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Identification and Characterisation of Mybbp1a as a Regulator of rRNA Synthesis

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1 Summary

The activity of rRNA gene transcription is the major determinant of cell proliferation and cell growth. In proliferating mammalian cells approximately 35 % of total transcription activity is dedicated to RNA polymerase I (Pol I) to produce the 47S rRNA precursor, which is subsequently processed to mature 18S, 5.8S and 28S rRNA by a number of endo- and exonucleolytic cleavage steps to provide the catalytical and structural core of the ribosomes.

The aim of the presented thesis was to identify novel regulators of rRNA gene transcription to gain further insights into the complex network of adaptive processes that match ribosome amounts with the physiological needs of the cell. For this purpose, factors interacting with the second largest subunit of the Pol I complex, RPA116, were purified by co-immunoprecipitation and subsequently analysed by mass spectrometry. Thereby, the protein Mybbp1a was identified as a promising candidate for further characterisation. Originally Mybbp1a has been described as an interacting partner of the proto-oncogene c-Myb and, since then, shown to interact with and to modulate the activity of several other regulators of Pol II-dependent transcription thereby modifying many different cellular key pathways. However, despite its predominant nucleolar localisation no function within this nuclear compartment has been characterised so far. The following Pol I reporter experiments as well as the assessment of endogenous levels of 47S rRNA precursor after siRNA-mediated depletion of Mybbp1a revealed the protein's ability to regulate rRNA gene transcriptional activity by a repressive mechanism. Unexpectedly for a repressor of rDNA transcription, depletion of Mybbp1a led to a strong retardation of cellular proliferation joined by a specific flattened phenotype of the cells. Therefore, an additional role for Mybbp1a in growth-related processes other than rDNA transcription was investigated and the requirement of the protein for efficient processing of the rRNA precursor could be determined by metabolic labelling of nascent rRNA precursors. Furthermore, the purification of Mybbp1a-associated factors determined several processing factors important for the maturation of both, the 40S and 60S ribosomal subunits, thereby confirming its presence in pre-ribosomal particles. Interestingly, the nucleolar localisation of Mybbp1a and the association with the identified processing factors was dependent on a RNA component as shown by immunofluorescence and size exclusion chromatography experiments with RNase-treated cells further emphasising a physical association of Mybbp1a with the processing machinery and a direct role of Mybbp1a in steps of ribosome biogenesis subsequent to rDNA transcription.

As revealed by this work, Mybbp1a integrates different functions with respect to rRNA synthesis. Its newly characterised role in rDNA transcription as well as rRNA processing discloses a potential mechanism to co-ordinate these both processes. Furthermore, the protein's involvement in various other cellular key pathways makes Mybbp1a a promising target for signalling pathways to adapt major cellular processes with the current condition of

the cell. The presented experimental data together with other published information suggest that in proliferating cells Mybbp1a is mainly associated with the pre-ribosomal complexes where it acts as a scaffold for rRNA processing and assembly factors and is functionally required to drive efficient ribosome biogenesis. Reduced levels of ribosome biogenesis, eg. caused by stress signals, potentially result in the disassembly of pre-ribosomal particles and subsequent release of Mybbp1a to the nucleoplasm. While remaining nucleolar Mybbp1a would repress RNA Pol I transcription released nuclear Mybbp1a would modulate the activity of RNA Pol II transcription regulators to cease cell cycle progression, proliferation and energy production. The further characterisation of Mybbp1a will not only be interesting with respect to its cellular role(s) but also regarding the mechanistic understanding of fundamental cellular processes such as transcription regulation and co-ordination with subsequent RNA maturation.

2 Introduction

The majority of transcriptional activity in growing cells is dedicated to the synthesis of ribosomal RNA (rRNA) to ensure sufficient supplies of ribosomes for the subsequent cell generation. This introduction will give a concise overview on the different steps of ribosome biogenesis and their regulation as well as their co-ordination with respect to the actual state of the cell. In general, it is restricted to review work on mammalian rRNA synthesis, however, key experiments from other systems will be discussed. Anticipating the identification of a novel regulator of rRNA synthesis during the here presented thesis work a short introduction on the factor Mybbp1a will be given. To start with the organisation of the rRNA genes and the nucleolus, the specialised nuclear compartment where most steps of ribosome biogenesis occur, will be described.

2.1 The ribosomal RNA genes

The synthesis of three out of the four rRNAs, the catalytical and structural core of the ribosome, is executed by the RNA polymerase I enzyme (Pol I) within a specialised nuclear compartment, the nucleolus. The multi-copy rRNA genes (rDNA) are organised in large clusters of tandem arrays on one or several chromosomes within the organism's genome. The number of rDNA repeats greatly varies between species, from ~150 (yeast, *S. cerevisiae*) and ~200 (mammals) gene copies per haploid genome to several thousands in most plants (Rogers & Bendich 1987; Warner 1999; Sanij & Hannan 2009). Intriguingly, only a portion of these genes is actively transcribed while the remainder persists in a silent transcriptional state (Conconi *et al.* 1989; Sanij *et al.* 2008). The fourth rRNA and the ribosomal proteins are provided by RNA polymerase III and II (Pol III and II), respectively. In general, this introduction is restricted to review work on mammalian rRNA synthesis, however, key experiments from other systems will be discussed.

2.1.1 The nucleolus - the site of ribosome biogenesis

The nucleolus is a distinct nuclear compartment and constitutes the site of rRNA gene transcription, rRNA processing and ribosome assembly. This highly dynamic structure assembles in the late telophase in several steps around rRNA gene clusters, which are therefore termed the nucleolar organiser regions (NOR). It persists throughout the interphase and disassembles when the cell enters mitosis (Leary & Huang 2001; Leung *et al.* 2004). The nucleolus is characterised by its unique density and easily visualised by light microscopy (Figure 1A). By electron microscopy three distinct nucleolar substructures can be distinguished. They include the fibrillar centres (FCs), which are enclosed by dense fibrillar components (DFCs), and further embedded in granular components (GCs). The distinct sub-compartments exhibit individual protein compositions, which are related to their specific

functions. While the FCs include factors exerting rDNA transcription such as Pol I subunits or the transcription factor UBF (upstream binding factor), the DFCs rather harbour proteins involved in rRNA processing like the 2'-O-methylase Fibrillarin (Figure 1B-D). Therefore it is suggested that rDNA transcription is initiated in the FCs or at the FC-DFC boarders and the nascent 47S rRNA precursors (pre-rRNA) extrude into the DFCs. There they are processed by the dedicated processing factors and subsequently assembled with ribosomal proteins on their further way to the GCs where late processing factors such as B23 are located (Figure 1E) (Lazdins *et al.* 1997; Mosgoeller *et al.* 2001; Lam *et al.* 2005).

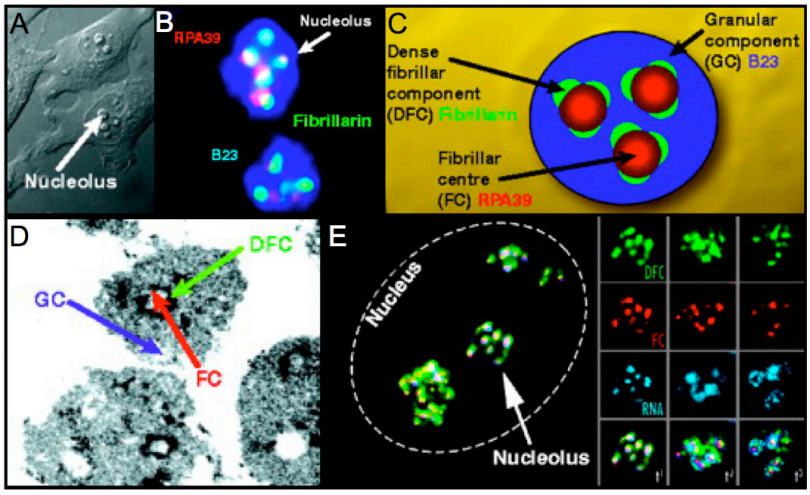


Figure 1: The nucleolus and its subcompartments.
(A) Light microscopic image; a nucleolus is indicated by an arrow. (B) Fluorescence microscopic image of nucleoli stained with antibodies against the Pol I subunit RPA39 and the processing factors fibrillarin and B23. (C) Schematic presentation of the nucleolar subcompartments with the respective marker proteins indicated. (D) Transmission electron microscopic image of nucleoli with its subcompartments indicated. (E) Sequential movement of rRNA. Cells were labelled with a short pulse of halogenated nucleotide, which reveals a wave of nascent rRNA spreading from the FC-DFC complexes to the GC regions. FC: fibrillar centre; DFC: dense fibrillar component; GC: granular component. (Lam et al. 2005)

Recent studies on the nucleolar proteome by mass spectrometry-based techniques have identified up to 700 proteins to be present in HeLa nucleoli (Andersen *et al.* 2002; Scherl *et al.* 2002; Andersen *et al.* 2005). More than 30% of the determined proteins are associated with one of the processes involved in ribosome biogenesis, which confirms the central role of the nucleolus in this process. However there are many proteins, which can be related to other processes such as cell cycle regulation, DNA damage repair, pre-mRNA processing, senescence and stress sensing emphasising the functional complexity of the nucleolus also beyond ribosome biogenesis (Pederson 1998; Olson *et al.* 2002; Mayer *et al.* 2005; Mayer & Grummt 2005; Yuan *et al.* 2005). A very recent genomic approach has furthermore sought to characterise nucleolus-associated chromatin domains (NAD) by sequencing and microarray technologies (Nemeth *et al.* 2010). Bioinformatical analysis identified 97 chromosomal regions including ~1000 genes and representing approximately 4% of the whole genome. One of several other gene families enriched in those domains is represented for example by

the 5SrRNA as well as the tRNA genes, which are both transcribed by Pol III, indicating that spatial regulation may play a role in co-ordinating the provision of components of the translation machinery and proofing former results on their co-localisation with the nucleolus (Thompson *et al.* 2003; Haeusler & Engelke 2006). Undoubtedly, the nucleolar structure integrates an extensive variety of functional scaffolds and coordinative responsibilities, of which the most prominent and well-characterised role is its contribution to the synthesis of mature rRNAs and their assembly into ribosomal particles.

2.1.2 The organisation of mammalian rRNA genes

The rRNA genes in human cells are clustered as head-to-tail tandem repeats on the short arms of the five acrocentric chromosomes (13, 14, 15, 21, and 22) termed nucleolar organiser regions (Henderson *et al.* 1972). The mammalian transcription units comprise ~43 kb in humans and ~45 kb in mouse and are built of a sequence encoding the 47/45S pre-rRNA (13-14 kb) separated by long intergenic spacers (IGS; ~30 kb)(Figure 2A) (Sanij & Hannan 2009). The mammalian rDNA promoter comprises 170 to 200 bp and has a bipartite structure including a core promoter sequence adjacent to the transcription start site and an upstream control element (UCE) approximately 100 bp upstream (Haltiner *et al.* 1986; Learned *et al.* 1986; Paule 1998) (Figure 2B). The pre-rRNA transcription unit encodes the 18S, 5,8S and 28S rRNAs, of which sequences are preceded by a 5' external transcribed spacer (5'ETS), separated by internal transcribed spacers 1 and 2 (ITS 1 and 2) and concluded by the 3' external transcribed spacer (3'ETS). The pre-rRNA coding region is concluded with 10 terminator elements, to which the Pol I-specific transcription termination factor I (TTF-I) binds (Grummt *et al.* 1985; Grummt *et al.* 1986; Henderson & Sollner-Webb 1986; McStay & Reeder 1986). An additional TTF-I binding site (T_0) is located upstream of the UCE (Paule 1998) and plays an important role in organising the chromatin structure at the promoter, which will be discussed in detail in the following chapters. The intergenic spacer has been originally termed non-transcribed spacer as it was thought to be transcriptionally inactive. However, several studies performed in different species have identified promoter sequences as well within the IGS (Coen & Dover 1982; Miller *et al.* 1983; Moss 1983; Kuhn & Grummt 1987). Subsequently different roles of IGS transcription have been suggested including a potential mechanism to enhance rRNA synthesis by trapping RNA Pol I and supplying it to the promoter (Moss 1983; Grimaldi *et al.* 1990) or to establish and maintain transcriptionally active or silent states of the rDNA repeats by modifying their chromatin structure (Mayer *et al.* 2006). Furthermore, an enhancer element is located adjacent to the spacer promoter, which helps to augment the probability of transcription from the linked gene (Moss 1983; De Winter & Moss 1987).

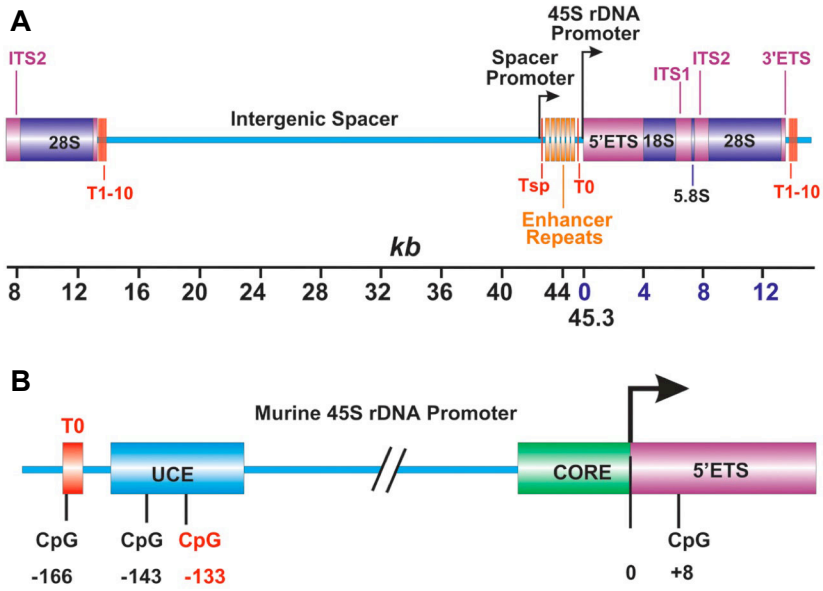


Figure 2: The organisation of mammalian rRNA gene repeats (Sanij & Hannan 2009).
(A) A schematic of a single mouse rDNA repeat. The 45S gene consists of sequences (13–14 kb) encoding the transcribed region followed by an intergenic spacer (IGS, blue line) of ~30 kb in length. Scale bars in kb are shown below; the site of transcription initiation of the 45S transcript is indicated at 0 kb. Terminator elements located downstream of the coding region (T_1-T_{10}), downstream of the spacer promoter (Tsp) and upstream of the 45S promoter (T_0) are indicated by the red bars. ETS: external transcribed spacer; ITS: internal transcribed spacer. (B) A schematic of the mouse 45S rDNA promoter. The rDNA promoter consists of two elements, an upstream control element (UCE) 100 nucleotides upstream of the transcription start site and the core promoter immediately adjacent to the start site. Transcription termination elements T_0 are located immediately before the UCE. The position of CpG residues at nucleotides -166, -143, -133 and +8 is indicated. The CpG di-nucleotide at -133, shown to be methylated by NorC is coloured in red.

2.1.3 The two different chromatin states of the rRNA genes

Early electron microscopic studies in amphibian oocytes displayed two different conformations of the rRNA gene copies (Miller & Beatty 1969). These chromatin preparations, which were from then on called ‘Miller spreads’, display a very characteristic structure for active gene units of several transcribing enzymes with extruding nascent pre-rRNA capped by a terminal knob termed ‘Christmas trees’. The transcriptionally active repeats are densely populated with Pol I complexes and altered by inactive, Pol I-lacking gene copies (Figure 3). While in yeast the stretches of different activity states seem to be arbitrarily distributed (Osheim *et al.* 1996; French *et al.* 2003) in mammalian cells, however, it is not yet clarified if the active genes are also randomly dispersed over the distinct rDNA cluster on the different chromosomes and whether these patterns are maintained throughout the cell cycle.

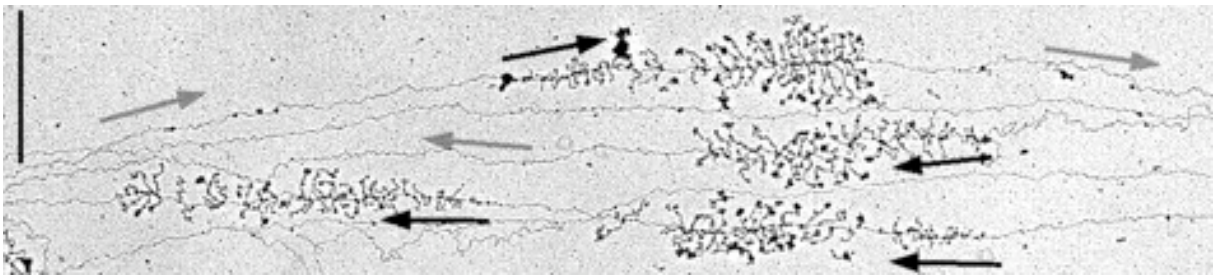


Figure 3: Miller spreads of yeast rRNA genes (French *et al.* 2003).
Active transcribed rRNA genes are occupied by Pol I with extruding nascent pre-rRNA capped by terminal knobs building the so-called 'Christmas tree' structures (black arrows in direction of transcription). Active genes are separated by inactive copies randomly (gray arrows). Scale bar = 1 µm.

These two activity states are mirrored by the co-existence of two distinct conformations of the rRNA gene-containing chromatin (r-chromatin), namely rDNA copies either packaged into regularly spaced nucleosomal arrays or lacking these structural features representing the transcriptionally inactive and active states, respectively (Prior *et al.* 1983; Dammann *et al.* 1993; Ahmad & Henikoff 2002; French *et al.* 2003; Nemeth *et al.* 2008). It is not yet clarified if active rDNA is completely devoid of nucleosomes or if it is rather wrapped into specialised non-canonical nucleosome structures leading to a highly dynamic and open chromatin conformation. The latter assumption is supported by different approaches in various model systems. In specific yeast strains, which contain only a limited number of rRNA genes and are therefore supposed to harbour only active rDNA copies, Proudfoot and colleagues have determined dynamic nucleosomal structures within the rDNA repeat by chromatin immunoprecipitation (ChIP) and biochemical fractionation methods (Jones *et al.* 2007). ChIP and immunofluorescence experiments in human, mouse and *Drosophila* cells revealed binding of antibodies recognising histone modifications associated with active transcription or the deposition of histone variant H3.3 within rDNA sequences (Ahmad & Henikoff 2002; Espada *et al.* 2007; Nemeth *et al.* 2008). Importantly, the study by Längst and colleagues additionally assessed the association of histone H3 within non-methylated rDNA repeats corresponding to the active gene portion and could show that approximately 50% of rDNA-associated histone H3 is bound to the active rDNA conformation (Nemeth *et al.* 2008). In contrast, different experimental approaches in yeast including electron microscopy and cross-linking of DNA by Psoralen, an agent supposed to intercalate only into nucleosome-free DNA regions, indicated that yeast may be different to mammals and active genes are devoid of nucleosomal structures (Dammann *et al.* 1993; Merz *et al.* 2008).

