polymerase to start transcription (Struhl, 1995).

Chromatin remodeling can occur partly by histone modification. Histones can be modified by acetylation, phosphorylation, methylation and ubiquitination, and the pattern of specific histone modifications determine gene activity (Jenuwein and Allis, 2001; Strahl and Allis, 2000). This marking system, known as the histone code, represents a fundamental regulatory mechanism that has an impact on most, if not all, chromatin mediated processes. In general, Highly acetylated histones are associated with euchromatic regions. In contrast, histone deacetylation is found in condensed chromatin. Histone acetylation is catalyzed by the histone acetylatransferases (HATs) and deacetylation by histone deacetylases (HDACs) which are recruited to promoters by transcription factors. Alternatively, ATP-dependent chromatin remodeling complexes are recruited for gene activation and repression. The ATP-dependent remodeling complexes use the energy of ATP hydrolysis to alter DNA-histone contacts and/or nucleosomes positioning and thereby control the exposure of regulatory elements in the chromatin (Whitehouse et al., 1999).

In recent years it has been shown that the components of the ubiquitin-proteasome pathway are also involved in regulation of transcription (Ottosen et al., 2002). Ubiquitin tagging has been shown to modulate stability of many transcription factors. Also histones, in particular H2A and H2B are ubiquitinated. Moreover, the ATPases of the 19S regulatory complex can associate with transcribed genes in vivo (Gonzalez et al., 2002) **(Fig. 7)**. Although the role of distinct proteasome subunits in transcription is not fully delineated some possibilities have been suggested. The 19S ATPases are suggested to generate the energy to drive the assembly, initiation or elongation activities of the transcription machinery, which might involve protein folding or remodeling of protein complexes (Ferdous et al., 2001). It has also been suggested that the 19S might recruit the rest of the proteasomes to active genes to control or down-regulate transcription by proteolysis.

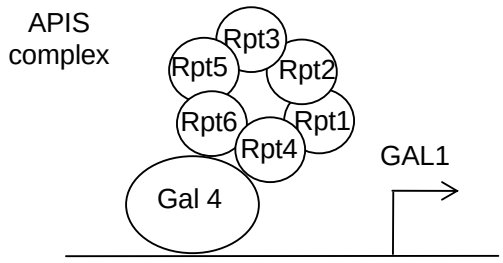

Figure 7. *The 19S RP ATPases are involved in pol II-mediated transcription in yeast.* The APIS (AAA proteins independent of 20S) complex is recruited to the active GAL1 promoter through interaction with the Gal4 transcription factor and functions as a transcriptional co-activator.

4.2.1 Pol II transcription

Pol II transcription by c-Myc is partly controlled through influencing the chromatin structure (Amati et al., 2001). Commonly, c-Myc recruits the chromatin remodeling factors to the proximity of the gene promoters which results in gene activation. The two main functional domains in c-Myc are the amino-terminal TAD with the conserved regions MBI and MBII, and the carboxy-terminal bHLHLZ dimerisation and DNA-binding domain. An intact bHLHLZ domain is indispensable for all known c-Myc's transcriptional and biological activities (Amati et al., 1992; Littlewood et al., 1992). c-Myc heterodimerisation with another HLHLZ protein, Max results in sequence specific DNA binding. c-Myc-Max heterodimers bind to the consensus DNA sequence CACGTG known as an E-box (Luscher and Larsson, 1999). c-Myc-Max heterodimers also recognise the noncanonical sites which are variations of the canonical E-box with the core TG or CG (Blackwell et al., 1993). In addition to c-Myc other HLHLZ-domain proteins such as Mad and Mnt can heterodimerise with Max and c-Myc has been shown to compete with these proteins for binding to Max and E-boxes. For example, the Mad/Max and Myc/Max heterodimers compete for binding to same E-boxes at the cyclin D2 promoter. While Mad/Max dimers repress transcription by recruiting

HDACs, Myc/Max dimers activate Cyclin D2 expression by recruiting HATs (Bouchard et al., 2001).

In contrast to the DNA binding domain, distinct regions in c-Myc TAD seem to mediate different transcriptional and biological activities. For example, MB II is necessary for transcription repression and cellular transformation, whereas MB I is unnecessary for these activities (Li et al., 1994). Various proteins have been shown to interact with distinct regions in the c-Myc TAD and it is likely that different regulatory effects are mediated through these interactions. The TAD-mediated interaction of c-Myc with the basal transcription factors TATA binding protein (TBP) and TFIID is believed to activate transcription by inducing the pre-initiation complex formation (Barrett et al., 2005; Chatterjee and Struhl, 1995; McEwan et al., 1996). The co-activator protein TRRAP (Transformation/transactivation domain-associated protein) interacts with c-Myc via MB II (McMahon et al., 1998). TRRAP mediates c-Myc activity by recruiting the histone acetyltransferases GCN5, PCAF and Tip60 (McMahon et al., 2000). Gene activation by c-Myc can also occur by mechanisms independent of histone acetylation. For example, interaction of c-Myc with the SWI/SNF co-activator complex which has ATPase/Helicase activity results in chromatin remodeling and transcription activation (Cheng et al., 1999; Flinn et al., 2002).