Although the definite chromatin conformation of active genes remains to be elucidated it is well established by different studies that in lower as well as higher eukaryotes no more than 50% of the rRNA gene copies are maintained in an active state at any given time, even in exponentially growing cells. (Conconi *et al.* 1989; French *et al.* 2003; Stefanovsky & Moss 2006; Sanij *et al.* 2008). Thus, the extent of rRNA synthesis is rather regulated on the level of transcription rates than by altering the dosage of active rRNA gene units per cell.

Nevertheless, the number of active rDNA units varies between certain cell types of one organism suggesting that the portion of active repeats alters during development and differentiation (Haaf *et al.* 1991). Interestingly, a very recent study by Hannan and colleagues revealed an increasing number of silenced rRNA genes during mammalian granulocyte differentiation, which might represent a mechanism to contribute to the propagation of reduced needs of rRNA synthesis during the special case of cellular differentiation (Poortinga *et al.* ; Sanij *et al.* 2008). However, why has such a large number of rRNA genes evolved, which is maintained silenced throughout a cell's life, if its purpose was not to regulate rRNA synthesis. Different hypotheses address this issue. Silenced repeats might constitute a landing platform for factors involved into the many nucleolar functions or restrict special factors from entering the nucleolus (Paule & White 2000; Moss & Stefanovsky 2002). Latest experimental results in yeast have further supported the well-accepted postulation that silenced repeats may serve as matrices for recombinational repair for the heavily transcribed rRNA genes, which are very likely to be targets of DNA damage due to their continuously accessible chromatin structure (Ide *et al.* 2010). In their study Kobayashi and colleagues have demonstrated that the loss of non-transcribed copies leads to an augmented sensitivity of cells to DNA damage, which is dependent on the level of rDNA transcriptional activity. According to their data additional (silenced) copies facilitate sister chromatid cohesion and thereby recombinational repair indicating that high concentrations of intensively transcribed genes might be toxic for the cell.

2.1.4 Epigenetic and topological features of active and inactive rRNA genes

Several recent studies investigated the additional layer of regulation involving modification of DNA and associated histones. These modifications are thought to be implied in the inheritance of transcriptional activity states through cell division and development and as they are not linked to DNA sequence are referred to as epigenetic. However, it is not finally resolved whether active and inactive states of specific r-chromatin stretches are maintained during cell cycle. Active and inactive rRNA gene populations are marked by specific DNA and histone modifications and distinct topological conformations (Santoro & Grummt 2005; Nemeth *et al.* 2008). The absence of DNA methylation and histone acetylation is characteristic for active repeats. Additionally, the transcribed genes exhibit a TTF-I-mediated DNA loop, which connects the gene promoter with the transcription terminator region (Nemeth et al. 2008) suggesting a mechanism to augment transcription rates by increasing the efficiency of transcription re-initiation. Inactive rRNA genes, however, are marked by CpG methylation, histone hypo-acetylation and methylation of specific lysine residues in histone H3 (H3) (Santoro & Grummt 2001; Santoro *et al.* 2002; Zhou *et al.* 2002). The establishment of these heterochromatic marks is dependent on the recruitment of NoRC, a remodeling

complex containing Tip5 (TTF-I interacting protein) and the ATPase SNF2h (Strohner *et al.* 2001), to the rRNA gene promoter. The recruitment of NoRC requires a non-coding (nc)RNA component originating from the IGS of the rDNA (Mayer *et al.* 2006) and TTF-I (Strohner *et al.* 2004), which is then bound to the promoter-proximal terminator element T_0. In the current view NoRC subsequently recruits different histone modifiers to deacetylate histone H4 and methylate H3 K9, H3K20 and H3K27 (Santoro *et al.* 2002; Zhou *et al.* 2002) and repositions a distinct nucleosome at the rDNA promoter (Li *et al.* 2006). NoRC-dependent nucleosome repositioning is a prerequisite for DNA methylation by DNA methyltransferases (DNMTs) and the modification of a specific CpG at position -133 relative to the transcription start site (+1) represents a hallmark of silenced rDNA repeats (Santoro & Grummt 2005; Li *et al.* 2006). This single modification has been shown to interfere with binding of UBF to the UCE element thereby impairing pre-initiation complex (PIC) assembly at the rDNA promoter on nucleosomal templates and be sufficient for repression of rDNA transcription *in vitro* (Santoro & Grummt 2001).

The chromatin modifications described above are thought to lead to long-term adjustments of transcriptional activity rather than flexible adaptations to the actual cellular conditions. However, it is still rather unclear to which extent the discussed chromatin modifications represent real epigenetic marks or must be rather regarded as a transient product of signal transduction pathways and restricted to a specific cell. These considerations are challenging not only with respect to pre-rRNA synthesis and r-chromatin modulation but obviously also for the understanding of these fundamental processes within the whole genome and are subject of intensive research (discussed in recent reviews by (Henikoff 2005; Ptashne 2007; Barth & Imhof 2010). While the mechanistic understanding with respect to the establishment and maintenance of silenced rRNA repeats increased continuously, the processes behind the construction of a highly mobile chromatin conformation to form transcriptionally active rRNA gene repeats are less understood. One of the most characterised factors in this regard is the Pol-I specific transcription factor UBF. The protein is associated with active NORs throughout mitosis and is absent from silent NORs (Roussel *et al.* 1993; Wright *et al.* 2006). UBF is a sequence-tolerant DNA-binding protein that interacts with the minor groove of DNA and binds to structured DNA. In its dimeric form the HMG-box-containing protein is able to bend DNA to a structure resembling a nucleosome in both DNA content and mass (Bazett-Jones *et al.* 1994; Copenhaver *et al.* 1994; Putnam *et al.* 1994). UBF is bound throughout the IGS and the pre-rRNA coding region (O'Sullivan *et al.* 2002) indicating that the protein plays an important structural and functional role in the establishment and maintenance of transcriptionally active r-chromatin. This specific function and its responsibilities related to pre-initiation complex assembly will be subject of the following chapter. Furthermore, next to its role in rDNA silencing TTF-I has been shown to contribute also to the establishment of an

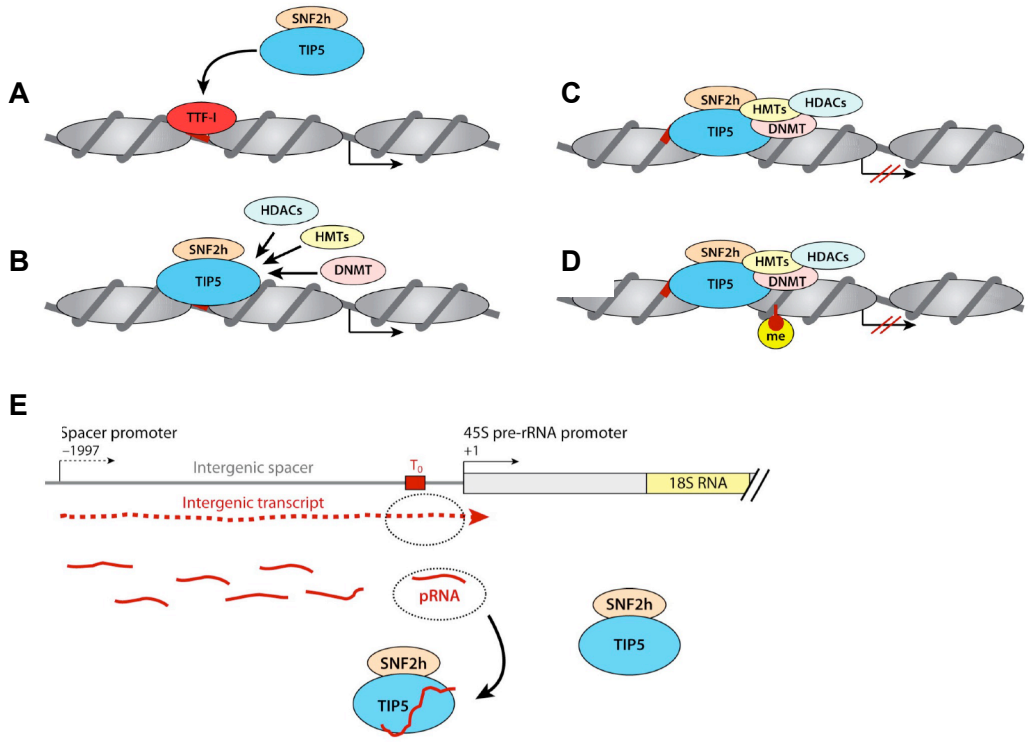


Figure 4: A model for the silencing mechanism of rRNA genes (McStay & Grummt 2008).
(A-D) Model depicting individual steps of rDNA silencing. (A) First, NoRC is recruited to the rDNA promoter by TTF-I bound to the promoter-proximal terminator T_0. (B) Next, NoRC interacts with the Sin3 corepressor complex, leading to deacetylation of histones H3 and H4, and with histone methyltransferases (HMTs) that methylate H3K9, H3K20, and H3K27. (C) These heterochromatic histone modifications may act as a signal for the ATPase SNF2h to shift the promoter-bound nucleosome 25 nt further downstream into a translational position that is unfavourable for pre-initiation complex formation. (D) The action of SNF2h may either relieve a steric constraint or expose CpG at –133 to methylation by DNA methyltransferases (DNMTs). Methylation of CpG at –133 in the context of chromatin impairs UBF binding and pre-initiation complex assembly. (E) Model depicting the role of promoter-associated RNA (pRNA) in rDNA silencing. Intergenic transcripts (dotted line) are synthesized from a spacer promoter located 2 kb upstream of the major 45S pre-rRNA promoter. The primary intergenic transcripts are degraded or processed by an as-yet-unknown mechanism. Transcripts of 150–300 nt that match the rDNA promoter (pRNA) bind to TIP5 (TTF-I-interacting protein 5) via the MBD (methyl-CpG-binding domain)-like TAM (TIP5/ARBD/MBD) domain. Association with pRNA is required for NoRC-mediated heterochromatin formation.

active r-chromatin conformation. TTF-I binding to its promoter-proximal binding site stimulates rDNA transcription *in vivo* (Henderson & Sollner-Webb 1986) and was subsequently shown to lead to a re-arrangement of nucleosomal positions on a rDNA promoter-containing template pre-assembled into nucleosomes *in vitro* (Langst *et al.* 1997; Langst *et al.* 1998).This correlated with transcriptional activation indicating that TTF-I recruits specific remodeling complexes to the rDNA promoter to induce nucleosome repositioning thereby allowing efficient transcription initiation. Interestingly, CSB (Cockayne syndrome protein B), an ATPase that is capable of chromatin remodeling and localised to the nucleolus, has been shown to interact with Pol I, TFIIH and Pol I-related basal transcription factors (Bradsher *et al.* 2002) and to stimulate rDNA transcription *in vivo* (Yuan *et al.* 2007). The observed transcriptional activation by CSB requires its ATPase activity and depends on TTF-I binding to its promoter-proximal binding site T_0. However, other factors might be additionally needed for the maintenance of an euchromatic conformation of active rDNA repeats.

2.2 rRNA gene expression by RNA polymerase I

In yeast almost 60 % of total transcriptional activity is dedicated solely to the production of ribosomal RNA by RNA polymerase I (Pol I) (Warner 1999). The following section will summarise current knowledge on the distinct steps and specificities of the process of mammalian rRNA gene expression.

2.2.1 Initiation of rRNA gene transcription

Pre-initiation complex assembly

In the classical view mammalian initiation complex formation is a step-wise assembly of at least four essential Pol I-specific factors, namely UBF, TIF-IA, - B and –C, and the Pol I enzyme complex at the dedicated promoter elements (Schnapp & Grummt 1991). The mammalian rDNA promoter has a bipartite structure including a core promoter element (CPE) adjacent to the transcription start site and an upstream control element (UCE). Pol I is a multi-protein complex comprising 14 core subunits, of which some are shared with one or both of the other two eukaryotic RNA polymerases (Moss *et al.* 2007). The upstream-binding factor UBF was shown to bind to the UCE upstream the core promoter, creating a favourable environment for the transcription initiation factor TIF-IB complex ((selectivity factor 1 (SL1) in humans) to associate with the CPE (Figure 5A) (Bell *et al.* 1990; Jantzen *et al.* 1992). UBF contains several HMG boxes, a motif known to bend DNA (Jantzen *et al.* 1990), which enables an UBF dimer to wrap the DNA such that it forms a loop of almost 360° every 140 bp. This conformation has been termed ‘enhancesome’ and suggests a model how the core promoter and UCE are brought in close proximity thereby supporting interaction with and parallel promoter recognition by TIF-IB (Figure 5B) (Bazett-Jones *et al.* 1994). The species-specific TIF-IB complex consists of the TATA-binding protein (TBP) and three Pol I-specific TBP-associated factors (TAF_Is), TAF_I95/110, TAF_I68 and TAF_I48, which, instead of TBP in the case of Pol II transcription, mediate DNA binding to the CPE (Comai *et al.* 1992; Heix *et al.* 1997). Subsequently Pol I is recruited to the rDNA promoter via the interaction of UBF with the Pol I subunit PAF53 (Hanada *et al.* 1996) and the binding of Pol I-associated TIF-IA to promoter-bound TIF-IB (Miller *et al.* 2001; Yuan *et al.* 2002). Pol I is a multi-protein complex comprising 14 core subunits, some of which are shared with one or both of the other two eukaryotic RNA polymerases (Moss *et al.* 2007). TIF-IA is a regulatory factor, which is conserved between human and yeast (with its homologue Rrn3)(Moorefield *et al.* 2000), and is associated only with the initiation-competent portion of Pol I complexes (Buttgereit *et al.* 1985; Schnapp *et al.* 1990; Bodem *et al.* 2000; Miller *et al.* 2001). The interaction of the fourth initiation factor TIF-IC with Pol I seems to be a prerequisite for Pol I-TIF-IA association (Schnapp & Grummt 1991) and is required for specific initiation and elongation (Schnapp *et al.* 1994; Paule 1998), however, its precise role is still elusive.

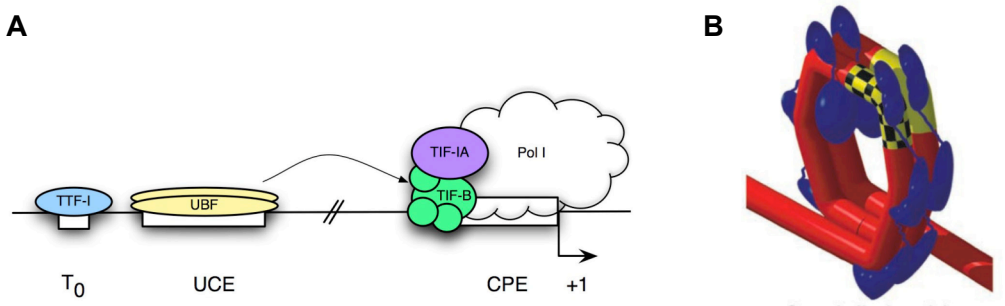


Figure 5: Basal transcription factors in rDNA transcription initiation and termination.
(A) The basal transcription factors required for rDNA transcription initiation. The ellipsoids represent the factors, which are associated with the rDNA promoter and/or Pol I, respectively. TTF-I is associated to the terminator binding site upstream of the promoter, T_0. Synergistic binding of UBF and TIF-IB to the promoter is required for the recruitment of Pol I (arrow). (B) Possible folding of the rDNA promoter by two adjacent enhancesomes induced by UBF binding (Moss *et al.* 2007). Model of two adjacent enhancesomes, of which the structure was determined in low-resolution by electron spectroscopic imaging, and a possible folding of rDNA promoter sequence. The UCE is shown checked black/yellow, the CPE in yellow, UBF in blue and DNA in red. Only the Core UBF region is shown and inter-HMG1 box linkers are shown generically.

The *Pol I holoenzyme* concept versus step-wise PIC assembly

Beginning of last decade, biochemical purification of Pol-I containing complexes in combination with mass spectrometry-based analysis revealed an association of initiation, elongation and processing factors with the Pol I subunits (Seither *et al.* 1998; Hannan *et al.* 1999; Fath *et al.* 2000; Iben *et al.* 2002). These findings raised the *Pol I holoenzyme* concept arguing for pre-assembled Pol-I containing complexes, which comprise the required activities to execute efficient rRNA synthesis. Thus, a huge ribonucleoprotein (RNP) complex purified from yeast contained next to Pol I and its initiation factors also rRNA processing factors such as Nop1p and Rrp5p along with small nucleolar RNAs (Fath *et al.* 2000). Furthermore this complex was able to support correct transcription, termination and pseudo-uridylation of rRNA supporting the idea of a specialised nucleolar domain, which serves as a scaffold for co-ordinated rRNA synthesis and processing. However, rather contrary to the *Pol I holoenzyme* concept were the results of a recent study, which assessed the movement of individual Pol I subunits and initiation factors by the FRAP (fluorescent recovery after photo bleaching) technique *in vivo* (Dundr *et al.* 2002) and rather indicated that the different Pol I components enter the nucleolus as distinct subunits.

Initiation and promoter clearance

Kinetic analysis of transcription initiation *in vitro* revealed that Pol I-dependent transcription is rate-limited in a step subsequent to recruitment and PIC assembly (Panov *et al.* 2001). The authors suggested that this step potentially reflects the process of the so-called promoter escape, a phenomenon known from Pol II-mediated transcription. Before Pol II enters a stable elongation phase the enzyme passes through several unproductive rounds of aborted synthesis and re-initiation (Dvir 2002). However, another comprehensive study on the

transition from initiation to elongation could not detect any short aborted transcripts anticipated in this case. Moreover, according to their assessment of Pol I occupancy at the transcribed rDNA regions and elongation rates with respect to resulting rRNA synthesis the authors rather define the processivity of the elongating Pol I enzyme as the rate-limiting step in the transcription process *in vivo* (Stefanovsky *et al.* 2006a). Still, limited data is available on which specific factors or signals are required for the initiation-competent complex to proceed with promoter clearance and switch from the initiation to the elongation mode. One prerequisite appears to be the phosphorylation of Pol I-associated TIF-IA at two specific serine residues by the casein kinase 2 (CK2), which triggers its release from the Pol I complex, as it was demonstrated by FRAP and CHIP experiments (Bierhoff *et al.* 2008). In support of this finding, inhibition of these modifications or the covalent fusion of TIF-IA to the Pol I subunit RPA43 impaired rDNA transcription.

2.2.2 rRNA gene transcription elongation

Approximations of RNA polymerase elongation rates by either computational analysis of FRAP data obtained in human cells or by directly counting Pol I molecules per DNA repeat with respect to rRNA transcriptional output as well as metabolic labelling and data modelling in yeast have ranged from approximately 30 to 100 nt per second (human and yeast, respectively) (Cavanaugh & Thompson 1985; Dundr *et al.* 2002; French *et al.* 2003; Kos & Tollervey 2010). However, so far relatively little is known on the components required for the constitution of an efficient elongation process. Notably, recent findings have attributed novel properties to UBF related to rRNA transcription elongation. Distinct chromatin immunoprecipitation (ChIP) studies revealed that UBF is not only associated with the rRNA gene promoter but also binds throughout the whole rRNA gene repeat (O'Sullivan *et al.* 2002; Nemeth *et al.* 2008). This finding is in conflict with a sole function in transcription initiation and rather argues for a superior, structural function in modulating the chromatin conformation at active rRNA genes. Accordingly Moss and colleagues could demonstrate that UBF is able to modify r-chromatin structure through its DNA binding qualities in a growth-dependent manner thereby modulating transcription elongation rates (Stefanovsky *et al.* 2006b). Remarkably, in growth-inhibited compared to growth-stimulated 3T3 cells no differences in Pol I loading could be detected by run-on or ChIP assays while the endogenous elongation rates of Pol I increased up to 5-fold and changed direct proportional to rRNA synthesis (Stefanovsky *et al.* 2006a). Thus, these data strongly suggest that the elongation process represents the rate-limiting step in Pol I transcription rather than PIC assembly or initiation confirming previous studies by Misteli and colleagues (Dundr *et al.* 2002). Another factor thought to influence transcription elongation by modifying rDNA chromatin conformation is CSB (Yuan *et al.* 2007). CSB has been shown to activate rDNA

transcription and CSB-mediated activation required its association with G9a, a histone methyl transferase, which is responsible for H3K9 di- and trimethylation (Tachibana *et al.* 2001; Tachibana *et al.* 2002). Importantly, H3K9 dimethylation and HP1γ, a protein harbouring a chromodomain that recognises H3K9 methylation (Font-Burgada *et al.* 2008), are present within the transcribed region of active rDNA repeats (Yuan *et al.* 2007). Hp1γ has been originally implied in heterochromatin formation but was recently also shown to bind to actively transcribed regions (Vakoc *et al.* 2005; Hediger & Gasser 2006). The association of both H3K9 dimethylation and HP1γ is dependent on ongoing Pol I transcription altogether suggesting a role of CSB in the establishment of an active r-chromatin conformation important for efficient elongation (Yuan *et al.* 2007). Further factors recently implied in the regulation of Pol I transcription elongation in yeast are represented by the PAF1C complex and Spt4 and 5, both originally known from Pol II elongation, which delivered additional clues on how Pol I transcription might be co-ordinated with respect to subsequent rRNA processing and nutrient availability (Schneider *et al.* 2006; Zhang *et al.* 2010a). In human cells TFIIH, a basal Pol II transcription factor with an essential role in nucleotide excision repair (Mydlikova *et al.* 2010), has been identified to interact with a subpopulation of Pol I and being essential for rRNA synthesis *in vitro* and *in vivo* at a post-initiation step in transcription (Iben *et al.* 2002).

2.2.3 Termination of rRNA gene transcription

Specific factors and sequence elements are required for Pol I transcription termination. These terminator sites called $T_{1\text{-}10}$ or Sal-boxes have a length of 18 bp in mouse and 11 bp in humans and are repeated 10 times downstream of the pre-rRNA coding region in the IGS of the rDNA gene (Grummt *et al.* 1985; Bartsch *et al.* 1988; Pfleiderer *et al.* 1990; Evers *et al.* 1995). Transcription termination is a multistep process including Pol I pausing, release of both the pre-rRNA and Pol I and 3' end-processing of the primary transcript. TTF-I binds sequence-specific to the terminator sites to form a barrier to Pol I elongation. The release factor PTRF (Pol I and transcript release factor) interacts with both TTF-I and Pol I, which leads to the dissociation of the transcript and Pol I from the DNA template. Notably, both a T-rich sequence located upstream of the T_1 terminator site and the orientation of the following terminator sites are crucial for the release and terminator functions of PTRF and TTF-I, respectively (Jansa *et al.* 1998; Jansa & Grummt 1999). Interestingly, recent experiments in yeast have shown a role for remodelling factors in transcription termination. Deletion of CHD1, ISW1 and ISW2 had no effect on transcription initiation, but caused an Pol I termination defect (Jones *et al.* 2007) indicating the requirement of a specialized chromatin structure directed by these remodeling factors for normal termination. A recent study employing the chromosome conformation capture (3C) assay revealed a TTF-I-mediated

interaction of the terminator and promoter sequences of active rDNA repeat adding a topological level of regulation and presenting a potential mechanism to enhance re-initiation events after successful transcription termination (Nemeth *et al.* 2008).

2.3 rRNA processing and ribosome assembly

The mammalian ribosome is a 4-MDa structure consisting of two distinct subunits referred to as the large (60S) and the small (40S) subunits (LSU and SSU, respectively). It is composed of ribosomal RNA representing the catalytic activity and ribosomal proteins (r-proteins) likely to be responsible for correct processing and folding of the rRNA and their proper assembly into the ribosome. The LSU contains three RNA species, the 28S, 5.8S and 5S rRNAs, and approximately 49 r-proteins, while the SSU contains a single RNA, the 18S rRNA, and approximately 33 r-proteins. Several steps of pre-rRNA processing by proper cleavage and nucleotide modifications are required before their assembly to gain a readily utilisable ribosome.

2.3.1 The cleavage events of the rRNA processing pathway

Most knowledge available on pre-rRNA processing has emerged from research in yeast. Studies in *S. cerevisiae* have revealed the extreme complexity of ribosome synthesis and assembly. While in mammalian cells the 47S precursor is the source to produce mature 18S, 5.8S and 28S rRNAs by a number of endo- and exonucleolytic cleavage steps in yeast the RNA Polymerase I synthesises a 35S pre-rRNA, which is processed into mature 25S, 18S and 5.8S rRNAs. A scheme of the pre-rRNA processing pathway including metabolic intermediates of both S. cerevisiae and mammals is depicted in Figure 6 (Gallagher *et al.* 2004; Prieto & McStay 2007). In *S. cerevisiae* proper ribosome assembly requires more than 170 non-ribosomal proteins, 70 small nucleolar RNAs (snoRNAs) and the proper covalent nucleotide modification such as 2'-O-ribose-methylation or pseudouridylation of about 100 different sites in the pre-rRNA by the site-specific snoRNPs complexes. Finally, the yeast ribosome is formed of 78 ribosomal proteins within a frame of properly folded rRNA molecules (Decatur & Fournier 2002; Tschochner & Hurt 2003). In a very recent study in yeast Tollervey and colleagues have employed a further optimised pulse-chase metabolic labelling approach integrating mathematical modelling to investigate the kinetics of rRNA gene transcription and subsequent processing (Kos & Tollervey 2010). Their results could not only confirm earlier studies on the Pol I transcription time needed to complete the pre-rRNA of ~170 s with a corresponding elongation rate of 40 nt/s but also reveal important insights related to the long-lasting dispute on the order of events involved in the generation of mature rRNAs. There data could thereby demonstrate that pre-rRNA processing clearly occurs co-transcriptionally.

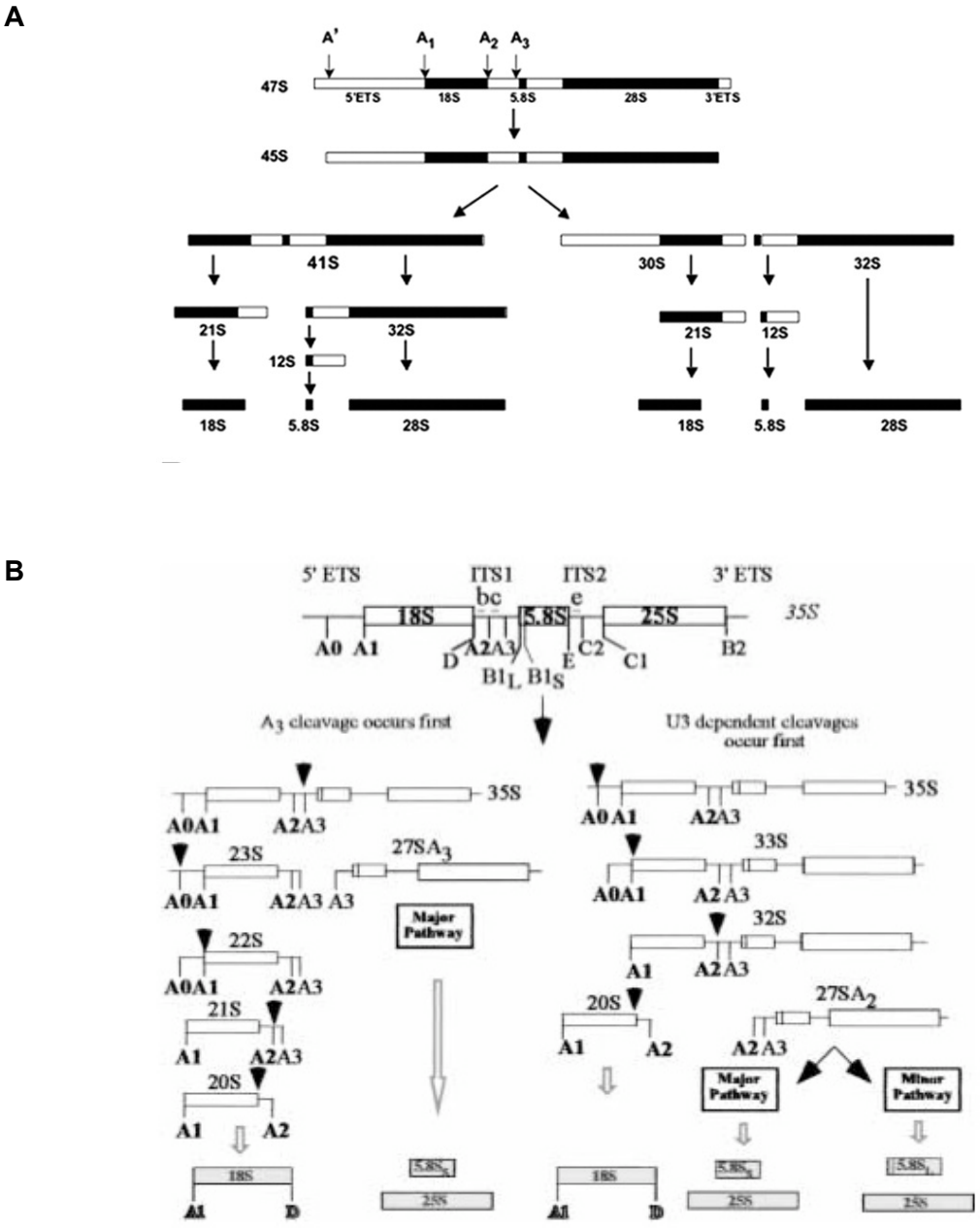


Figure 6: rRNA processing pathway.
(A) The human rRNA processing pathway (Prieto & McStay 2007). (B) The rRNA processing pathway of *S. cerevisiae* (Gallagher *et al.* 2004).

2.3.2 Co-transcriptional processing and ribosome assembly

Early kinetic studies of yeast ribosome biogenesis have indicated that pre-rRNA processing starts only at a time when the precursor is fully completed (Udem & Warner 1972; Trapman & Planta 1975). This assumption supported preceding electron micrographs of active rRNA genes in amphibian oocytes, also known as Miller spreads or Christmas trees, which displayed nascent transcripts constantly increasing in size over the rDNA repeat (Miller & Beatty 1969). However, about 20 years later it was proposed that the terminal knobs visible on these Christmas tree structures include factors of the pre-rRNA processing machinery providing the first hints on the possibility of co-transcriptional processing (Kass *et al.* 1990; Mougey *et al.* 1993). Subsequently, a recent affinity-tag purification followed by mass spectrometry-based analysis has identified a particle including pre-rRNAs, U3 snoRNA, processing factors, ribosomal proteins and several novel proteins, which were accordingly termed Utps (U three proteins) (Dragon *et al.* 2002). Depletion of components of this complex termed the SSU processome leads to disruption of the terminal knobs and impairment of 18S rRNA biogenesis finally giving evidence on the functional nature of the for long-known knob structures.

The integration of several other experimental results has further shaped the current view of pre-rRNA processing as described in the following. First a common precursor particle of both ribosomal subunits with a sedimentation constant of 90S assembles on the pre-rRNA (Grandi *et al.* 2002). Early co-transcriptional rRNA processing is initiated after the full association of the SSU processome components, which mainly overlap with those of the 90S pre-ribosome and include the U3 snoRNA (Mougey *et al.* 1993; Dragon *et al.* 2002) that base pairs with sequences near the 5' end of the pre-rRNA (Beltrame *et al.* 1994; Beltrame & Tollervey 1995). SSU processome-dependent processing leads to pre-rRNA cleavage in the ITS1, subsequent release of most of the pre-40S components from the pre-ribosomal structure and assembly of 60S factors on the pre-rRNA (Osheim *et al.* 2004). According to a detailed kinetic study in yeast by Tollervey and colleagues 70% of rRNA precursors are affected by such co-transcriptionally cleavage events and the majority of released 20S rRNA precursor has undergone methylation already as nascent transcript (Kos & Tollervey 2010). Interestingly, the ordered integration of ribosomal proteins into the SSU processome forming several stable intermediates has also shown to be crucial for early pre-rRNA cleavage as well as for subsequent efficient SSU export and the final cytoplasmic pre-rRNA maturation step to yield mature 18S and a translation-competent 40S subunit (Ferreira-Cerca *et al.* 2005; Rouquette *et al.* 2005; Ferreira-Cerca *et al.* 2007). Hence, this co-transcriptional phase of assembly and step-wise maturation probably represents a mechanism to control the fidelity of ribosome biogenesis. In contrast to the pre-40S particle the pre-60S complex is exported to the cytoplasm in an almost mature state and cytoplasmic biogenesis steps just

include the facilitated release of several non-ribosomal proteins, yielding fully functional 60S subunits (Zemp & Kutay 2007). Also, the maturation pathways of the individual subunits seem to be mostly independent, as impairment of one in most cases still allows completion of the other leading to a mature subunit (Zemp & Kutay 2007; Holzel *et al.* 2010). A wide range of factors involved in either SSU or LSU maturation and nuclear export pathways have been described with their according function (Dez & Tollervey 2004; Krogan *et al.* 2004). However, it remains unclear to which extent the components of the SSU and LSU pre-ribosomal particles overlap. There is growing evidence that some factors are shared components of both complexes and/or are employed in both of the respective maturation and export pathways (Venema & Tollervey 1996; Moy & Silver 1999; Bassler *et al.* 2001; Milkereit *et al.* 2001; Oeffinger *et al.* 2004; Oeffinger *et al.* 2007) indicating a potential mechanism to coordinate the two processes and prevent nuclear export of incorrect or only partially assembled precursors of ribosomal subunits. Importantly, over the past years various factors have been determined, which are involved in several steps of the entire ribosome production, from transcription initiation to ribosome nuclear export, supporting an anticipated co-ordination of these processes. Such functional interdependencies represent a potential mechanism for quality surveillance of ongoing ribosome biogenesis. Recent research on this topic will be summarised within the next chapter, and its interconnections with other cellular pathways to adapt ribosome biogenesis to the actual conditions of the cell will be discussed.

2.4 Co-ordination of ribosome biogenesis

In yeast more than 75% of total transcription activity is dedicated to supply the RNA and protein components of the ribosomes, of which approximately 60% is committed solely to rRNA gene transcription by Pol I (Warner 1999). In proliferating mammalian cells still 35% of nuclear transcription is used for the synthesis of rRNA whereas in non-growing cells the transcription of rRNAs is greatly reduced. Therefore the production of ribosomes has to be tightly regulated with respect to the actual cellular conditions. Recent studies have demonstrated that such co-ordinative mechanisms reacting on energy status, cellular stress or nutrient availability directly affect the various phases of ribosome biogenesis, from the transcription process to ribosome nuclear export. Importantly, also reverse mechanisms exist, which communicate defects in ribosome biogenesis to induce appropriate cellular processes such as proliferation stop and eventually apoptosis. Furthermore over the past years several proteins have been identified, which mutually fine-tune the kinetics of the distinct sub-processes of ribosome biogenesis.

2.4.1 Co-regulation of rRNA gene transcription, processing and ribosome assembly

Recently, a subset of seven SSU processome proteins, the t-Utps (transcriptional-Utps), were determined to be also required for efficient rDNA transcription in yeast, linking rDNA transcription with rRNA processing and ribosome assembly (Gallagher *et al.* 2004). McStay and colleagues have identified the human orthologues of the t-Utps and confirmed their requirement for both efficient transcription and processing of the 47S pre-rRNA (Prieto & McStay 2007), indicating that this coordinated mechanism is conserved throughout evolution. In the following Ke and colleagues have identified human ALP (acetyltransferase-like protein) to be a novel t-Utp displaying several features of the classical t-Utps factors such as association to U3 snoRNA and rDNA and requirement for efficient rDNA transcription and 18S pre-rRNA processing (Kong *et al.* 2010). Moreover they could show that the factor acetylates the Pol I-specific factor UBF thereby enhancing its association to the Pol I subunit PAF53 a pre-requisite for productive Pol I transcription initiation and suggesting a mechanism by which t-Utps might positively regulate transcriptional activity of Pol I. Targeting another step in rDNA transcription, Moss and colleagues have recently suggested a role for transcription elongation in rRNA processing and ribosome assembly (Stefanovsky *et al.* 2006a). Erk-dependent phosphorylation of UBF directly influences RNA Pol I elongation rates by inducing an UBF-dependent rearrangement of the chromatin environment. This effect could serve as a mechanism to coordinate transcription and the assembly of pre-ribosomal complexes on nascent rRNA by modulating the Pol I elongation rate. Further insights into how these processes might be coordinated came from Nomura and co-workers

who demonstrated a physical interaction of Pol I with the elongation factors Spt4 and Spt5 and an rRNA processing defect upon depletion of the latter (Schneider *et al.* 2006). An interrelationship between transcription elongation and rRNA processing was further confirmed by using a yeast strain with a mutated RNA Pol I enzyme with reduced elongation rates (Schneider *et al.* 2007). Reduced elongation rates lead to severe defects in rRNA processing and ribosome assembly, suggesting an intimate link between elongation rates and rRNA maturation. Moreover, evidence has been reported by different laboratories on a regulated interplay of proper pre-ribosome assembly, correct pre-rRNA processing and efficient nuclear export of the pre-ribosomal particles to the cytoplasm depending on different ribosomal proteins and other factors (Ferreira-Cerca *et al.* 2005; Ferreira-Cerca *et al.* 2007; Zhang *et al.* 2007; Robledo *et al.* 2008; Oeffinger *et al.* 2009). However, still little is known on actual mechanisms, which recognise aberrant pre-rRNAs or deficient pre-ribosomal particles, and subsequently induce their elimination. Very recent studies in yeast and mammals have demonstrated that failures in proper SSU processome assembly caused by siRNA-mediated depletion of some of its components or accumulation of aberrant rRNA transcripts in cells treated with Actinomycin D indeed trigger a nucleolar surveillance pathway (Wery *et al.* 2009). This switch leads to polyadenylation of pre-rRNAs by the poly(A) polymerase-containing TRAMP complex and degradation by 3' to 5' exoribonucleolytic activities of the exosome. Importantly, Trf5, a component of the TRAMP complex, colocalised with nascent pre-rRNPs indicating that this control mechanism is acting co-transcriptionally. Finally, to ensure efficient ribosome biogenesis also transcription processes by Pol II and Pol III are anticipated to be co-regulated with Pol I transcription to provide the ribosomal and the various non-ribosomal proteins as well as the fourth rRNA, 5S, respectively, in required amounts. To test this postulation Chédin and colleagues have constructed a mutant yeast strain expressing a Pol I enzyme, which remains constitutively initiation-competent under stress conditions, and monitored any co-regulated actions between the three polymerase enzymes by metabolic labelling and genome-wide gene expression profiling (Laferte *et al.* 2006; Chedin *et al.* 2007). Intriguingly, they could demonstrate that Pol I transcriptional derepression leads to derepression of Pol II gene transcription that is restricted to those genes encoding ribosomal proteins and rRNA processing factors, as well as concomitant deregulation of 5S rRNA expression. Taken together, ribosome biogenesis appears to be controlled by feedback mechanisms between its different steps, which ensure proper assembly occurs on all synthesis levels, from transcription initiation to ribosome export, as well as between the different involved RNA polymerase enzymes.