Another transcriptional co-activator of c-Myc is the Skp2 Ubiquitin-ligase protein. Skp2 binds to c-Myc via MBII and the bHLHLZ domain and promotes c-Myc ubiquitination and proteasomal degradation. However, Skp2 is also a potent stimulator of c-Myc and defects in Skp2 reduce c-Myc transcriptional activity (Kim et al., 2003; von der Lehr et al., 2003). The dual action of Skp2 indicates that ubiquitination and possibly proteasome-mediated degradation might be required for c-Myc-activated transcription.

c-Myc can also repress transcription. For example, transcription of the cell cycle-inhibitory proteins p21 and Ink4b is repressed by c-Myc (Staller et al., 2001; Wu et al., 2003). A number of c-Myc repressed genes contain initiator elements (Inrs). Inrs are usually found in TATA-less promoters and are binding sites for c-Myc interacting proteins such as Yin-Yang1 (YY1) and Miz1. c-Myc represses transcription by sequestering these positively acting transcription factors (Peukert et al., 1997; Shrivastava et al., 1993).

4.2.2 Pol III transcription

Pol III is the largest nuclear RNA polymerase and has the greatest number of subunits. It transcribes the tRNA and the 5S rRNA genes (Geiduschek and Tocchini-Valentini, 1988). Pol III-transcribed genes often contain internal promoters, located downstream of the transcription start site. The promoter is recognised by the DNA-binding factor TFIIIC2. TFIIIC2 recruits another factor TFIIIB, which in turn recruits pol III and positions it over the transcription start site (Schramm and Hernandez, 2002). The activity of TFIIIB is a major determinant of protein synthesis capacity. Its activity is controlled by several tumor suppressors and oncogenes including p53, RB and E1A (Chesnokov et al., 1996; White et al., 1996; Whyte et al., 1988).

c-Myc is also a direct and potent activator of pol III transcription (Gomez-Roman et al., 2003). This has been shown by retroviral transduction of the estrogen receptor-fused c-Myc (MycER) in primary human fibroblasts. Induction of c-Myc by Hydroxytamoxifen (HOT) activated pol III-derived transcription. Deletion of residues 106-143 in the c-Myc transactivation domain which also contains the MBII prevented induction of tRNA genes. c-Myc was shown to be a strong activator of pol III since the activity of tRNA genes increased 12-fold or more which significantly exceeds the influence of c-Myc on the activity of pol II-transcribed genes. Inhibition of c-Myc by RNA interference (RNAi) or in genetic knockouts caused reduction in pol III transcription in both human and rodent cells, indicating that pol III transcription is sensitive to c-Myc at physiological concentrations. Furthermore, c-Myc could associate with the tRNA and 5S rRNA genes in vivo. However these genes do not contain E-box sequences that are recognised by c-Myc. Instead, c-Myc seems to be recruited to these genes through interaction with TFIIIB (Gomez-Roman et al., 2003).

4.2.3 Pol I transcription

In the mid 1900s it was discovered that the ribosomal RNA genes are transcribed by Pol I, in the nucleoli (Tocchini-Valentini and Crippa, 1970). Nucleoli are formed around the rDNA post-mitosis, with the onset of rRNA transcription (Scheer and Weisenberger, 1994; Warner et al., 1973). In higher eukaryotes multiple copies of rRNA genes are arranged as tandem repeats. In humans, the rRNA genes are organized as clusters of 43kb repeats on five different chromosomes. Each repeat unit is comprised of a transcribed region flanked by a non-transcribed intergenic spacer. The transcribed region contains the genes encoding the 18, 5,8 and 28S subunits of the ribosome (Sylvester et al., 1986).

Pol I-mediated transcription of the ribosomal genes is a well regulated process and adjustment of the rRNA synthesis rate in response to extracellular signals is believed to control ribosome production and the potential for cell growth (Grummt, 2003; Russell and Zomerdijk, 2006). It was shown early that in growing cells only a subpopulation of the rRNA genes is active at any given time, and that activity is tightly related to the structure of the rDNA chromatin (Flavell, 1986; McKnight and Miller, 1976). As shown later by psoralen DNA-crosslinking experiments, two classes of rDNA coexist in cycling cells. One that is free of regularly spaced nucleosomes, and associated with active rRNA synthesis, and the other that displays regularly spaced nucleosomes and corresponds to inactive genes (Conconi et al., 1989). DNA and histone modifications and ATP-dependent chromatin-remodeling are suggested to be involved in establishment and maintenance of the active and inactive state of the rRNA genes (Grummt and Pikaard, 2003).