2.4.2 Adapting ribosome biogenesis to the metabolic state of the cell

The activity of rRNA gene transcription in a given cell is the major determinant of cell proliferation and cell growth and is tightly regulated to match ribosome amounts with the current physiological needs of the cell. Accordingly, various regulatory mechanisms target the different steps of rRNA gene synthesis, from pre-initiation complex assembly to transcription termination, to adapt the transcriptional output of Pol I to the current metabolic state of the cell. Several qualitatively different mechanisms have been described over the past years. Accordingly modulation of rRNA synthesis levels with respect to the cellular conditions can be achieved by:

- changing the activity of factors required for rRNA synthesis through posttranslational modification.

The essential transcription initiation factor TIF-IA is a central target of a number of signalling pathways such as the JNK (c-Jun N-terminal kinase), the AMP-activated protein kinase (AMPK), the mTOR (mammalian target of rapamycin) or ERK (extracellular signal-regulated kinase) pathway to enhance or interfere with the pre-initiation complex formation. By modulating the factor's activity through reversible posttranslational modifications these mechanisms directly translate actual cellular conditions such as stress stimuli, energy supply, nutrient availability and cell growth, respectively, into the required rRNA synthesis activity (Zhao *et al.* 2003; Mayer *et al.* 2004; Mayer *et al.* 2005; Hoppe *et al.* 2009). Also other auxiliary factors are modified according to the actual state of the cell. Thus, acetylation of TAF_I68 by PCAF augments its DNA binding properties leading to increased rDNA transcription activity *in vitro*, while deacetylation by the HDAC Sir2a, which needs NAD^+ as cofactor and is thereby dependent on the intracellular energy status, has the opposite effect (Muth *et al.* 2001). Probably by directly changing the conformation of the Pol I enzyme the PAF1C complex, originally known to have a role in Pol II elongation, was recently described to also influence Pol I transcription elongation *in vitro (Zhang et al. 2010b)*. The positive effect of PAF1C on rRNA synthesis is dependent on nutrient availability and TOR signalling suggesting an additional mechanism to modulate Pol I activity with respect to metabolic conditions.

There is also evidence that cell-cycle-dependent proteins target rDNA transcription activity. For example the activity of the Pol I-specific transcription factors UBF and TIF-IB/SL1 has been shown to depend on the respective cell cycle phase (Heix *et al.* 1998; Klein & Grummt 1999). Thus, the cell-cycle-regulated cdc2/Cyclin B kinase phosphorylates the transcription initiation factor TIF-IB/SL1 subunit TAFI110 thereby impairing its interaction with UBF and productive pre-initiation complex formation. Furthermore, the retinoblastoma protein (Rb) has been shown to interfere with Pol I *in vitro* transcription by inhibiting UBF activity through direct interaction (Cavanaugh *et al.* 1995). Subsequent investigation revealed the underlying

mechanism with Rb disturbing the interaction of UBF and TIF-IB/SL1 in vitro and in vivo, which is prerequisite for efficient Pol I transcription initiation (Hannan *et al.* 2000).

- modification of the r-chromatin structure.

Furthermore mechanisms, which target rDNA chromatin conformation, can also be considered to alter Pol I transcriptional activity for example by modulating its initiation competency or elongation rate. As described earlier phosphorylation of the Pol I-specific factor UBF by the ERK kinase leads to a structural rearrangement of the r-chromatin, in which UBF is directly involved. The subsequent changes in Pol I elongation rates thereby reflect an immediate response of rDNA transcription to growth factor signalling (Stefanovsky *et al.* 2001; Stefanovsky *et al.* 2006a). Recently Yanagisawa and colleagues have described a mechanism linking nutrient availability to heterochromatin formation and rDNA silencing (Murayama *et al.* 2008). They demonstrated that an altered NAD^+/NADH ratio as a result of glucose deprivation modulates the activity of eNOSC (energy-dependent nucleolar silencing complex), a complex including the newly identified Nucleomethylin, the NAD^+-dependent histone deacetylase SIRT1 and the histone methyltransferase SUV39H1, thereby leading to r-chromatin modification and transcriptional silencing.

- by adapting the supply of factors required for ribosome biogenesis.

c-Myc is one of the best-characterised transcription factors, which is not least due to its role in tumorigenesis. The proto-oncogene product is a global regulator of various cellular processes, which include proliferation, growth, differentiation and apoptosis (Oster *et al.* 2002). Recently c-Myc has been shown to directly and indirectly regulate rRNA synthesis. Firstly, the protein binds to the rDNA repeat and activates Pol I transcription by the recruitment of transcription factor TIF-IB/SL1 and modulation of chromatin composition and higher-order structure in response to mitogenic signals (Arabi *et al.* 2005; Grandori *et al.* 2005; Shiue *et al.* 2009). Additionally, in its role as transcription factor c-Myc has been not only shown to positively regulate rRNA synthesis by Pol I directly but also the expression of genes coding for ribosomal proteins and non-ribosomal proteins engaged in ribosome production as well as the fourth rRNA 5S transcribed by Pol II and Pol III, respectively (Gomez-Roman *et al.* 2003; Schlosser *et al.* 2003). Hence, c-Myc plays a central role in the regulation of ribosome biogenesis as the protein controls the supplies of all required resources from an elevated hierarchic level.

Recently, a mechanism has been described that targets solely the level of specific rRNA processing factors. Zhang and colleagues have observed an interaction between the tumor suppressor ARF and Nucleophosmin/B23, a riboendonuclease involved in 28S maturation (Savkur & Olson 1998) and demonstrated that B23 gets polyubiquitinated in an ARF-dependent manner leading to its proteosomal degradation (Itahana *et al.* 2003). As expected decreased levels of B23 result in the inhibition of ribosome biogenesis and, importantly, also

to an induction of apoptosis suggesting a feedback mechanism between regulation of cellular proliferation and ribosome biogenesis.

2.4.3 Feed-back mechanisms from ribosome biogenesis to cellular proliferation

Several cellular stresses lead to inhibition of rRNA gene transcription and thereby subsequent ribosome biogenesis comes to a halt. Over the past years different studies have demonstrated that the fidelity of ribosome biogenesis is monitored by the cell cycle regulator p53. Defects in either of the maturation pathways of the 40S and 60 S ribosomal subunits lead to p53 accumulation and cell cycle arrest. Here, various factors of both pathways have been identified to play a role in targeting the p53 pathway including Bop1 (Pestov *et al.* 2001), human UTP18 (Holzel *et al.* 2010), several ribosomal proteins of the LSU, namely L5, L11 and L23, and S7 belonging to the SSU (Lohrum *et al.* 2003; Zhang *et al.* 2003; Dai & Lu 2004; Jin *et al.* 2004; Chen *et al.* 2007). L11, and subsequently also the other ribosomal proteins, were characterised as inhibitors of Hdm2 (and Mdm2 in mouse), an E3 ubiquitin ligase, which targets p53 for degradation (Harris & Levine 2005). By this mechanism increased levels of unbound ribosomal proteins as a result of impaired ribosome assembly lead to a p53 response and proliferation stop.

2.5 The Myb-binding protein 1a

In the course of the project Myb-binding protein 1a (Mybbp1a) was identified as an interacting factor of RNA Pol I and its implication in rDNA transcription regulation and rRNA processing was characterised. The following chapters give a short overview on the actual knowledge on this protein.

2.5.1 Characteristics and structural features of Mybbp1a

Mybbp1a has been originally identified as an interaction partner of the c-Myb protein, which plays a critical role in regulating proliferation and differentiation of hematopoietic cells (Favier & Gonda 1994; Tavner *et al.* 1998; Sakamoto *et al.* 2006). The 160-kDa protein is ubiquitously expressed and localizes predominantly, but not exclusively, to the nucleolus (Tavner *et al.* 1998). Lower protein levels are also detectable in the nucleoplasm and cytoplasm and it has been shown that Mybbp1a is shuttling between these cellular compartments by using CRM1-dependent and independent nuclear export pathways (Keough *et al.* 2003).

Mybbp1a is widely conserved throughout evolution, and potential orthologues have been identified in animals, plants and fungi (Tavner *et al.* 1998; Keough *et al.* 1999; Yang *et al.* 2003). The human Mybbp1a protein (Accession number: gi:6959304; AF147709.1) is highly similar to the mouse orthologue (Accession number gi:31982724; NP058056.2) with 69% of identical amino acids (assessed by BLASTP 2.2.23, (Altschul *et al.* 1997)) . Expectedly the two orthologues share several structural features such as leucine charged domain (LCD) motifs, an acidic domain and several basic amino acid repeats as summarised in Figure 7 (Keough *et al.* 1999). The strict conservation of a number of polar residues in two regions (CR1 and 2) of the protein throughout species argues for their implication in preserved, highly specific interactions (Yang *et al.* 2003). Interestingly, the potential yeast homologue Pol5p has been shown to bind to the rRNA gene promoter and to have a role in rRNA synthesis (Shimizu *et al.* 2002; Yang *et al.* 2003; Nadeem *et al.* 2006). A database search employing the Conserved Domain algorithm ((Marchler-Bauer *et al.* 2009); version v.2.22) determined two conserved domains within the human protein, the DNA Pol phi domain (pfam 04931; E-value: 1,54e-138), which is named after the potential yeast homologue Pol5p and is also present in the potential Mybbp1a homologuesin other species, and the transcription initiation factor IIF alpha subunit domain (TFIIF-a; pfam 05793; E-value: 6,55e-03), which is only found conserved in the human protein. TFIIF directly binds to Pol II and is responsible for its site-specific transcription initiation (Orphanides *et al.* 1996).

Interestingly, in certain cell lines, a specific portion of mouse Mybbp1a is processed by proteolytic cleavage to generate the N-terminal fragment $p67^{MBP}$ (Tavner *et al.* 1998). The existence of such post-translational cleavage products has also been confirmed in human

cell lines. After exposing HeLa cells to UV light or chemicals that impair transcription, Ishii and colleagues observed the appearance of N-terminal fragments of Mybbp1a with molecular weights of 67 kDa and 140 kDa ($p67^{MBP}$ and $p140^{MBP}$, respectively) and a translocation of the full-length protein (partially) and its cleavage products from the nucleolus to the nucleoplasm (Yamauchi *et al.* 2008). Human Mybbp1a and its post-translational cleavage products are summarised together with their structural features in Figure 7.

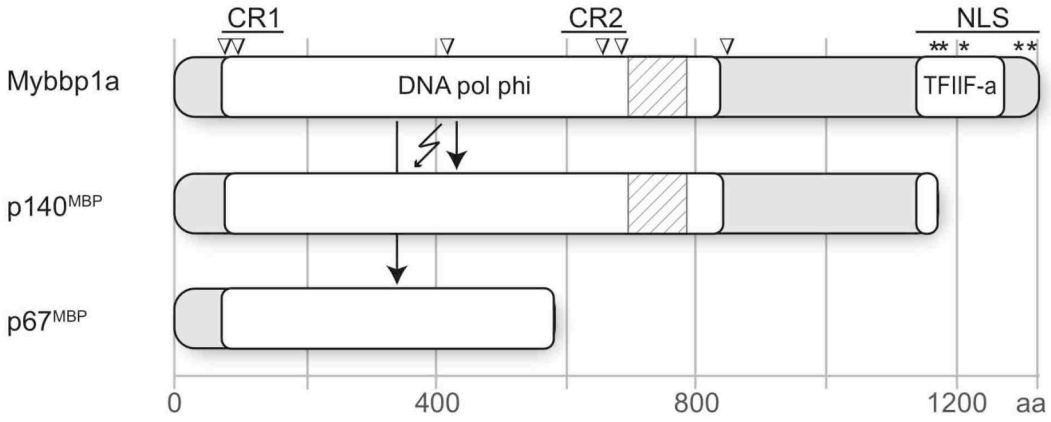


Figure 7: Analysis of Mybbp1a sequence and complexes.
(A) Schematic representation of the conserved regions of human Mybbp1a and its proteolytic cleavage products $p140^{MBP}$ and $p67^{MBP}$. Sequence analysis of Mybbp1a (gi 6959304) with the Conserved Domain database search (Marchler-Bauer *et al.* 2009) (version v.2.22) revealed two conserved domains: the DNA polymerase phi domain from amino acid (aa) 70 - 836 (DNA pol phi; pfam 04931; E-value: 1,54e-138) and the transcription initiation factor IIF alpha subunit from aa 1147 - 1282 (TFIIF-a; pfam 05793; E-value: 6,55e-03). Their corresponding E-values with the Mybbp1a sequence are indicated. Conserved regions (CR) 1 (aa 70 - 106 and 130 - 166) and 2 (aa 595 - 624 and 644 - 693) contain strictly conserved polar residues (Yang *et al.* 2003). The putative leucine charged domain motifs (∆; aa 74-78; 96-100; 419-423; 656-659; 685-693; 849-853), the basic amino acid repeats (*; aa 1167-1172; 1179-1185; 1210-1219; 1297-1301; 1317-1324) and the acidic domain (dashed box; 696 - 784) are also present in the mouse orthologue (Tavner *et al.* 1998; Keough *et al.* 1999). Upon transcription impairment by chemical stress (indicated by the arrow ↯) Mybbp1a gets partially proteolyzed to generate the N-terminal fragments $p140^{MBP}$ and $p67^{MBP}$ (Yamauchi *et al.* 2008). NLS: Nuclear Localisation Signal (Keough *et al.* 2003). The length of the different proteins is indicated on the bottom.

2.5.2 Functional properties of Mybbp1a

Despite its predominant localisation to the nucleolus no function of Mybbp1a had been determined in this compartment so far. However, several publications connect Mybbp1a to transcriptional regulation by acting as a co-repressor on Pol II-mediated gene expression. Mouse Mybbp1a and $p67^{MBP}$ bind to the leucine zipper motif within the negative regulatory domain (NRD) of c-Myb and $p67^{MBP}$, but not Mybbp1a, inhibits trans-activation by c-Myb (Favier & Gonda 1994; Tavner *et al.* 1998). In subsequent studies several other interaction partners of Mybbp1a were identified. Thus, both proteins, Mybbp1a and $p67^{MBP}$, bind to the transcriptional co-activator PPAR gamma coactivator 1α (PGC-1α) (Fan *et al.* 2004), which is a key regulator of several aspects of mammalian metabolic processes such as mitochondrial biogenesis and respiration in muscle and gluconeogenesis in liver (Puigserver & Spiegelman 2003). By binding to PGC-1α Mybbp1a represses its transcriptional co-

activation activity at its target genes. The Mybbp1a protein has also been characterised as a co-repressor of NF-κB, a transcriptional regulator of a wide range of genes including regulators of proliferation, cell cycle and apoptosis (Pahl 1999; Owen *et al.* 2007). By binding to the transcription activation domain of its subunit RelA/p65 Mybbp1a competes with the histone acetylation transferase co-activator p300 thereby repressing the trans-activating capability of NF-κB (Owen *et al.* 2007). A further piece of evidence underlining Mybbp1a's capacity to act as a transcriptional co-repressor was described in the context of Period2 gene expression, which produces PER2, a core oscillator for maintenance of circadian clock (Hara *et al.* 2009). Initially the authors have found Mybbp1a to be interacting with another core oscillator, CRY1, and subsequently tested Mybbp1a's function in circadian gene expression. Mybbp1a represses transcription from a Per2-luciferase reporter construct and it was shown to bind to the endogenous Per2 promoter. Mybbp1a also negatively regulates the transcriptional activity of the Prep1-Pbx1-heterodimer by competing with Pbx1 for the Prep1 binding site (Diaz *et al.* 2007), both of which are homeodomain-containing transcription factors and co-regulators of Hox gene products (for review, see (Moens & Selleri 2006). Furthermore the Mybbp1a protein is part of a co-repressor complex, which contains also several chromatin-modulating factors like HDAC1, 2 and 3, and inhibits the activity of photoreceptor cell-specific nuclear receptor (PNR) (Takezawa *et al.* 2007). In one case Mybbp1a has been also characterised as a co-activator related to aromatic hydrocarbon receptor (AhR)-dependent gene expression (Jones *et al.* 2002). Moreover the protein is part of the B-WICH complex, which contains next to its core proteins, the Williams syndrome transcription factor (WSTF) and Snf2h, Pol I and III transcribed RNAs. Figure 8 summarises the known interaction partners of Mybbp1 with respect to the time of their identification as well as any connected functional data if available.

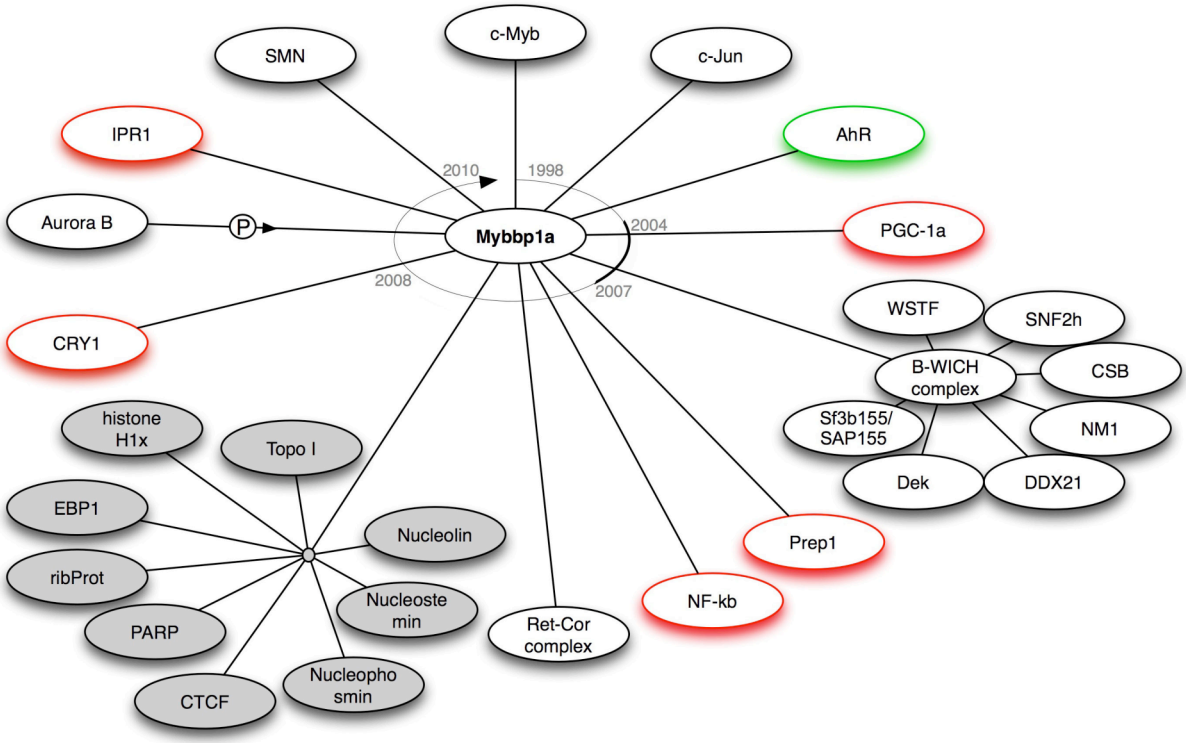


Figure 8: The interaction network of Mybbp1a.
Schematic overview of so far identified proteins interacting with Mybbp1a. The respective year of data publication is indicated in the centre. Interaction partners, which have been identified with Mybbp1a as bait, are highlighted in light grey. Positive and negative regulatory impact of Mybbp1a is indicated by green and red colour, respectively. AhR: aromatic hydrocarbon receptor; WSTF: Williams syndrome transcription factor; CSB: Cockayne syndrome protein B; NM1: nuclear myosin 1; ribProt: ribosomal proteins; Topo I: Topoisomerase I: P: phosphorylation mark. As the accomplishment of this thesis description was delayed with regard to the completion of laboratory work, the period of laboratory work is marked within the centre of the diagram (bold line) with respect to the publication years of Mybbp1a-related information.

2.6 Objectives

A mammalian genome comprises around 400 rRNA gene copies. On approximately half of these genes a transcriptional repressive environment is established and maintained during cell division. The remainder is actively transcribed but tightly regulated according to the metabolic state of the cell. The aim of this work was to gain more insights into the regulatory mechanisms, which take effect on rRNA gene transcription particularly on the level of transcription initiation with focus on chromatin-dependent processes.

First aim

Several recent studies suggest the Pol I complex being a so-called 'holoenzyme' bringing every activity needed for transcriptional initiation and regulation to the rDNA promoter by its own. It is known from the Pol II complex that several initiation factors and elongation factors are more or less stably associated with the enzyme (ref). The first aim of this thesis was to purify Pol I-associated factors with the help of a newly established mouse cell line stably expressing a tagged version of a Pol I subunit.

Second aim

The purification was expected to identify several proteins, which either have not been characterised in the context of rRNA gene transcription or found associated with the Pol I complex so far. Thus, the second aim of this work was to characterise these factors with respect to their function in rRNA gene transcription and/or associated processes thereby gaining further insights into the involved regulatory networks.

3 Materials and Methods

3.1 Material

3.1.1 Solutions, buffers and media

Stock solutions and buffers were made according to standard protocols (Ausubel 1989; Sambrook *et al.* 1989; Hoffmann-Rohrer & Labaere 2000; Sambrook & Rusell 2001). Protease Inhibitors (either Complete® EDTA-free (Roche) or a mix of Leupeptin, Pepstatin, Aprotinin (all 1 µg/ml) and PMSF (0.2 mM) as well as DTT (1 mM) were freshly added. The most common solutions are listed below.

EX-x buffers
20 mM Tris-HCl pH 7.6
1.5 mM MgCl2
0.5 mM EGTA
10% glycerol
x mM KCl

AM/A-x buffers (with/without $MgCl_2$)
20 mM Tris-HCl pH 7.9
5 mM $MgCl_2$
0.1 mM EDTA
10% or 20% glycerol
x mM NaCl

Phosphate Buffered Saline (PBS)
140 mM NaCl
2.7 mM KCl
8.1 mM Na_2HPO_4
1.5 mM KH_2PO_4
pH 7.4

TE buffer
10 mM Tris-HCl pH 7.6
1 mM EDTA

TBE buffer
90 mM Tris-HCl pH 7.6
90 mM boric acid
2 mM EDTA

TAE buffer
40 mM Tris-HCl pH 7.6
40 mM acetate
1 mM EDTA

DNA sample buffer (10x)
50% glycerol
50 mM Tris-HCl pH 7.6
10 mM EDTA
0.05% bromophenol blue
and xylene cyanol
or 0.05% Orange G

SDS protein sample (Laemmli) buffer (6x)
350 mM Tris-HCl pH 6.8
10% SDS
30% glycerol
5% β-Mercaptoethanol
0.2% bromphenol blue

Stacking gel buffer (4x)
0.5 M Tris-HCl, pH 6.8
0.4% SDS

Separating gel buffer (4x)
1.5 M Tris-HCl, pH 8.8
0.4% SDS

SDS-PAGE running buffer
192 mM glycine
25 mM Tris
0.1% (w/v) SDS

Flag peptide stock solution
5 mg/ml Flag peptide (Sigma)
10 mM Tris-HCl, pH 7.4
150 mM NaCl

MOPS buffer
40mM 3-(N-morpholino)propanesulfonic acid, pH 7
10 mM sodium acetate
1 mM EDTA

Polyethylenimine (PEI) stock solution
1 mg/ml PEI was dissolved in 150 mM NaCl solution and the pH was adjusted to 7.4 using HCl. The stock solution was stored in aliquots at -20 °C.

For maintenance of mammalian cell lines the following commercially available media and solutions were used: MEM (Earle's with Glutamax), Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids, sodium pyruvate, newborn/fetal calf serum, Trypsin/EDTA (all Invitrogen) and Penicillin/Streptomycin stock solution (Pen/Strep, 10000 U/ml penicillin, 10 mg/ml streptomycin, C. C. Pro)

Any additional buffers are described in the individual method sections.

3.1.2 Consumables and equipment

Unless otherwise stated, all common chemicals and materials were ordered by Amersham/Pharmacia (Freiburg), E. Merck (Darmstadt), NEN/Perkin Elmer (Rodgau), Pierce (Bonn), Promega (Mannheim), Roche (Mannheim), Roth (Karlsruhe), Serva (Heidelberg) and Sigma (München). Radioactive-labelled nucleotides were ordered from Amersham.

Enzymes	
restriction endonucleases	NEB, Fermentas, Promega, Roche
Klenow enzyme	NEB
Shrimp alkaline phosphatase	NEB
T4 polynucleotide kinase (PNK)	Promega
T4 DNA ligase	NEB
Taq DNA polymerase Expand	Promega
M-MLV reverse transcriptase	Invitrogen
RNase A	Roche
MNase (S7 Nuclease)	Roche
DNase I (RNase-free)	Roche
Chromatographic material	
M2-agarose freezer-safe (Flag-beads)	Sigma
Protein G sepharose 4FastFlow	NEB, Amersham
Sephadex G25 spin column	Roche
Blotting material	
Hybond N+ membrane	Amersham
Whatman 3MM paper	Whatman
Other	
Flag peptide	Sigma
Polyfect transfection reagent	Qiagen
Oligofectamine transfection reagent	Invitrogen
RNasin	Promega
random primer	Promega
Peqgold protein marker II, IV (prestained)	Peqlab
DNA marker 10kb ladder	NEB
dialysis membranes	Spectra Por
filtration units	Merck
Strata Clean Resin	Stratagene
Distamycin A hydrochloride (#D6135)	Sigma
Equipment	
Superose 6 HR 10/30 gelfiltration column	Pharmacia
Chromatography system (ÄKTA)	Pharmacia
Trans-Blot SD Semi-Dry Transfer Cell	Biorad
Optima-LE 80K ultracentrifuge	Beckman Coulter
Bioruptor sonicator	Diagenode
ABI PRISM 7000 Sequence detection system	Applied Biosystems
Nanodrop ND-1000	Peqlab
table centrifuge 5415R	Eppendorf

3.1.3 Antibodies

Antibody	Dilution Western Blot	Dilution Immunofluorescence	Immunoprecipitation (per 300 µl extract and 15 µl Prot G-Sepharose)	Reference
α-mouse-p160, rabbit, polyclonal	1:1000	1:200/400		(Tavner *et al.* 1998)
α-human-p160, rabbit, polyclonal	1:1000	1:200/400	12 µl	(Tavner *et al.* 1998; Hochstatter *et al.* unpublished)
α-RPA116, rabbit, polyclonal	1:1000	1:200/400		kindly provided by I Grummt (Seither & Grummt 1996)
α-PAF53, rabbit, polyclonal	1:1000	-		kindly provided by I Grummt (Seither *et al.* 1997)
α-TIF-IA, mouse, monoclonal	1:500	-		kindly provided by I Grummt (Bodem *et al.* 2000)
α-mTTF-I, C7, rabbit, polyclonal	1:2500	1:100		kindly provided by I Grummt (Evers *et al.* 1995)
α-hSnf2h, rabbit, polyclonal	1:2000	-		kindly provided by I Grummt (Santoro *et al.* 2002)
α-Pes1, rat, monoclonal	1:2000	-		kindly provided by D. Eick (Holzel *et al.* 2005)
α-Fibrillarin (P2G3), mouse, monoclonal	1:1000	1:400		(Christensen & Banker 1992)
α-EBP2, rabbit, polyclonal	1:1000	-	3 µl	kindly provided by L Frappier (Wu *et al.* 2000)
α-rpS2, rabbit, polyclonal	1:1000	-	3 µl	kindly provided by M. Bedford (Swiercz *et al.* 2005)
α-DDX21, rabbit, polyclonal	1:1000	-	30 µl	PTG
α-Nol1, rabbit, polyclonal	1:1000	-	30 µl	PTG
α-hnRNP A1 (4b10), mouse, monoclonal	1:1000		3 µl	kindly provided by G. Dreyfuss
α-Flag M2, mouse, monoclonal	1:1000	1:400		Sigma
α-Flag, rabbit, polyclonal	1:1000	1:200/400		Sigma
α-BrdU, mouse, monoclonal	-	1:200		Roche
α-rabbit IgG secondary antibody (HRP-conjugated)	1:10000	-		Amersham

Antibody	Dilution Western Blot	Dilution Immunofluorescence	Immunoprecipitation (per 300 µl extract and 15 µl Prot G-Sepharose)	Reference
α-mouse IgG secondary antibody (HRP-conjugated)	1:5000	-		Amersham
α-rat IgG secondary antibody (HRP-conjugated)	1:5000	-		Dianova
α-mouse IgG secondary antibody (Cy2/3-labeled)	-	1:1000/2000		Jackson
α-rabbit IgG secondary antibody (Cy3/2-labeled)	-	1:1000/2000		Jackson

3.1.4 Vectors

Vector	Description	Reference
pBS-RPA 116 6-2	Contains the cDNA of mouse RPA116.	(Seither & Grummt 1996)
pEGZ-fRPA116	Derived from the pEGZ/MCS vector (see Appendix 6.1 for vector map). Transcript encodes mouse full-length RPA116 with N-terminal Flag-tag.	See chapter 3.2.1
pact-Flag-hMybbp1a	Derived from the pact-c-myb vector (see Appendix 6.1 for vector map). Transcript encodes full-length human Mybbp1a.	(Keough *et al.* 1999; Hochstatter *et al.* unpublished)
pact-Flag-mMybbp1a	Derived from the pact-c-myb vector (see Appendix 6.1 for vector map). Transcript encodes full-length mouse Mybbp1a.	(Tavner *et al.* 1998; Keough *et al.* 2003)
pact Flag-p67*NLS	Derived from the pact-c-myb vector (see Appendix 6.1 for vector map). Transcript encodes the N-terminal 580 aa of mouse Mybbp1a fused to SV40NLS.	(Tavner *et al.* 1998; Keough *et al.* 2003)
pcDNA3.1-FLAG-Tip5	Derived from the pcDNA 3.1 vector (Invitrogen). Transcript encodes full-length mouse Tip5 with N-terminal Flag-tag	Kindly provided by I Grummt (Zhou *et al.* 2002)
pHrD-IRES	Derived from pGL3 Luciferase reporter vector encoding the firefly luciferase (Promega). Pol II-specific sequences were replaced by the human Pol I-specific promoter sequence from bp -410 to +314 (relative to the transcription start site) and an internal ribosome entry site (IRES).	Kindly provided by ST Jacobs (Ghoshal *et al.* 2004)
pRL-TK	HSV-thymidine kinase (TK) promoter. Transcript encodes the *Renilla* luciferase.	Promega
pMrWT-T	Artificial 'rDNA minigene'. It contains the mouse rDNA promoter sequences from -170 to +155 including the upstream terminator T_0 at position -170 and a 3.5 kb 3´-terminal rDNA fragment with terminator elements (T_1-T_8), spaced by 686 bp of plasmid sequences (pUC plasmid).	(Strohner *et al.* 2004)

3.1.5 Oligonucleotides

Name	Sequence	Description
Cloning of the Flag-tagged RPA116 -expression vector		
Flag-RPA116-fw	ATG GAT CCA TGG ACT ACA AGG ACG ACG ATG ACA AG*G ATG TCG ACG GCC GG*	fw-primer for Flag-tagging (bold); Bam HI restriction site 5' of start codon; RPA116 ORF (4-18 bp, italic)
Flag-RPA116-rev	GAG GAT CC*T CAG ATG ACA TCC AGT TTC ACT*	rev-primer with Bam HI restriction site 3' of stop codon; RPA116 ORF (1288-1308 bp, italic)
Oligos for quantitative real-time PCR		
Hs5-F	CCT GCT GTT CTC TCG CGC (+132/+149)	primer for TaqMan quantitative PCR PCR product: +132/+198 bp[1]
Hs5-R	GGT CAG AGA CCC GGA CCC (+198/+181)	
Hs5-Taqman	6FAM-AGC GTC CCG ACT CCC GGT GC (+155/+174)	
HsBac-F	TGC CGA CAG GAT GCA GAA G (+927/+945)	primer for TaqMan quantitative PCR PCR product: +927/+1026 bp[1]
HsBac-R	GCC GAT CCA CAC GGA GTA CTT (+1026/+1006)	
HsBac-Taqman	TCA AGA TCA TTG CTC CTC CTG AGC (+980/+1003)	
rDNAP-F	ATG GTG GCG TTT TTG GGG	primer for SYBR quantitative PCR; PCR product: -133/+116 relative to the transcription start site[1]
rDNAP-R	AGG CGG CTC AAG GCA GGA G	
IGS-F	CGC TGT CCA TCT CTG TCT TTC TAT G	primer for SYBR quantitative PCR; PCR product: +22730/+22906 relative to the transcription start site[1]
IGS-R	ATA CAC CGA GTG GGG AAG CC	

3.1.6 siRNA sequences

Name	Sequence	Target mRNA
Control (Ctrl)	5'-CUU ACG CUG AGU ACU UCG AdTdT	Firefly luciferase
Mybbp1a.1	5'-GCC GAC UUG AAU AUA AUA CdTdT	human Mybbp1a
Mybbp1a.2	5'-UGG AUC AUC UUU CGA UUG GdTdT	human Mybbp1a
Mybbp1a.3	5'-AUA CGC AAG CUG UUU CUA AdTdT	human Mybbp1a
Pes1	5'-AGG UCU UCC UGU CCA UCA AdTdT	human Pes1
TIF-IA	5'-CAA AGG ATC TAT ATC GCG AdTdT	human TIF-IA

3.1.7 Bacteria strains and cell lines

XL1-Blue (Stratagene) and DH5α (Invitrogen) *E. coli* strains were used for DNA plasmid amplifications. Media and plates for bacteria were prepared according to standard protocols (Sambrook and Russell, 2001).

[1] human ribosomal DNA complete repeating unit; GenBank: U13369.1; GI:555853

The following mammalian cell lines were used: the mouse lymphoblast cell line MB III (ATCC® Number: CCL-32™), the human epithelial carcinoma HeLa cell line and the human embryonic kidney (HEK) 293T cell line.

3.2 Methods

3.2.1 Generation of a mouse Flag-RPA116 expression construct

For creation of a mouse Flag-RPA116 expression construct the coding sequence of RPA116 was amplified from the vector pBS-RPA 116 6-2 (kind gift of I. Grummt; (14)) with the Expand High Fidelity PCR system (Roche) employing the primer pair flag-RPA116-fw and flag-RPA116-rev. This primer pair additionally introduced a Flag-tag coding sequence upstream of the RPA116 start codon as well as flanking BamHI restriction sites. The PCR product was ligated into the EcoRV blunt end restriction site of the vector pT7Blue3 (Novagen) and subsequently digested with BamHI. The products were separated by agarose gel electrophoresis and the DNA band representing the Flag-RPA116 construct was extracted from the agarose gel. The purified DNA was then ligated into the BamHI restriction site of pEGZ/MCS (plasmid map see Appendix 6.1). The proper direction of the inserted construct was verified by restriction digest analysis. The plasmid was sequenced and named pEGZ-fRPA116.

3.2.2 Cell culture and generation of a MB III cells stably expressing Flag-RPA116

The mouse lymphoblast cell line MB III (ATCC® Number: CCL-32™) was cultured in MEM (Earle's, with Glutamax) with non-essential amino acids (1%, v/v), sodium pyruvate (110 mg/l) and 10% heat-inactivated newborn calf serum. To create a MB III cell line stably expressing Flag-RPA116, cells were transfected with pEGZ-fRPA116, selected with Zeocin (Invitrogen), cloned by serial dilution and maintained in medium supplemented with 100 µg/ml Zeocin. HeLa and HEK293T cells were cultured in DMEM containing 10% heat-inactivated fetal calf serum and Penicillin/Streptomycin (10000 U/ml/10 mg/ml). The cell lines were cultured at 37 °C in 5% CO_2.

3.2.3 DNA transfection of mammalian cells

MBIII, HeLa and HEK 293T cells were transfected with DNA constructs either by using the Polyfect reagent or Polyethylenimine (PEI). Cells were seeded at least 14 h prior transfection and were approximately 60% confluent at the starting point. If not stated otherwise DNA amounts were chosen according to the manufacturer's instruction.

For transfection with Polyfect in the 6-well plate format $4x10^5$ cells were seeded per well and transfected with 1.5 µg of DNA. In brief, DNA was diluted with serum-free DMEM medium to a final volume of 100 µl, 12 µl Polyfect reagent was added, mixed and incubated for 10 min at RT. Then 600 µl DMEM supplemented with 10% FCS/Pen/Strep were added, mixed and dispersed on one well, which contained 1.5 ml fresh DMEM/10% FCS/Pen/Strep. For transfection with the PEI protocol cells were accordingly transfected with 1.5 µg of DNA per

well (6-well plate format). DNA was diluted in serum-free DMEM to a final volume of 200 µl, 4.5 µl of PEI stock solution were added and incubated at RT for 10 min. The mixture was then dispersed on one well, which contained 1.8 ml fresh DMEM/10% FCS/Pen/Strep. Cells were split 1:2 after 24 h and harvested at different time points for protein, DNA and/or RNA extraction.