The level of rRNA expression can in principal be controlled either by varying the ratio of active and silent genes or by modulating the initiation frequency in active genes. The latter seems to regulate rRNA transcription in response to growth signals in mammals (Conconi et al., 1989). Several components of the pol I transcriptional machinery are responsive to growth stimulation **(Fig. 8)**. One is the transcription factor I-A (TIF-IA). TIF-1A aids the assembly of the preinitiation complex (PIC) by binding to the promoter selectivity factor (SL1) and pol I. Activation of TIF-IA downstream of the growth signal-activated TOR/S6K and RAS/ERK pathways activates rRNA transcription (Grummt, 2003; Mayer et al., 2004; Zhao et al., 2003). In addition, the PIC contains another basal transcription factor namely, the upstream binding factor (UBF). UBF is proposed to function as an architectural protein at the rDNA promoter (Jantzen et al., 1992; Pikaard et al., 1989; Wright et al., 2006). The activity of UBF is also responsive to growth signals and mainly regulated by phosphorylation (Drakas et al., 2004; Stefanovsky et al., 2006; Stefanovsky et al., 2001). Phosphorylation-dependent interaction of the tumor suppressor proteins RB and p53 with UBF and SL1 down regulates rRNA transcription through interference with the assembly of the basal transcription machinery (Cavanaugh et al., 1995; Hannan et al., 2000; Zhai and Comai, 2000).

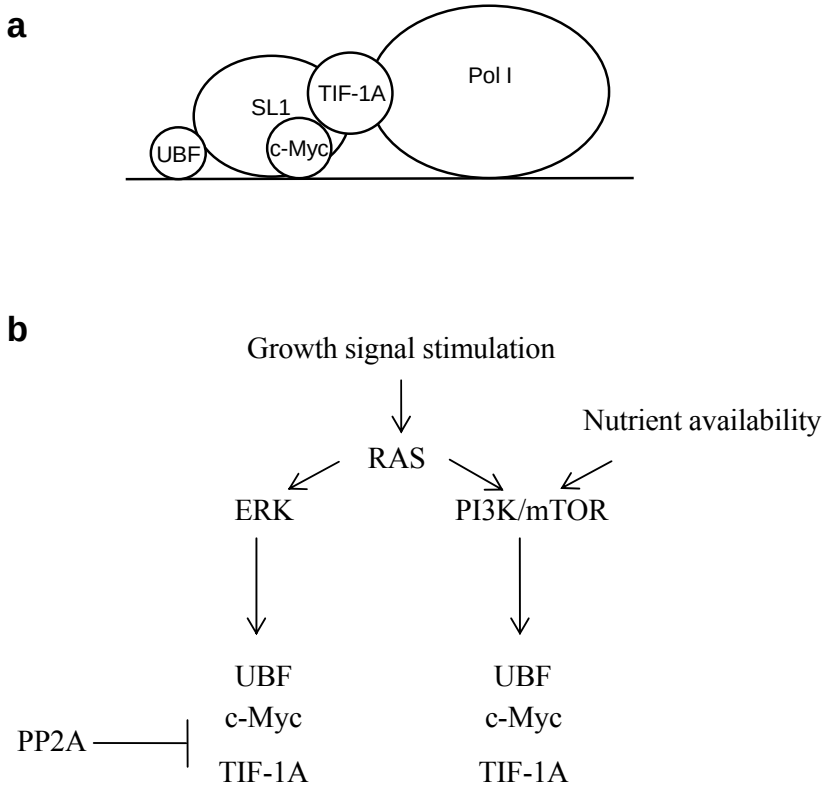

Figure 8. *Regulation of pol I transcription.* **a)** The main components of the pol I transcription machinery are the SL1 complex (assembled of TBP and TAFs), the TIF-1A and the transcription factor UBF. SL1 binds to and recruits the TIF-1A-associated pol I to the rDNA promoter. SL1 is thereby essential for PIC-formation and transcription initiation. The SL1 complex also interacts with c-Myc at the rDNA promoter. **b)** Both the PI3K and the ERK signal transduction pathways downstream of the activated RAS control the activity of the components of pol I transcription. UBF and TIF-1A and c-Myc are activated downstream of these pathways. The tumor suppressor PP2A (protein phosphatase 2A) is involved in down-regulation of c-Myc and TIF-1A upon withdrawal of growth stimulation.

In mammalian cells c-Myc is a direct activator of pol I-mediated transcription (**paper II** and (Grandori et al., 2005)). Initially, the nucleolar localisation of c-Myc and the requirement of its DNA binding motif for this accumulation led us to investigate whether c-Myc could interact with the rRNA gene-repeats in the nucleoli. The human rDNA gene contains numerous E-boxes which are putative c-Myc binding sites and c-Myc can associate directly with the rDNA via some of the E-box-containing sequences in both human cell-lines and primary cells. c-Myc can activate rRNA activation following serum stimulation and inhibition of c-Myc leads to a decrease in rRNA transcription in both mitogen-stimulated and cycling cells.

Association of c-Myc with the rDNA is accompanied by the recruitment of TRRAP and pol I and correlates with rDNA histone H4 acetylation **(paper II)**. Thus, similarly to the mechanism of pol II activation, histone acetylation seems to be involved in activation of c-Myc-mediated pol I activation. Furthermore, c-Myc associates with the SL1 complex and binding of SL1 to the rDNA correlates with c-Myc activity. Hence, c-Myc might facilitate the recruitment or assembly of the basal pol I machinery on rDNA (Grandori et al., 2005). c-Myc is also shown to bind to additional sites in the rDNA both up- and downstream of the rRNA coding region and might also influence transcription through rearranging the rDNA. In agreement with this hypothesis, formation of DNA loops has been shown to facilitate rDNA transcription (Stefanovsky et al., 2006).