3.2.4 Extract preparation

3.2.4.1 Nuclear extract from MB III and HEK293T cells

Nuclear extracts were prepared from exponentially growing MB III control cells or MB III cells stably expressing Flag-RPA116 (pEGZ-fRPA116) as well as from HEK 293T cells 48 h after transfection with pact-Flag-hMybbp1a or vector control. Cells were harvested, washed with PBS and resuspended in three packed cell volumes (PCV) of buffer A (20 mM, HEPES pH 7.9; 0.2% NP-40; 10 mM KCl; 1 mM EDTA; 10% Glycerol; 1mM DTT; protease inhibitors) and incubated for 10 min on ice. Cell lysis was monitored by microscope. Nuclei were washed in Buffer A, resuspended in three PCV of Buffer B (420 mM NaCl; 20 mM; HEPES pH 7.9; 10 mM KCl; 1 mM EDTA; protease inhibitors) containing 2% (v/v) Distamycin A hydrochloride (only for the extract preparation from MBIII cells) and incubated on a rotating wheel for 40 min at 4°C. After centrifugation with 17000g for 10 min the nuclear fraction was collected and dialysed against AM100 (100 mM NaCl; 20 mM Tris HCl, pH 7.9; 5 mM $MgCl_2$; 0.1 mM EDTA; 20% Glycerol; 1mM DTT; protease inhibitors).

3.2.4.2 Whole Cell extract from MBIII cells

The whole cell extract was prepared according to the Manley protocol (10). Cells were harvested by centrifugation for 5 min with 210 g, washed with 1x PBS/5 mM $MgCl_2$, resuspended in four PVC of buffer Homog I (10 mM Tris/HCl pH 7.9, 1 mM EDTA, 25 % Saccharose, 50 % Glycerol, 5 mM DTT, PMSF) and incubated for 20 min on ice. All following reactions were performed on ice or at 4°C. Cells were lysed by approximately eight strokes with a dounce homogenizer with pestle “B”, efficient lysis was verified in the microscope and four PCV of buffer Homog II (50 mM Tris/HCl pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 20% Glycerol, 2 mM DTT, PMSF) were added slowly while stirring on a magnetic stirrer. Then one PCV of saturated $(NH_4)_2SO_4$ solution was slowly dropped in the solution, incubated for 30 min with gentle stirring and centrifuged for 3 h at 50000 rpm in a Ti 70 rotor in a Optima LE-80K ultracentrifuge (Beckman). The supernatant was carefully removed, slowly mixed with 0.33 g solid, pounded $(NH_4)_2SO_4$ per ml supernatant. After adding 10 µl 1 M NaOH per g $(NH_4)_2SO_4$ the solution was stirred for 30 min and centrifuged for 20 min at 10000 rpm in a SW34 rotor in a Sorvall RC5C centrifuge. The pellet was then resuspended in 1/10 volume of the supernatant AM100 (supplemented with PMSF, 1 mM DTT), dialysed once for 2 h and

subsequently overnight against AM100 (PMSF, 1 mM DTT). After centrifugation for 10 min at 17000 g the extract was aliquoted, shock frozen in liquid N_2 and stored at -80°C.

3.2.4.3 Nuclear extract from HeLa cells

HeLa nuclear extracts were prepared 48 h after transfection with pact-Flag-hMybbp1a or vector control. Cells were grown on 15 cm diameter (∅) dishes to approximately 80% density, washed with PBS and either harvested in 3 ml PBS by scraping or detached by Trypsin-EDTA and centrifuged at 210 g at 4°C. All subsequent steps were conducted on ice and with solutions and equipment cooled to 4°C. Cells were resuspended in PBS, counted, pelleted at 2500 g for 6 min, resuspended in five PCV of hypotonic Buffer C (10 mM KCl; 10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 1 mM DTT; protease inhibitors). After incubation for 15 min on ice the samples were centrifuged at 2.000 g for 8 min and the cell pellet was resuspended in two PCV of Buffer C. The cells were lysed with the help of a dounce homogenizer (pestle B) and cell lysis was followed by light microscopy. After two centrifugation steps at 1.000 g and 4.000 g, each for 10 min, the pellet was resuspended in 3 ml of Buffer D (20 mM HEPES, pH 7.9; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 20% (v/v) Glycerol; 1 mM DTT; protease inhibitors) per 10^9 cells. After incubation for 30 min at 4°C on a rotating wheel the extract was sonicated 2x 20 sec in a volume of 200 µl (alternatively 3x 20 sec in a volume of 250 µl) with the Bioruptor (H, Diagenode) and centrifuged at 17000 g for 15 min. After centrifugation the nuclear fraction was collected and dialysed against Buffer D containing 300 mM NaCl.

3.2.5 Immunopurification of proteins

For immunoprecipitation experiments extracts were incubated with pre-equilibrated anti-Flag M2 agarose in the presence of 0.02% NP-40 or 0.25% Triton X-100, protease inhibitors and 1 mM DTT. After three washing steps (15 min at 4 °C on a rotating wheel) in the respective extract buffer proteins were eluted with 0.25 mg/ml Flag-peptide. Flag-RPA116-containing complexes were additionally washed with AM300 buffer [300 mM NaCl] before elution. If needed proteins were concentrated with Strata Clean resin (Stratagene) for 15 min at RT on a rotating wheel or by TCA-precipitation. Proteins were separated by SDS-PAGE and analysed by Western blotting. For the identification of novel associated proteins gels were silver- or Coomassie-stained (see chapter 3.2.19.3 and 3.2.19.4), protein bands were cut out and subjected to MALDI-TOF mass spectrometry (Zentrallabor für Proteinanalytik, Adolf-Butenandt-Institut, LMU, Germany).

3.2.6 Co-immunoprecipitation of Flag-Mybbp1a-associated factors

Antibodies directed against human Mybbp1a, Nol1, DDX21, EBP2, ribosomal protein S2 and hnRNPA1 (respective volumes see chapter 3.1.3) were incubated with 300 µl HeLa nuclear

extract supplemented with protease inhibitors for 5 h at 4° C on a rotating wheel. Then 15 µl of Protein-Sepharose beads (1:1 slurry, Amersham) and 0.025% (v/v) Triton X-100 were added and the samples were further incubated for 1.5 h. Subsequently the beads were washed 3x in washing buffer (20 mM HEPES, pH 7.9; 300 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 20% (v/v) Glycerol; 1 mM DTT; protease inhibitors; 0.025 Triton X-100) for 10 min, resuspended in 15 µl SDS-PAGE sample buffer and analysed by SDS-PAGE and Western Blotting. An equal amount of rabbit IgG was treated in parallel as control.

3.2.7 Immunofluorescent staining and image capture

Cells were grown on coverslips or in Labtech chambers overnight and fixed in ice-cold methanol/acetone (1:1, v/v) for 1 min. Alternatively, cells were fixed in PBS/2% (w/v) paraformaldehyde for 15 min on ice and permeabilized in PBS/0.25% Triton X-100/1% (w/v) paraformaldehyde for 10 min on ice. Cells were washed with PBS/0.01% Tween (PBS-T) and unspecific binding was blocked with PBS-T containing 2% BSA and 5% goat serum for 1 h. Primary antibodies (dilutions see chapter 3.1.3) were incubated in blocking solution at RT for 1 h or at 4° C in a humidified chamber over night. Cells were washed with PBS-T and incubated with Cy2- or Cy3-labeled secondary antibodies in blocking solution at RT for 1 h. DNA was counterstained with Hoechst 33342 stain (Sigma) diluted in PBS (1:10000) and mounted in Vectashield (Axxora). Fluorescence images were acquired with a Zeiss Axiovert 200 inverse microscope.

3.2.7.1 RNA-dependent-localisation of proteins

To assess RNA-dependent localisation of proteins by immunofluorescence HeLa cells were washed with PBS and incubated with PBS containing 0.1% Triton X-100 for 10 min and subsequently incubated with 1 mg/ml RNase A (Sigma) in PBS for 20 min at RT prior fixation. Control cells were incubated with either 3 µl/ml RNase-free DNase I (Roche) and 3 mM $MgCl_2$ or both, RNase A and DNase I. Cells were washed with PBS, fixed and prepared for immunofluorescence microscopy as described above.

3.2.7.2 BrdU labeling of newly synthesised DNA

Prior immunofluorescent labeling HeLa cells were incubated in DMEM containing 10 µM BrdU for 30 min at 37°C, washed with PBS-T and fixed with PBS/4% formaldehyde for 10 min at RT. Cells were washed, permeabilized with PBS/0.2% Triton X-100 and incubated with PBS/2N HCl for 10 min. After two washing steps with PBS cells were analysed for the presence of BrdU incorporation by immunostaining with antibodies recognising BrdU.

3.2.8 siRNA-mediated protein depletion

The day before transfection $2x10^5$ Hela cells were seeded per well (6 well plate format). 20 µM siRNA was transfected using the Oligofectamine reagent (Invitrogen) according to the

manufacturer's instruction. Before transfection cells were washed 2x with DMEM supplemented with 10% FCS and finally 0.8 ml were added. Per reaction the following solutions were prepared:

Solution A:		**Solution B:**	
Oligofectamine	4 µl	siRNA [20µM]	10 µl
OptiMEM	11 µl	OptiMEM	175 µl

Solution A and B were mixed, incubated for 15 min at RT and added to the cells. Protein and RNA were extracted at different time points.

3.2.9 Analysis of the rRNA precursor and β-actin mRNA by quantitative real-time PCR

3.2.9.1 Cellular RNA extraction

RNA was extracted from HeLa cells with the QiaShredder and RNeasy kit either with or without additional on-column DNase I treatment (both Qiagen) according to the manufacturer's instruction. RNA concentration was measured either with the Genequant II (Pharmacia Biotech) or the Nanodrop ND-1000 (Peqlab).

3.2.9.2 Reverse transcription of cellular RNA

125 to 1000 ng of cellular RNA was used to produce cDNA by reverse transcription. The 20 µl reaction contained 2 µl of random primer (500 µg/ml, Promega), 2 µl of dNTPs (2.5 mM each, Bioline) and sterile water as added to a total volume of 12 µl. RNA was added and incubated for 5 min at 65°C. After chilling on ice the reaction was supplemented with 5x First-Strand-Buffer (Invitrogen), 2 µl 0.1 M RNase-free DTT (Invitrogen) and 1 µl RNasin (Promega) and incubated for 2 min at 37°C. 200 U of M-MLV Reverse Transcriptase (Invitrogen) were added and the reactions were incubated for 1 h at 37°C and subsequently heat-inactivated for 15 min at 70°C. A control reaction was performed in absence of reverse transcriptase. The linearity of the reaction was analysed by titration of RNA input for each primer pair used in subsequent real-time PCR quantification.

3.2.9.3 Quantitative PCR

Quantitative PCR was carried out using the ABI PRISM 7000 Sequence detection system (Applied Biosystems). 0.5 – 1.5 µl of the reverse transcription reaction were used as template for the PCR reaction. Taqman 2xPCR Master Mix (Applied Biosystems) was used according to the manufacturer's instructions. To detect the 47S rRNA precursor oligos recognising its 5'ETS (external transcribed spacer) were used (oligos: Hs5-F/-R/-Taqman). A β-actin-mRNA amplicon was used for normalisation (oligos: HsBac-F/-R/-Taqman). For both

amplicons the recommended annealing temperature of 60°C was applied and the following PCR cycles were used

1x 2 min, 50°C
1x 10 min, 95°C
40x 15 sec, 95°C and 1 min, 60°C.

3.2.10 Metabolic labeling of nascent RNA in HeLa cells

HeLa cells were transfected with the respective siRNAs in the 6-well plate format as described above (chapter 3.2.8). Metabolic labeling was essentially performed as described previously (8). Four days after siRNA transfection the cells were incubated in phosphate-free DMEM supplemented with 10% FBS (Sigma) for 30 min and then supplied with 15mCi/ml ^{32}P-orthophosphate for 6 h. Total RNA was isolated using the RNeasy Mini kit (Qiagen). 1.5 µg of metabolically labeled total RNA was separated on a 15 cm long agarose gel (1% agarose; 5.5% formaldehyde; 1xMOPS, pH 7) with 110 V at RT. The gel was dried on Whatman paper using a regular gel dryer (Bio-Rad) for 2–3 h at 80 °C. Metabolically labeled RNA was visualised and quantified by autoradiography on a PhosphoImager (FLA 3000, Fujifilm). In parallel efficiency of protein depletion was monitored by Western blotting.

3.2.11 Proliferation assay

To monitor proliferation rates HeLa cells were detached with Trypsin-EDTA and diluted in PBS. Subsequently cells were stained with trypan blue and living cells were counted in a Neubauer hemocytometer in triplets at the indicated time points.

3.2.12 Chromatin immunoprecipitation (ChIP)

3.2.12.1 Preparation of nucleolar chromatin

Formaldehyde cross-linked nucleolar chromatin was prepared as described previously (O'Sullivan *et al.* 2002). Briefly, exponentially growing HeLa cells were cross-linked by formaldehyde (0.25%, 10 min, RT). After quenching with 125 mM glycine cells were harvested by being scraped. After centrifugation the cell pellet was resuspended in 0.5 ml of high-magnesium buffer (10 mM HEPES [pH 7.5], 0.88 M sucrose, 12 mM $MgCl_2$, and 1 mM DTT, protease inhibitors) and nucleoli were released by sonication on ice (two of 10 s each at full power) using a Bioruptor device. The release of nucleoli was monitored by microscope. Nucleoli were pelleted by centrifugation in a microfuge (15000 x *g* for 20 sec) and the pellet was resuspended in 0.5 ml of low-magnesium buffer (10 mM HEPES [pH 7.5], 0.88 M sucrose, 1 mM $MgCl_2$, and 1 mM DTT, protease inhibitors). Nucleoli were subject to further sonication on ice (10 s, microtip, amplitude 40%, Branson: digital sonifier 250D) and pelleted as before. Nucleoli were resuspended in 0.1 ml of 2x TE (20 mM Tris [pH 8.0], 2 mM EDTA) and 10 µl of 20% SDS was added. After incubation for 15 min at 37°C, disappearance of nucleolar structures were verified by microscopy. 0.4 ml 2x TE was added and the solution

was sonicated (three bursts of 30 s at full power, Bioruptor). The resulting sheared nucleolar chromatin was centrifuged in a microfuge (15000 x *g* for 1 min), and the supernatant was used immediately in nucleolar ChIP assays. To verify the size range of sonicated chromatin an aliquot of the supernatant was digested with RNase A followed by proteinase K digestion and formaldehyde cross-links were reversed by incubation at 65°C overnight. DNA was precipitated and fragment size was determined around 500–1000 bp as analysed by agarose gel electrophoresis. Per reaction 100 µl of nucleolar chromatin supernatant was combined with 1.4 ml PBS containing 0.1% BSA, 0.01% Triton X-100 and protease inhibitors and subjected to chromatin immunoprecipitation.

3.2.12.2 Preparation of chromatin from whole cell extract

Formaldehyde cross-linked whole nuclear chromatin was prepared as described previously (Peters *et al.* 2003; Martens *et al.* 2005). Exponentially growing HeLa cells were cross-linked by formaldehyde (0.5%, 10 min, RT). After quenching with glycine in a final concentration of 125 mM cells were washed with PBS, 750 µl lysis buffer (1% SDS, 10mM EDTA, pH 8.0, 50mM Tris-HCl, pH 8.0, protease inhibitors) was added to each 15 cm dish and incubated for 5 min at 4°C. Cells were harvested by scraping and sonication (3x15 sec, microtip, amplitude 45%, Branson: digital sonifier 250D) was used to disrupt cellular membranes and fragment chromatin. Sufficient fragmentation of chromatin was monitored as described above. Per reaction 420 µl of supernatant was centrifuged in a microfuge (15000 x *g*, 5 min), 400 µl of supernatant was combined with 3.6 ml of a mixture of dilution buffer (1% Triton X-100, 150mM NaCl, 2mM EDTA, pH 8.0, 20mM Tris-HCl, pH 8.0, protease inhibitors) and lysis buffer (9:1) in a 15 ml Falcon tube and subjected to chromatin immunoprecipitation.

3.2.12.3 Immunoprecipitation of chromatin

Supernatant prepared as above was incubated with 5 µg of the indicated antibodies (polyclonal rabbit α-RPA116, α-TTF-I (C7), α-hMybbp1a, α-Flag, normal rabbit IgG) on a rotating device at 4°C overnight. Simultaneously, per reaction 50 µl (or 100 µl) of a 50% slurry of protein G-Sepharose beads (Amersham) were pre-equilibrated with BSA (100µg/ml) and yeast tRNA (100 µg/ml). After incubation of the beads with the immune complexes on a rotating device for 1 h at 4°C, beads were recovered by gentle centrifugation (600 x *g* for 15 sec) and washed as follows (nucleolar chromatin): twice with 2xTE containing 0.2% SDS, 0.5% Triton X-100 and 150 mM NaCl; twice with 2xTE, 0.2% SDS, 0.5% Triton X-100, and 500 mM NaCl; and twice with 2xTE. Immunoprecipitated material was eluted twice from the beads with 50 µl of 2xTE containing 2% SDS at 37°C for 10 min. Eluates were pooled and subjected to RNase and proteinase digestion. Formaldehyde cross-links were reversed by incubation at 65°C overnight, DNA was precipitated and resuspended in 100 µl 1xTE. Bead-immunoprecipitates derived from nuclear chromatin were washed 3x with washing buffer (1%

Triton X-100, 0.1% SDS, 150mM NaCl, 2mM EDTA, pH 8.0, 20mM Tris-HCl, pH 8.0, with protease inhibitors) and 1x with final washing buffer (1% Triton X-100, 0.1% SDS, 500mM NaCl, 2mM EDTA, pH 8.0, 20mM Tris-HCl, pH 8.0, protease inhibitors). Immunoprecipitated material was eluted with 450 µl elution buffer (1% SDS, 100 mM $NaHCO_3$) and DNA was prepared as described above.

3.2.12.4 Quantitative PCR

Purified DNA derived from the chromatin immunoprecipitates and chromatin input were analysed by quantitative real-time PCR using the SYBR Green (Applied Biosystems) quantification method according to the manufacturer's instruction on an ABI PRISM 7000 Sequence detection system (Applied Biosystems). Results were calculated relative to standard curve values, corrected for non-specific binding (normal rabbit IgG ChIP) and presented as percentage of input DNA. The primer pairs rDNAP-F/-R and IGS-F/-R were used to detect rDNA promoter (product -133 to +116 relative to the transcription start site) and intergenic spacer (IGS, product +22730 to +22906 relative to the transcription start site) sequences, respectively.

3.2.13 Extract fractionation by density gradient centrifugation

A 10% - 45% (v/v) glycerol gradient was prepared in AM100 by using a Gradient Master 105/106 (BioComp) set at 2:05 min/81.8° angle/speed 15. 500 µl of MB III whole cell extract was applied and centrifuged with 35 K (Sw 41 Ti rotor) in an Optima-LE 80K ultracentrifuge (Beckman Coulter) at 4°C for 12 h (no break). In parallel, Thyroglobulin (Pharmacia) was used as a molecular weight marker. Subsequently 500 µl fractions were collected and 15 µl of each fraction were separated on a 7% SDS-PAA gel and analysed by Western blotting or Commassie staining.

3.2.14 Extract fractionation by gel filtration chromatography

200 µl HeLa nuclear extract was either incubated with 10 µg/ml RNase A (Sigma) or its solvent for 2 h at 4 °C prior to Superose 6 HR 10/30 (Pharmacia) fractionation on a Äkta chromatography system. 500 µl fractions were collected and proteins were concentrated by incubating each fraction with 5 µl of Strata Clean resin (Stratagene) with gentle rotation for 15 min at RT. After centrifugation the resin was resuspended in 2x Laemmli-buffer, proteins were separated by SDS-PAGE and analysed by Western blotting. The calibration of the column with the marker proteins Thyroglobulin (669 kDa), Apoferritin (443 kDa), BSA (67 kDa) (all Pharmacia) was used for size estimation.

3.2.15 RNA Pol I transcription reporter assay

HeLa cells were transfected with Polyfect reagent (Qiagen) in the 6-well plate format as described above (chapter 3.2.3) with the indicated amounts of expression construct together

with empty vector DNA to adjust DNA concentration and the pHrD-IRES reporter construct (kind gift of ST. Jacob, (6)) as a reporter for RNA Pol I transcription activity (each reaction was done in duplet). The plasmid pRL-TK carrying the renilla luciferase gene under the control of a HSV-thymidine kinase (TK) promoter (transcribed by RNA polymerase II) was co-transfected in each experiment to normalize for differences in transfection efficiency. 48 h after transfection (if not indicated otherwise), luciferase activity was measured using the Dual Luciferase Assay kit (Promega) according to the manufacturer's instructions. Cells were washed in PBS, harvested in 500 µl PBS by scraping and centrifuged for 5 min with 3000 rpm in a table centrifuge. Cells were incubated in 100 µl 1x lysis buffer for 15 min on ice, vortexed and centrifuged with 13000 rpm in a table centrifuge for 5 min. 20 µl of the supernatant were supplemented with 100 µl luciferase substrate (firefly) and subsequently 100 µl Stop and Glo buffer (renilla substrate) and luminiscence was measured after each additiion by a luminometer. The firefly luciferase counts derived from the RNA Pol I reporter construct were divided by the renilla luciferase counts and compared to control transfections with empty vector. Protein levels were monitored by Western blotting.

3.2.16 *In vitro* Pol I transcription assay

The pMrWT-T construct (rDNA minigene), containing a fusion of the rDNA promoter (-170 - +155 including the upstream terminator/TTF-I binding site T_0) and terminator region (3.5 kb 3'-terminal rDNA fragment including the terminator sites T_1 – T_{10}) served as template for the transcription reaction (a schematic is depicted in Figure 10). A typical transcription reaction was prepared with a final volume of 25 µl containing transcription buffer (20 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.5 mM DTT, 5 mM $MgCl_2$, 80 mM KCl, 10 mM creatine phosphate, 10% glycerol, 0.66 mM of each nucleotide ATP, UTP and GTP, 12.5 µM CTP and 1.5 µCi of α-^{32}P CTP) and either 10 ng of naked DNA or 40 ng chromatinised template. To start the transcription reaction 1 or 2 µl of MB III whole cell extract were added as source for transcription factors in parallel with recombinant TTF-I Δ185 (see text chapter 3.1.2.1; kindly provided by R. Strohner) or its solvent. The samples were incubated for 60 min at 30 °C and the reactions were stopped by addition of 25 µl stop buffer (10 µg glycogen, 2% SDS, 10 µg proteinase K, 100 mM EDTA) and further incubated for 1 h at 40 °C. For transcript precipitation 25 µl 7.5 M NH_4Ac and 200 µl pure ethanol were added, incubated for 15 min on ice and centrifuged with 13000 rpm (table centrifuge) for 15 min at 4 °C. After washing once in 70% ethanol the pellet was dissolved in 9 µl 80% formamide containing bromphenol blue and shaked for 5 min at RT. Transcripts were separated on a 4.5% polyacrylamide gel in 0.4x TBE at 250 V for approximately 1 h. The gel was dried on a Whatman paper for 2 h at 80 °C on a gel dryer (BioRad) and documented by autoradiography on a phosphoimager or x-ray film (Fuji).

3.2.17 Nucleosome mobility assay

The nucleosome mobility assay allows the visualisation of nucleosome movements on a short DNA fragment catalysed by ATP-dependent remodelling enzymes. The reactions were performed in EX-75 buffer with a final volume of 12 µl containing 30 to 60 fmol of mononucleosomes end-positioned on a radiolabeled 247 bp DNA fragment (kindly provided by R. Strohner and G. Längst), 1mM ATP, 1mM DTT and 200 ng/µl CEA using siliconised tubes (Biozym). The nucleosomes were incubated with different dilutions of protein fractions immunoprecipitated with α-Flag-agarose either from MB III or Flag-RPA116-MB III whole cell extracts (chapter 3.2.4.2 and 3.2.5) for 90 min at 30 °C in absence or presence of ATP. Different dilutions of the remodelling factor ACF were used as positive control. The reactions were stopped by the addition of 0.4 µg plasmid competitor DNA and further incubated for 5 min at RT. Resulting nucleosome positions were separated by native gel electrophoresis with 4.5% polyacrylamide gels in 0.4x TBE, which were pre-electrophoresed for 1 hour. After electrophoresis for approximately 2 hours at 100-130 V the gels were dried on Whatman paper with help of a gel dryer and analysed by autoradiography.

3.2.18 Standard protocols in molecular biology

Preparation of competent bacteria, transformation of bacteria, DNA purification, concentration determination of DNA, restriction enzyme analysis, polymerase chain reaction (PCR), ligation of DNA fragments, agarose gel electrophoresis etc. were performed essentially as described in (7, 12). Furthermore plasmid DNA was extracted from bacteria with help of Plasmid Purification kits, DNA fragments were isolated from agarose gels with the Gel Extraction kit and RNA was isolated from mammalian cells with the QiaShredder and RNeasy Mini kit (all Qiagen).

3.2.19 Standard protocols in protein analytics

Protein analysis was performed according to standard protocols (7, 12). In general, proteins were kept on ice (4°C) and in buffers containing protease inhibitors and reducing agents.

3.2.19.1 Determination of protein concentrations

Protein concentration was determined with the colorimetric assay described by Bradford (Bradford 1976).

3.2.19.2 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was employed for the separation of denatured proteins and was performed with the Novex system (pre-assembled gel cassettes). Resolving and stacking gels were prepared according to standard protocols using ready-to-use polyacrylamide solution (Rotigel, 30%, Roth). For electrophoresis, protein

samples were mixed with SDS-PAGE sample buffer, denatured for 5 min at 95°C and directly loaded on the gel. Proteins were separated at 100 to 150 V. In parallel the molecular weight of proteins was estimated with the help of non- or pre-stained marker proteins (peq-gold II and IV, Peqlab). Following electrophoresis the gels were either Coomassie- or Silver-stained or subjected to Western blotting.

3.2.19.3 Coomassie blue staining of protein gels

Polyacrylamide gels were fixed for 30 min in fixing solution (50% methanol, 10% acetic acid) and stained for at least 60 min on a slowly rocking platform with Coomassie staining solution (0.025% (w/v) Coomassie Blue R in 10% (v/v) acetic acid). To visualize proteins, gels were de-stained in 10% (v/v) acetic acid. After documentation, the gels were dried on a Whatman paper for 2 h at 80 °C on a gel dryer (BioRad). If gels were prepared for subsequent mass spectrometry analysis gels were stained with colloidal Coomassie (Colloidal Blue Stain, Invitrogen) according to the manufacturer's instruction, protein bands were cut out and handed over to the analysing facility (Zentrallabor für Proteinanalytik, Adolf-Butenandt-Institut, Munich).

3.2.19.4 Silver staining of protein gels

The staining of proteins with silver nitrate solution was done essentially according to the protocol of Blum (2). The gel was fixed in 40% (v/v)ethanol/10% (v/v) acetic acid for at least 2 h and washed 3x for 20 min in 30% (v/v) ethanol. After incubation in 0.02% (w/v) sodium thiosulfate ($Na_2S_2O_3$) for 1 min the gel was washed 3x with water for 20 sec and stained with 0.2% (w/v) $AgNO_3$ solution for 1 h. Subsequently the gel was washed with water (3x 20 sec) and incubated with developing solution (3% (w/v) Na_2CO_3, 0.05% (v/v) H_2CO, 0.0004% (w/v) $Na_2S_2O_3$) until the protein bands were visible (approximately 5 to 10 min). After a short wash in water the reaction was stopped by incubating the gel in 0.5% (w/v) glycine solution for at least 5 min. Finally, gels were documented and dried on a Whatman paper for 2 h at 80 °C on a gel dryer (BioRad).

3.2.19.5 Semi dry Western analysis

Proteins were separated by SDS-PAGE and transferred to a nitrocellulose or PVDF membrane (Amersham) at 14 V for 1 h by using the Trans-Blot SD chamber (Bio-Rad). For protein transfer the gel was packed between gel-sized Whatman papers either soaked in anode or cathode buffers.

Anode buffer I	**Anode buffer II**	**Cathode buffer**
300 mM Tris	25 mM Tris	70 mM CAPS, pH 10.5
15% methanol	15% methanol	15% methanol

After transfer membranes were incubated in blocking solution (PBS, 5% dry milk, 0.1% Tween) for 1 h at RT to reduce unspecific binding and subsequently with the primary

antibody diluted in blocking buffer for 1 h at RT or overnight at 4 °C (dilutions are summarised in chapter 3.1.3). Membranes were washed 3x in PBS supplemented with 0.1% Tween (PBS-T) for 10 min and incubated with the respective secondary antibody (horseradish peroxidase-coupled) in blocking solution for 1 h at RT. After three further washes for 10 min in PBS-T the membranes were analysed by using the ECL reagent (Amersham) and exposure to x-ray film (Fuji).

4 Results

4.1 Purification of RNA Polymerase I (Pol I)-associated factors

4.1.1 Establishment and characterisation of a MBIII cell line stably expressing Flag-tagged RPA116

The expression of the rRNA genes is regulated at different levels of the rRNA metabolic pathway, at the stage of transcription initiation (reviewed in (Grummt 2003)), transcription elongation (Schneider *et al.* 2006; Stefanovsky *et al.* 2006a), rRNA processing and assembly (Milkereit *et al.* 2001; Ferreira-Cerca *et al.* 2007; Holmstrom *et al.* 2008). To reveal potential regulators that act at the level of transcription initiation and elongation, a murine MB III cell line stably expressing Flag-tagged RPA116 (Flag-RPA116-MB III), the second-largest subunit of RNA Pol I (Seither & Grummt 1996), was established for immunopurification of Pol I-associated factors. Therefore a Flag-RPA116 expression construct was generated, transfected into MB III cells and Flag-RPA116-MB III clones were obtained. The cloning strategy and establishment of the stable cell line is described in detail in Material and Methods, 3.2.1). Subsequent characterisation by immunofluorescence microscopy showed constitutive expression at low levels of the N-terminally tagged protein and its nucleolar localisation similarly to the endogenous protein as revealed by immunostaining with α-RPA116 and α-Flag (Figure 9A and B). Furthermore Flag-RPA116 was fully incorporated into the RNA Pol I complex as shown by co-immunoprecipitation analysis and glycerol gradient centrifugation. Flag-immunoprecipitation co-purified PAF53, a bona-fide subunit of the RNA Pol I complex, from Flag-RPA116-MB III but not from MB III nuclear extracts (Figure 9C, lane 3). Moreover, when Flag-RPA116-MB III whole cell extract was analysed by glycerol gradient (10 - 50%) centrifugation endogenous and Flag-tagged RPA116 eluted in the same fractions and no Flag-RPA116 was detected in additional fractions as confirmed by Western blotting with α-RPA116 and α-Flag (Figure 1D).

4.1.2 Purification of Flag-RPA116-containing complexes

4.1.2.1 Chromatin-specific activities in extracts and purifications

For the purification of regulators of Pol I-mediated rDNA transcription a major focus was laid on the identification of chromatin-modulating factors, which modify the chromatin environment suchlike to enable transcription initiation and elongation. Therefore different extract preparations to obtain whole cell or nuclear fractions were additionally characterised with respect to chromatin-specific activities to select the most appropriate protocol for their purification. Firstly, extracts were tested in a Pol I *in vitro* transcription system (Langst *et al.* 1998; Strohner *et al.* 2004) to investigate their transcriptional activity from different templates

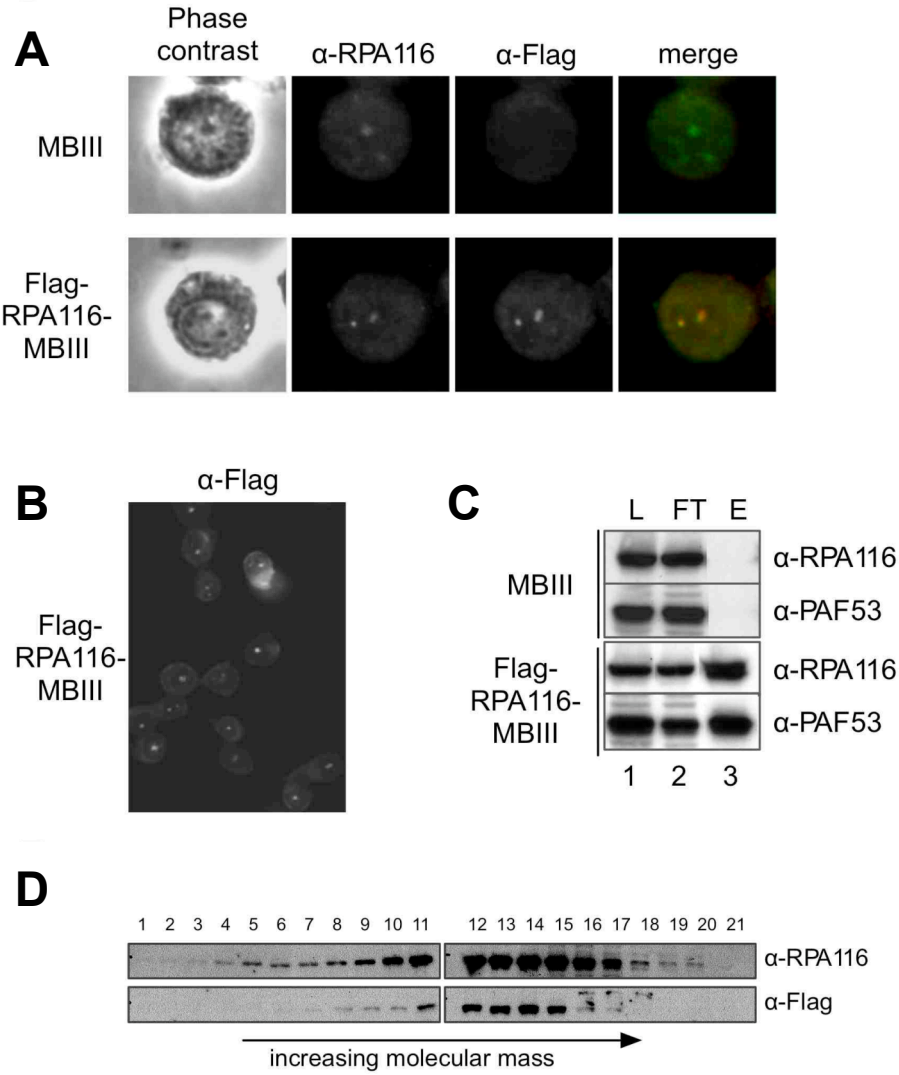


Figure 9: Characterisation of the MB III cell line stably expressing Flag-RPA116.
(A) Co-immunostaining of control MB III cells and MB III cells stably expressing Flag-RPA116 (Flag-RPA116-MB III) with α-RPA116 (green) and α-Flag (red) antibodies to detect the endogenous and tagged RPA116 proteins. (B) Immunostaining of a broader section of cells as shown in (A) with α-Flag. (C) Western Blot of immunoprecipitations from nuclear extracts of control MB III and Flag-RPA116-MB III cells with α-Flag agarose. Load (L; 6%), flow-through (FT; 6%) and eluted proteins (E) were analysed with the indicated antibodies. (D) Western Blot of glycerol gradient fractions. Whole cell extract was separated by glycerol gradient (10 – 50%) centrifugation and fractions were analysed with the indicated antibodies.

such as 'naked' DNA or DNA reconstituted into chromatin. For this purpose the pMrWT-T construct containing a rDNA minigene with promoter and terminator elements including the Transcription Termination Factor I (TTF-I) binding sites T_0 upstream of the transcription start site (+1) and T_1 to T_{10} downstream of the rDNA minigene was used as template (upper cartoon; Figure 10A). The DNA was packed into nucleosomes by the salt assembly method and the quality of the assembly reaction was monitored by MNase digestion (for details see Material and Methods, 3.2.16). TTF-I is required for the termination of transcription from both the DNA and chromatin templates as well as for transcription initiation from the chromatin template. For efficient initiation and specific termination a N-terminally truncated form of TTF-

I lacking the N-terminal amino acids 1-185 (TTF-IΔN185) was additionally supplemented to the *in vitro* system. The lower cartoon in Figure 10A gives an overview on the expected transcripts from chromatin and DNA templates in presence or absence of TTF-IΔN185 (marked in black). While transcription is inhibited from the chromatin template in absence of TTF-IΔ185 a read-through transcript is made from the DNA template. On the other hand if TTF-IΔN185 is present a specifically terminated transcript is synthesised from both templates. For the *in vitro* transcription reaction the two different templates were incubated with extracts in absence or presence of TTF-IΔN185 and the radioactively labelled transcripts were subsequently separated by gel electrophoresis and analysed by autoradiography (for detail see Material and Methods, 3.2.16). Figure 10B depicts the resulting transcripts when the MB III whole cell extract was used as a source for Pol I and the other required factors. As expected long, heterogeneous read-through transcripts were synthesized from the naked DNA template (lane 1, 2), but transcription was suppressed on the chromatin template (lane 5, 6). Consistent with the published data a specifically terminated transcript was detected when transcription was carried out with DNA and chromatin template in the presence of TTF-IΔN185 (lane 3, 4, 7, 8). The minor fraction of terminated transcripts in the reaction with DNA template without TTF-IΔN185 (lane 1, 2) is probably due to the presence of DNA-binding-competent TTF-I in the MB III extract. These observations prove the transcription activity of the MB III extract on free DNA and on chromatin templates confirming the presence and activity of factors required for Pol I transcription initiation and elongation in chromatin. Between several analysed extract preparations the MB III whole cell extract showed the most promising results with respect to the *in vitro* transcription assay as well as to initial immunoprecipitation trials and was therefore used for subsequent purifications and confirmatory experiments.To test whether chromatin remodelling activities would co-purify with the Pol I complex, the fractions immunoprecipitated with Flag-agarose from control MB III and Flag-RPA116-MBIII were analysed in a nucleosome mobility assay. In this approach a mono-nucleosome reconstituted on a radioactively labelled DNA fragment is used as a template. Incubation with enzymes capable of ATP-dependent chromatin remodelling would lead to re-positioning of the nucleosome, which can be resolved by its electrophoretic mobility. In this experiment isolated end-positioned mono-nucleosomes were utilised as template (for detail see Material and Methods, 3.2.17) and incubated with different dilutions of the fractions immunoprecipitated from Flag-RPA116-containing or control MB III extracts in presence of ATP (Figure 10C). As negative control reactions were incubated in absence of ATP (lane 1, 2, 9, 10, 17). The recombinantly expressed and reconstituted remodelling complex ACF, containing the ATPase ISWI and the Acf1 subunit, served as positive control (lane 17 - 22). In several experiments an enrichment of remodelling activities could be detected after co-immunoprecipitation with Flag-RPA116 (Figure 10C; lane 15, 16) compared

to the control (Figure 10C; lane 7, 8). However, only weak remodelling activitiy was measured. Taken together, the outcome of these chromatin-related assays show that MB III whole cell extract preparation and immunoaffinity purifications were performed under conditions that preserve complex integrity and generate transcriptionally active RNA Pol I complexes, which, presumably, also contain nucleosome remodelling activities.