Our results presented in **paper II** also indicate that pol I-mediated rRNA transcription requires functional proteasomes. Despite nucleolar accumulation of c-Myc, rRNA transcription is reduced rather than further stimulated following proteasome inhibition. Therefore, similar to pol II activation, simultaneous proteasomal degradation might be necessary for c-Myc-dependent pol I activation. A shorter-term treatment with proteasome inhibitor does not cause a detectable change in pre-rRNA levels (Grandori et al., 2005). Thus, the reduction in rRNA transcription seems to be detectable after a longer term proteasome inhibition. The mechanisms of activation of rRNA transcription by c-Myc will be further discussed in the results and conclusions section in this thesis.

5 ROLE OF c-MYC IN CANCER

Alterations in the c-MYC gene and protein are frequently detected in human cancers and are often associated with poorly differentiated and invasive tumors. One of the best studied c-Myc related cancers is the Burkitt's lymphoma where c-MYC gene translocation and amplification can be detected in virtually every case (Spencer and Groudine, 1991). c-MYC gene rearrangements has also been detected in other cancers like non-Burkitt's lymphomas, prostate, breast and gastrointestinal cancers (Nesbit et al., 1999). Generally, gene rearrangements result in elevated levels of c-Myc expression. Mutations in the coding sequence of c-MYC have been detected in some cancers such as Burkitt's lymphomas (Axelson et al., 1995). Hot-spots for these mutations which are Thr58 and Ser62 interfere specifically with the rapid turn-over of the c-Myc protein (Bahram et al., 2000; Salghetti et al., 1999). Moreover, mutations in non-protein coding regions that interfere with control of c-Myc translation have been detected and shown to be significant in deregulating c-Myc (Bentley and Groudine, 1988)Thus, the oncogenic activation of c-Myc might result from gene alterations or from disruption of any one of the multiple regulatory steps that control c-Myc levels in normal cells.

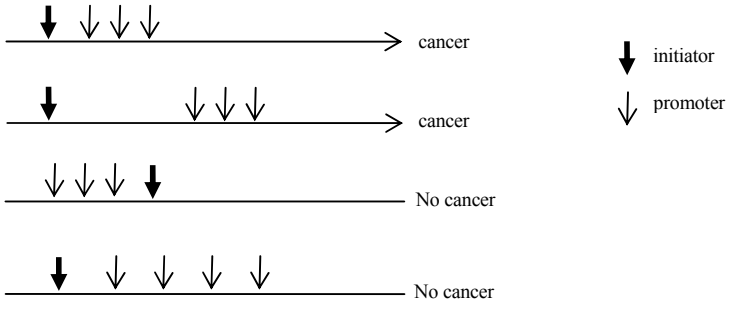

Figure 9. *Mechanisms of cancer initiation and progression*. The outcome of exposure to tumor initiators (mutagens) and promoters is shown. Cancer develops only if the exposure to the promoter follows the exposure to the initiator and the intensity of exposure to the promoter exceeds a certain threshold. Although naturally occurring cancers arise through random sequence of initiation and promotion steps their evolution is believed to be governed by similar principals Alberts et. al. (1994) Molecular biology of the cell.

The tumor-causing capacity of c-Myc has been studied both in vitro and in vivo. In vitro transformation assays which assess the oncogenic potential of proteins by parameters such as focus formation, anchorage independent growth and ability to give rise to tumors when injected into a host show that c-Myc can transform cells when co-expressed with a cooperating oncogene such as activated Ras (Land et al., 1983; Ruley, 1990). In addition, transgenic animal models have been used to study the oncogenic activity of c-Myc (Morgenbesser and DePinho, 1994). It has been shown that transgenic mice expressing a c-MYC gene under the control of the immunoglobulin enhancer element develop early pre-B cell lymphoid malignancies, indicating that in these B-cells activated c-Myc alone might be sufficient to initiate tumors (Adams et al., 1985).

Although the link between c-Myc and cancer is well established the molecular mechanisms through which the oncogenic c-Myc acts are not fully known. Some studies support a role for c-Myc in both tumor initiation and progression **(Fig. 9)**. For example, in transgenic mice inducible over-expression of c-Myc is sufficient to cause the rapid development of some tumors, which then require persistent c-Myc over-expression throughout their development (Felsher and Bishop, 1999). Moreover, acceleration of tumorigenesis in these animals is observed when c-Myc induced apoptosis is suppressed by over-expression of anti-apoptotic proteins such as Bcl2, or loss of p53 (Blyth et al., 1995; Jacobs et al., 1999).

It is commonly accepted that the critical outcome of c-Myc deregulation in tumorigenesis is uncontrolled proliferation. Proliferation control by c-Myc is mainly via promoting growth and oncogenic activation of c-Myc might couple deregulated growth to uncontrolled cell division **(Fig. 10)**. Indeed, deregulation of different c-Myc-controlled steps in ribosome biogenesis and protein synthesis which adjust the rates of growth are often associated with cancers (Ruggero and Pandolfi, 2003). For example, increased rRNA transcription is associated with cancer. Moreover, an increase in production or activity of r-proteins is detected in many cancers (Ferrari et al., 1990; Kondoh et al., 2001). It is unknown whether deregulation of growth is associated with cancer progressing or sufficient for cancer initiation. Neither is it known whether c-Myc targets involved in growth directly mediate its oncogenic effects. Thus the relative contributions of different components of the protein synthesis machinery, their precise effect within the context of a multi-step pathway that leads to cancer and the mechanisms that couple cell cycle regulation to growth remain to be determined.

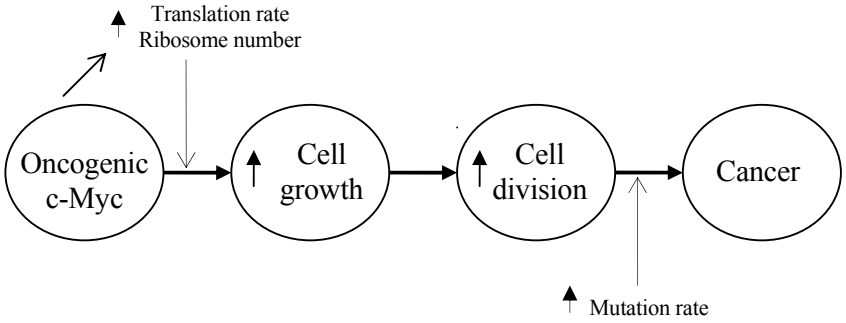

Figure 10. A *possible pathway that connects activated c-Myc to deregulated proliferation and cancer*. c-Myc controls ribosome biogenesis and translation via its pol I, II and III-transcribed targets. Deregulation of c-Myc can lead to an increase in ribosome number, protein synthesis rate and growth. Deregulated growth might in turn induce uncontrolled cell division.

Structural alterations of the nucleoli are characteristic of a number of malignancies (Maggi and Weber, 2005). In fact, nucleolar alterations have now proven to be the best indicator of survival and response to treatment in various human cancers (Maggi and Weber, 2005; Pich et al., 2000). It is generally assumed that in cancers, increased proliferation would result in an increased need for growth and ribosome production and consequently larger and a greater number of nucleoli. However, the mechanisms that cause nucleolar alterations and whether nucleolar alterations are the cause or the consequence of cancer are unknown (Fischer et al., 2004).

Several types of human cancers which often display nucleolar alterations are also associated with deregulated c-Myc **(Table 2)**. In these cancers often both c-Myc deregulation and nucleolar alterations predict an aggressive course of disease (Fischer et al., 2004; Pich et al., 2003; Smetana, 2002). Investigation of neuroblastomas suggests that there is a link between nucleolar changes and N-Myc, a protein functionally closely related to c-Myc (Kobayashi et al., 2005). In this study it is shown that only tumors with the amplified N-MYC gene displayed prominent nucleoli, a feature that is associated with poor prognosis. The mechanisms that induce nucleolar enlargement and possible involvement of c-Myc in this process have not been addressed. c-Myc is a central regulator of ribosome biogenesis and oncogenic c-Myc

might induce nucleolar alteration by deregulating the nucleolar processes of rRNA transcription and subsequent steps that lead to ribosome assembly.

Since high levels of c-Myc are associated with its nucleolar localisation it can be speculated that in cancer cells, c-Myc accumulates in nucleoli. However, in blood and breast cancer cell-lines which express high levels of c-Myc constitutively, c-Myc does not accumulate in nucleoli **(paper III)**. Nucleolar accumulation of c-Myc is associated with a decrease in rRNA transcription **(paper II),** and thus is unfavorable for growth**.** Therefore, we speculate that nucleoli in these cancers might be altered to sustain and possibly increase rRNA transcription in the presence of excessive c-Myc levels.

Table 2. *Human tumors that are generally associated with both deregulated Myc and nucleolar alterations.*

Disease	Nucleolar Alterations	Myc gene alterations
Lymphomas	Enlarged and increased number of nucleoli[1]	c-Myc gene translocations amplifications, mutations[2]
Breast carcinomas	Enlarged nucleoli[3]	c-Myc gene amplifications[2]
High grade prostatic intraepithelial neoplasia	Enlarged nucleoli[4]	c-Myc gene amplifications[5]
Neoroblastomas[6]	Enlarged nucleoli	N-Myc gene amplification

References: [1]Smetana K. et al. (2002) Eur. J.Histochem 46:125-32
[2]Nesbith CE. et al. (1999) Oncogenen 18:30004-16
[3]Pich A. et al. (2003) Oncol. Pep. 10:1329-35
[4]Fischer. et al. (2004) J. Cell Biochem. 91:170-84
[5]Qian J. et al. (1997) Mod.Path. 10:1113-9
[6]Kobayashi. et al. (2005) cancer 103:174-80

6 COMMENTS ON METHODOLOGY

6.1 MICROSCOPY

In the studies in this work immunostaining along with conventional or confocal fluorescent microscopy were used to study and compare protein localisation. The confocal microscope filters out the out-of-focus light from above and below the point of focus in the object. By scanning sequential planes in the Z-direction, this technique allows creation of three dimensional images. In specimens double-labeled for different molecules, the three dimensional information obtained by confocal sectioning, can more accurately show colocalizations of the signals. We used this technique to study the localisation of c-Myc in relation to fibrillarin in the nucleoli.

The intracellular dynamics of c-Myc was studied by photobleaching-based techniques, fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP). These techniques are based on the irreversible bleaching of fluorescent molecules by a high-powered laser beam and are used to measure the dynamics of fluorescently-labeled molecules in living cells. In FRAP the rate of recovery of fluorescence in a defined region of a cell after a bleaching is determined. This recovery results from the movement of unbleached fluorophores from the surroundings into the bleached area. Two kinetic parameters of a protein can be determined by quantitative FRAP the mobile fraction which is the fraction of the protein which can diffuse into the bleached region and the diffusion constant which is a measure of the rate of protein movement. In FLIP the decrease/disappearance of fluorescence in a defined region adjacent to a repetitively bleached region is measured. Loss of fluorescence will occur if the protein can diffuse between these areas (Lippincott-Schwartz et al., 2001).

6.2 IN-VIVO BINDING ASSAY

The Chromatin immunoprecipitation (ChIP) technique was used to study the interaction of c-Myc with the rDNA in vivo. In this technique the chromatin-associated proteins can be cross-linked to the DNA. The protein-DNA complexes are then isolated by immunoprecipitation and the DNA can be analysed by PCR to determine the binding of the protein to a specific DNA sequence. ChIP is a sensitive technique by which transient and dynamic interactions between transcription factors and DNA can be detected.

6.3 TRANSCRIPTION ASSAYS

To measure transcription ^{3}H-uridine incorporation assays were performed. In these assays the amount of TCA-precipitable ^{3}H-uridine incorporated to the newly synthesized RNA is quantified by scintillation counting. The in vitro nuclear run-on assays were carried out to study the transcription of the rRNA genes after mitogen stimulation. By this assay it is possible to assess whether changes in the RNA levels are due to transcription or to other processes, such as RNA degradation. Run-on assay is done by isolation of the intact nuclei from cells. Isolated nuclei contain transcription complexes "stalled" on the DNA template, due to the acute loss of ribonucleotides. Transcription thus halted can be restarted by the addition of labeled ribonucleotides. The in vitro transcripts are analyzed by hybridization to specific cDNAs corresponding to different regions in the transcribed gene and it can thus be determined whether the in vitro transcripts are elongated from the transcription start site or from the downstream elements. In these assays α-amanitin was used to assess pol I transcription specificity. At specific concentrations α-amanitin inhibits Pol III and/or pol II, whereas pol I activity is not sensitive to α-amanitin.

7 RESULTS

7.1 PAPER I

In **paper I** we show that in Cos cells both the endogenous c-Myc and the transiently transfected c-Myc tagged with green fluorescent protein (GFP) are evenly distributed in the nucleoplasm. As shown by FRAP experiments, c-Myc is highly mobile and diffuses rapidly within the nucleoplasm. Furthermore, we show by FLIP analysis that c-Myc shuttles continuously between the nucleus and the cytoplasm. Although in most cells c-Myc could not be detected in the nucleoli, a small fraction of cells contained observable levels of nucleolar c-Myc under physiological conditions of growth. To assess whether c-Myc levels could influence its nucleolar distribution we performed transfection analysis. In transfected cells the fraction of cells with nucleolar c-Myc increased progressively as c-Myc expression levels increased. Moreover, following 4 hours treatment of Cos7 cells with the proteasome inhibitors ALLN or MG132 c-Myc accumulated in the nucleoli in nearly all cells. To determine the dynamics of nucleolus-associated c-Myc we performed FLIP and could show that nucleolar c-Myc was mobile. The residual mobility of nucleolar c-Myc suggests that c-Myc accumulation was not attributed to formation of protein aggregates. The relative mobility of the nucleolar c-Myc was quantified using FRAP. The average recovery half-time for nucleolar c-Myc was calculated to 6 minutes. The relatively slow recovery kinetics of the nucleolar c-Myc is indicative of a stable biological interaction between c-Myc and nucleoli.

In addition, localisation of the proteasome was studied by immunostaining, using antibodies to the 20S CP. Proteasomes were recruited to and co-localised with the nucleolar c-Myc both on proteasome inhibitor treated cells and in cells expressing ectopic c-Myc. In the cells that expressed high levels of c-Myc the subset of nucleoli that accumulated c-Myc also accumulated proteasomes.

7.2 PAPER II

In **paper II** we focus on the function of c-Myc in nucleoli. We show that the nucleolar localisation of c-Myc is mediated by its DNA-binding domain and that the nucleolar c-Myc is heterodimerised to Max. As shown by ChIP assay in HeLa cells, the c-Myc/Max heterodimer binds directly to the rRNA genes within an E-box-containing sequence between nucleotides 931-918, downstream of the transcription start site.

Binding of c-Myc to this region was enhanced after treatment of cells with proteasome inhibitor, indicating that c-Myc binding to rDNA might be stabilized by proteasome inhibition. c-Myc is also recruited to this rDNA region in primary human lymphocytes, upon mitogenic stimulation by phytohaemaglutinin (PHA) and interleukin-2 (IL2) and in the inducible human U2OS-MycER cells. In all of the examined cells association of c-Myc with the rDNA correlates with histone H4 acetylation of the rDNA chromatin.

Recruitment of c-Myc to the 931-918 region in stimulated lymphocytes is followed by association of pol I and TRRAP with this region and increase in rRNA expression, measured by 3H-uridine incorporation. Furthermore, nuclear run-on assays show that increased association of c-Myc with the rDNA is parallel with an increase in pol I transcription which is seen throughout the transcribed region of the rDNA. Inhibition of c-Myc by 10058-E4 treatment or through siRNA expression causes a significant decrease in rRNA transcription both in PHA/IL2-induced lymphocytes and in serum-induced U2OS cells.

We also show that inhibition of proteasomes down-regulates rRNA transcription. This was shown by BrU pulse-labeling of newly synthesized RNA in intact Cos cells. Inhibition of rRNA transcription was observed in the nucleoli that also accumulated c-Myc. Northern blot analysis of the pre-rRNA transcript in HEK293 cells treated with proteasome inhibitor for 4 and 8 hours shows that the rRNA levels decrease in a time dependent way. Although in cycling HEK293 cells proteasome inhibition has a clear inhibitory effect on rRNA synthesis, this effect is relatively modest. Serum-induced rRNA expression was efficiently blocked by proteasome inhibition as shown by northern blot analysis of the pre-rRNA in serum stimulated U2OS-MycER cells.

7.3 PAPER III

In **paper III** we investigate the localisation of endogenous c-Myc in BL and MCF-7 cancer cells lines, which express high levels of c-Myc constitutively. As shown by immunostaining in these cells excess c-Myc does not accumulate in the nucleoli in these cells. We also examined the distribution of c-Myc following 4 hours of proteasome inhibitor treatment. In BL cells we could not detect nucleolar c-Myc, whereas in MCF-7 cells c-Myc could be detected in nucleoli in a small subpopulation of cells.

We also studied the effect of c-Myc- and proteasome-inhibition on nucleolar organisation by using fibrillarin distribution as a marker for nucleolar integrity.

Normally fibrillarin is dispersed throughout the nucleoli but perturbations in nucleolar function cause redistribution of fibrillarin. In these experiments cells were treated with the transcription inhibitor Actinomycin D, c-Myc inhibitor (10058-F4) or proteasome inhibitor. As expected, Actinomycin D treated nucleoli were smaller than nucleoli in untreated cells. In c-Myc or proteasome inhibitor treated cells we could not detect any significant change in the number or size of the nucleoli. Moreover, fibrillarin was redistributed in nearly all Actinomycin D treated cells and a small fraction (5%) of the untreated control cells. Inhibition of c-Myc resulted in a 4-fold increase in the number of cells with redistributed fibrillarin compared to untreated control cells. Similarly, proteasome inhibition resulted in a 2-fold increase in the number of cells with redistributed fibrillarin. Inhibition of c-Myc through transient transfection of Cos cells with dominant negative mutants corresponding to amino acids 270-439 and 1-148 in c-Myc caused severe nucleolar changes. In these cells fibrillarin was either redistributed to the nucleolar periphery or ejected from the nucleoli.

Furthermore we show that during proteasome inhibition when the 20S CP of the proteasome accumulates in nucleoli, the 19S RP was detected in nuclei but not nucleoli, suggesting that the 20S CP might be recruited without any attached 19S.

8 CONCLUSIONS AND FUTURE PERSPECTIVES

Although it is well established that c-Myc is an essential regulator of cell growth, the mechanisms through which c-Myc controls growth are not fully delineated. The main observation in this thesis is that c-Myc is a direct activator of rRNA transcription by pol I **(paper II)**. The rRNAs and the ribosomal proteins are assembled into the ribosomes which are the protein-making units in cells. In addition to activating ribosomal RNAs and proteins, c-Myc regulates many other essential components of the protein synthesis machinery, such as translation factors and tRNAs through activation of pol II and III (Gomez-Roman et al., 2006). Coordinated regulation of these components is necessary for efficient protein synthesis and adequate rates of protein synthesis are a prerequisite for growth (Ruggero and Pandolfi, 2003). Thus, regulation of protein synthesis might be the basis for growth promotion by c-Myc.

The rate of growth is highly responsive to the extracellular environment. The cell response to growth stimulation is initiated by transcription activation of the rRNA genes, which also is a rate-limiting step in growth (Grummt, 2003; Russell and Zomerdijk, 2006). The RAS/ERK and the RAS/PI3K/mTOR signal transduction pathways are central mediators of rRNA transcription regulation in response to extracellular signals. These pathways control the activity of the essential components of the pol I-machinery such as TIF-1A and UBF (Mayer et al., 2004; Zhao et al., 2003). c-Myc is also a target of these signaling pathways. Activation of c-Myc downstream of these signals coordinates rRNA transcription with activation of genes required in the subsequent steps of protein synthesis. We show that c-Myc plays a decisive role in activation of pol I following growth stimulation of resting cells **(paper II)**. This is consistent with the function of c-Myc in inducing transition of resting cells to cycling in response to growth stimulation. Although it is clear that in organisms like yeast growth is directly connected to cell division, the evidence is still inconclusive in higher eukaryotes. Thus, it remains to be determined whether growth stimulation is a critical means by which c-Myc can drive the cell cycle.

The exact mechanism of c-Myc-mediated rRNA transcription activation need to be determined. However, there are implications for a number of possible mechanisms. First, association of c-Myc with the rDNA correlates with rDNA histone acetylation. This suggests that c-Myc might activate pol I transcription by recruiting HATs and remodeling the chromatin (paper II and (Grandori et al., 2005). But, the fine-

tuning of rRNA transcription control is believed to be through modulating transcription levels from genes that already exist in an "open" conformation with accessible chromatin (Conconi et al., 1989). In addition, c-Myc was shown to activate pol I-transcription on a non-nucleosomal template in vitro (Grandori et al., 2005). Thus, the role of histone acetylation in rDNA gene activation is unclear. Further investigation is needed to clarify if and how histone acetylation contributes to rRNA gene activation in vivo. The second possible mechanism by which c-Myc might activate pol I transcription is via facilitating the preinitiation-complex formation by binding to SL1. c-Myc can interact with TBP and TAF components of the SL1 complex and association of TBP with the rDNA promoter correlates with levels of c-Myc (Grandori et al., 2005). A third suggested mechanism by which c-Myc might activate the rDNA is through inducing DNA-loop structures. Formation of rDNA loops by e.g. UBF has been shown to enhance rDNA transcription. c-Myc-Max heterodimers can bind to multiple sites in the rDNA (Grandori et al., 2005) and form bivalent heterodimers (Nair and Burley, 2006).

Further investigation is needed to determine whether c-Myc can be directly coupled to induction of the nucleolar abnormalities in disease. Nucleolar alterations are accurate indicators of prognosis in several types of cancers. But the mechanisms that cause them and the physiological consequence of nucleolar alterations are largely unknown. Nucleolar enlargement in cancer might be induced by an increase in the ratio of active to inactive rRNA genes. We observed that in cancer cell-lines which display nucleolar alterations and express high levels of c-Myc, c-Myc does not accumulate in nucleoli **(paper III)**. Evading nucleolar accumulation is likely to be favorable for growth, since excess nucleolar c-Myc seems to downregulate rRNA transcription **(paper II)**. c-Myc accumulation could be circumvented by specific nucleolar modifications that act in synergy with deregulated c-Myc.

We also conclude that the proteasomes are needed for pol I-mediated transcription **(paper II)**. Proteasomes can be recruited to and enriched in nucleoli **(paper I)** and inhibition of the proteasomes disturbs the nucleolar organisation **(paper III)**. The role of proteasomes in the multiple steps of the ribosome biogenesis has been studied recently (Stavreva et al., 2006). In agreement with our results, it has been shown that inhibition of proteasomes induces specific and well-defined molecular changes in the nucleoli. This is demonstrated by changes in the localisation of nucleolar proteins involved in rRNA transcription and processing during proteasome inhibition. It was also shown by electron microscopy that after a 3.5h proteasome inhibitor treatment

the normal nucleolar structure becomes completely disrupted. We detected a reduction in rRNA transcription after 4 to 8h of proteasome inhibition (**paper II**). However, in another study no change in pre-rRNA levels could be observed after 3h of inhibition (Grandori et al., 2005). In the study by Stavreva et. al a 4h treatment caused a small but reproducible reduction in the rRNA transcription. In agreement with our results, these authors suggest that considering the block of rRNA processing and failure to accumulate pre-rRNA it is likely that pol I transcription is affected by proteasome inhibition.

The mechanism that involves proteasomes in pol I-mediated transcription regulation is unclear. Proteasome subunits are suggested to be involved in the activation of pol II-mediated transcription via regulation of both folding and ubiquitin-mediated degradation of transcription factors and similar mechanisms might regulate pol I transcription. We suggest that the 20S subunit of the proteasome is present in nucleoli without the 19S RP, since we were able to detect the S6 component of the 19S RP in nuclei but not nucleoli **(paper III)**. However, since no 20S subunit is known to be involved in specific protein recognition it remains to be answered how the 20S proteasomes are recruited to and retained in nucleoli. Another issue to be addressed is the function of nucleolar proteasomes. The 20S proteasome by itself is a latent enzyme. It is possible that the 19S particles are detached from the nucleolar 20S following proteasome inhibition, during which localisation of 19S was determined. Alternatively, a distinct activator complex, such as the PA28 might activate the nucleolar 20S proteasomes. To address these issues the association of the proteasomes with nucleoli and rDNA under physiological conditions should be investigated.

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