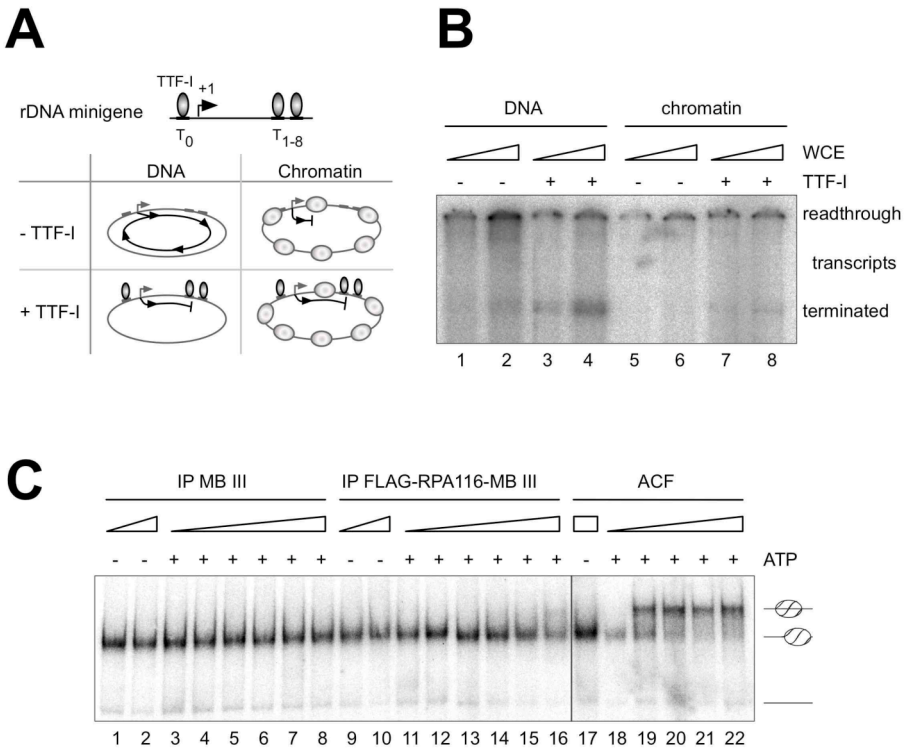
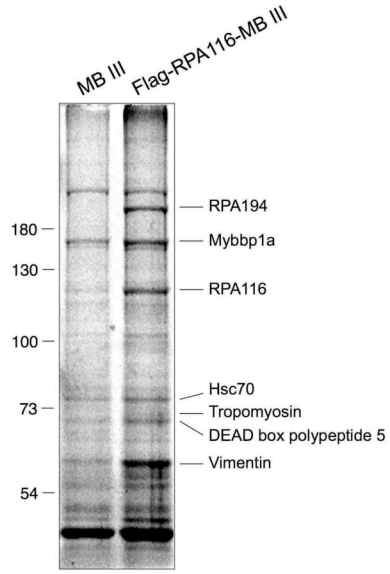


Figure 10: Characteristics of the extract for purification of Flag-RPA116-associated proteins.
(A) Schematic representation of the TTF-I dependent Pol I *in vitro* transcription system. The pMrWT-T construct (rDNA minigene), containing the rDNA promoter and terminator region and serving as template in the transcription reaction, is shown on the top. The Pol I-specific Transcription Termination Factor I (TTF-I) is required transcription termination and as well for transcription activation from chromatin but not from naked DNA templates. The table shows the expected transcripts (black; upper left: readthrough, upper right: no transcript, lower left and right: terminated transcript) in dependency on the respective template (grey; DNA or DNA pre-assembled into chromatin) and the absence or presence of TTF-I (shaded ellipse). (B) The MB III whole cell extract has *in vitro* transcription activity from DNA and chromatin templates. The pMrWT-T construct, either as naked DNA (10 ng; lanes 1-4) or pre-assembled into chromatin (40 ng; lanes 5-8), was incubated with increasing amounts of MB III whole cell extract (WCE) in absence (lane 1, 2, 5, 6) or presence (lane 3, 4, 7, 8) of TTF-I Δ185 for the in vitro transcription reaction. The radioactively labelled transcripts were purified, separated by polyacrylamide electrophoresis and visualized by autoradiography. The positions of the non-terminated readthrough transcripts and the terminated transcripts are indicated. (C) Nucleosome mobility assay with Flag-RPA116-associated proteins. Whole cell extracts of MB III or MB III cells stably expressing Flag-RPA116 (Flag-RPA116-MB III) were used for immunoprecipitation (IP) with α-Flag agarose. 4 µl of a 1:3 step dilution series of co-purified protein fractions from the IP with MB III (IP MB III; lane 1-8) or with Flag-RPA116-MB III extracts (IP Flag-RPA116-MB III; lane 9-16) were incubated with mononuclesomes end-positioned on a radiolabelled 247 bp DNA fragment in absence (lane 1,2,9,10,17) or presence of ATP. Different dilutions of the remodelling factor ACF (lane 17-21/22) were used as positive control. Resulting nucleosome positions were analysed by native gel electrophoresis. The positions of free DNA and end- or center-positioned nucleosomes are indicated on the right.

4.1.2.2 Immunoprecipitation of Flag-RPA116-containing complexes

To purify Flag-RPA116-containing complexes nuclear cell extracts were prepared from control MB III and Flag-RPA116-MB III cells growing in suspension. The control and Flag-RPA116-containing extracts were incubated with α-Flag agarose, the precipitations were thoroughly washed with buffer containing 300 mM NaCl to reduce unspecific binding and subsequently eluted with Flag peptide. The eluted fractions were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE), the gel was stained with Coomassie, protein bands were sliced out and subsequently analysed by MALDI-TOF mass spectrometry.

A



B

accession number	polypeptide	biological process	component
gi|34328146	RPA194	rRNA transcription	RNA Pol I, n, no
gi|31982724	Mybbp1a	transcription regulation	no, n, c
gi|6677789	RPA116	rRNA transcription	RNA Pol I, n, no
gi|476850	Hsc 70	chaperone, stress response	c
gi|111212	Tropomyosin 5	filament	c, cytoskeleton
gi|6681157	DEAD box polypeptide 5	helicase activity, transcription cofactor activity	RNP complexes, n
gi|2078001	Vimentin	type III intermediate filament	c, cytoskeleton

Figure 11: Purification of Flag-RPA116-associated factors.
(A) Coomassie-stained SDS gel of immunoprecipitations from nuclear extracts of MB III or MB III cells stably expressing Flag-RPA116 (Flag-RPA116-MB III) with α-Flag agarose. Eluted protein fractions were separated by SDS-PAGE, Coomassie stained, protein bands were cut and subjected to MALDI-TOF mass spectrometry. Proteins identified are indicated on the right. (B) Summary of the RPA116-associated proteins identified by mass spectrometry. The table shows the repective accession numbers and gene ontology terms (biological process, component) of the identified polypeptides.

Next to RPA116 and the largest Pol I subunit RPA194 five other proteins were identified as enriched in the Flag-RPA116-containing elution (Figure 11A; lane 2) compared to the control (lane 1): the transcription regulator Mybbp1a, the chaperone Hsc70, the filaments Tropomyosin 5 and Vimentin, and the helicase DEAD box polypeptide 5. The different factors are summarised in Figure 11B with their respective accession number identified by MALDI-TOF and gene ontology (GO) terms related to biological process and component. Despite extensive washing steps in the presence of 300 mM NaCl a significant amount of unspecific binding was detected when comparing the Flag-RPA116-containing and control elution (Figure 11A). For that reason different immunoprecipitation conditions were tested and the protocol was optimised related to efficiency and background for the subsequent confirmatory experiments (data not shown).

4.1.2.3 Mouse Mybbp1a interacts with the Pol I complex

One of the proteins interacting with Flag-RPA116 was identified as mouse Mybbp1a (mMybbp1a) by MALDI-TOF analysis (Figure 11). This protein has been described as Pol II transcription regulator in several cases earlier (Fan *et al.* 2004; Diaz *et al.* 2007; Owen *et al.* 2007; Hara *et al.* 2009) and chromatin-modifying enzymes have been implied in its mode of action as transcriptional co-repressor (Fan *et al.* 2004). These characteristics made it an interesting candidate protein and therefore its association with the Pol I complex was further investigated in detail. Firstly, the interaction of mMybbp1a and RNA Pol I was verified by repeated co-immunoprecipitation experiments (Figure 12). MB III and Flag-RPA116-MB III whole cell extracts were incubated with α-Flag agarose, the precipitates were thoroughly washed and eluted with Flag peptide. Load, flow-through, eluted and agarose bead fractions were separated by SDS-PAGE and analysed with α-RPA116 and α-Flag by Western Blotting to control the efficiency of Flag-RPA116 enrichment from Flag-RPA116-MB III compared to control extracts.

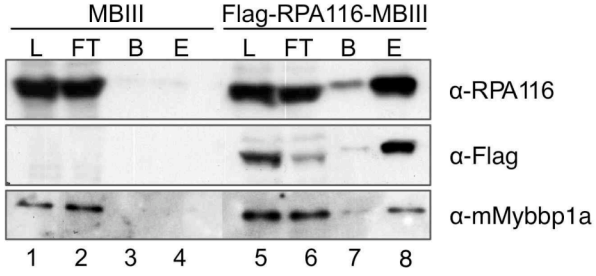


Figure 12: Co-immunoprecipitation of Flag-RPA116 and Mybbp1a.
Flag-RPA116-containing complexes were immunoprecipitated with α-Flag agarose from whole cell extracts of MB III and Flag-RPA116-MB III cells. For the detection with α-RPA116 and α-Flag load (L, 40%), flow-through (FT, 40%), bead-bound (B) and eluted (E) proteins were analyzed by Western blotting. For the detection with α-Mybbp1a antibodies 0,8% of the load and flowthrough were loaded on the gel.

The detection with α-mMybbp1a revealed an enrichment of mMybbp1a in the immunopurified and eluted fractions from RPA116-MB III extracts (lane 7, 8) compared to control extracts (lane 3, 4) confirming the association of mMybbp1a with the Pol I complex.To investigate whether Mybbp1a and RPA116 are present together in distinct complexes in the cell MB III whole cell extract was analysed by Superose 6 size exclusion column for separation by the fast protein liquid chromatography (FPLC) system (Äkta, Amersham) (Figure 13A) and glycerol gradient (10 - 45%) centrifugation (Figure 13B), which both allow separation of complexes according to their native size. Size markers were treated in parallel to determine the respective complex size corresponding to each fraction. Subsequently proteins were precipitated and separated by SDS-PAGE and analysed by Western blotting. The detection with antibodies against the Pol I subunits RPA116 and PAF53 and mMbybbp1a revealed a co-migration of the proteins over several fractions. The analysis by Superose 6 size exclusion showed at least two peeks of co-migration at different molecular weights, one in the high molecular range (around and eventually above 2 MDa; fraction 14 - 19) and one in the middle molecular range (700 kDa; fraction 23 - 25)(Figure 13A).

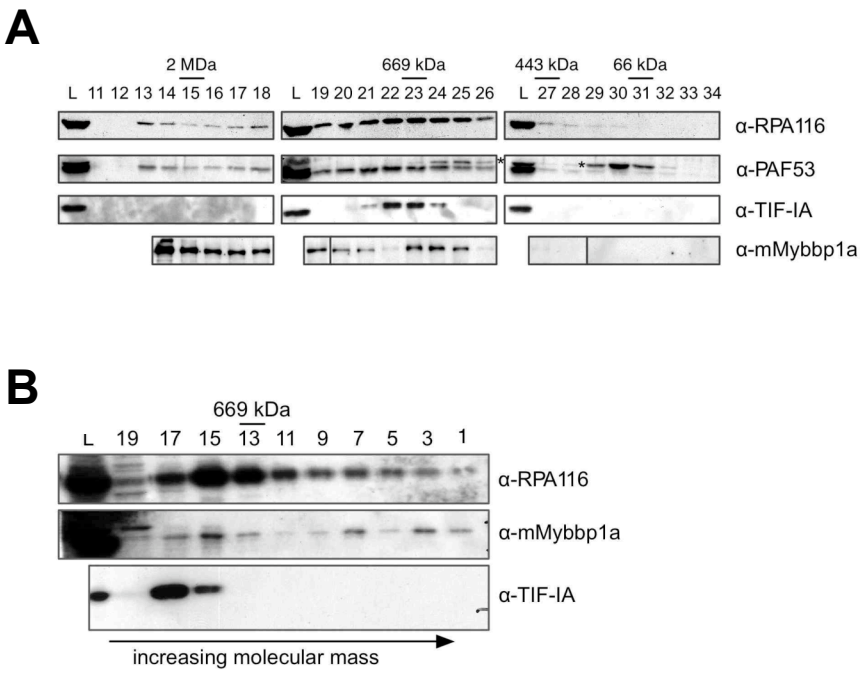


Figure 13: Analysis of RPA116 and Mybbp1a by (A) gelfiltration analysis and (B) glycerol gradient centrifugation.
(A) MB III whole cell extract was separated on a Superose 6 gelfitration column. Load (L, 4%) and collected fractions (4%) were analyzed by Western blotting with the indicated antibodies. Unfortunately, no sufficient amounts of fraction 11 to 13 and load were available for the time-wise shifted investigation of mMybbp1a. (B) MB III whole cell extract was applied on a 10 – 45% glycerol gradient. The load (L, 2%) and every second fraction (3%) was analysed by Western blotting with the indicated antibodies. The size of 2 MDa, 669 kDa, 443 kDa and 66 kDa corresponds to Dextran, Thyroglobulin, Apoferritin and bovine serum albumine, respectively.

The evaluation by glycerol gradient revealed three distinct peeks in faction 1/3, 7 and 15 (Figure 13B) with fraction 15 corresponding to the middle molecular range identified also by Superose 6 size exclusion. The peaks in fraction 1/3 and 7 of the glycerol gradient probably

reflect the enrichment in the high molecular weight range detected by size exclusion and with a qualitatively different resolution additionally indicate the existence of two distinct complexes. In parallel the Pol I-specific Transcription Initiation Factor TIF-IA was analysed as an example of a known Pol I complex-associated factor and co-migration with RPA116 was detected by both approaches at around 600 kDa, which is the size were the core Pol I subunits are the most enriched. Taken together these data show that mMybbp1a is indeed associated with the Pol I complex. The protein is part of differently composed and sized complexes in the cell, which might also contain the Pol I enzyme, potentially reflecting different steps of a ‘holoenzyme’ composition.

4.2 Analysis of Mybbp1a function in the nucleolus

So far Mybbp1a function has been only characterised in the context of Pol II and III transcription. After the confirmation of Mybb1a being a novel interactor of the Pol I complex next steps were to analyse if the protein exhibits also regulatory activity on Pol I transcription. For this purpose human cells were chosen as model system as several tools to study Pol I transcription in this regard were already established and, eg concerning transfection efficency, better to handle.

4.2.1 Mybbp1a localises to the nucleolus in human cell lines

Prior functional investigation the localisation of human Mybbp1a and its anticipated association with the Pol I complex were confirmed by immunostaining and co-immunoprecipitation assays in the human system. To monitor cellular localisation HeLa cells, either wildetype or cells transfected with a human Flag-Mybbp1a expression construct or control plasmid, were fixed with 1:1 (v/v) acetone/methanol 48 hours after transfection and incubated with α-Mybbp1a or α-Flag. For visualisation of the nucleoli the cells were either co-stained with α-RPA116 or phase contrast images were taken. Confirming previous studies (Yamauchi *et al.* 2008; Perrera *et al.* 2010), human Mybbp1a was found to preferentially localise to the nucleolus (Figure 14). Furthermore two additional mouse expression constructs, which were intended for later use in functional assays, were tested for their localisation in human cells, mouse Flag-Mybbp1a (Flag-mMybbp1a) and the C-terminally truncated form Flag-mp67^{MBP}*NLS coding only for the first 585 N-terminal aa of the protein. As the truncated protein does not naturally localise to the nucleus the coding sequence of the SV40-large-T nuclear localisation signal was C-terminally fused to the protein coding sequence for artificial tethering (Tavner *et al.* 1998). While transiently transfected Flag-mMybbp1a consistently showed a predominant nucleolar localisation, Flag-mp67^{MBP}*NLS visualisation exhibited a more diffuse nuclear staining pattern but with preference for the nucleolus. These observations indicate that mouse Mybbp1a might be able to complement the endogenous protein whereas the C-terminally truncated mutant seems to have lost certain functional properties.

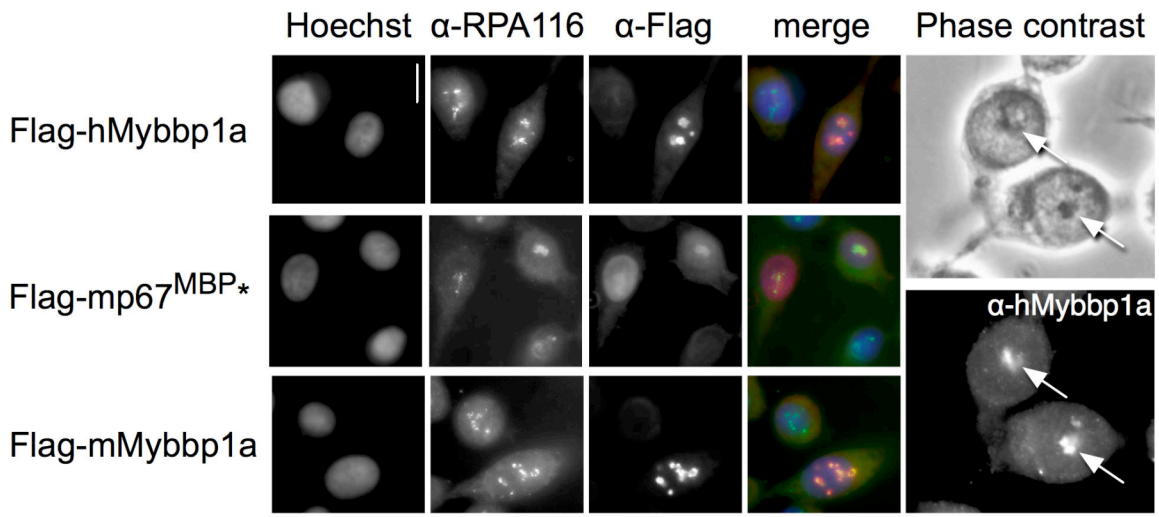


Figure 14: Localization of RNA Pol I and Mybbp1a in HeLa cells.
48 h after transfection with human Flag-Mybbp1a (Flag-hMybbp1a), mouse Flag-Mybbp1a (Flag-mMybbp1a) or mouse Flag-p67^{MBP}*NLS (Flag-mp67^{MBP}*) expression constructs cells were fixed and stained with antibodies against RPA116 (Cy2, green) and Flag (Cy3, red). Nuclei were stained by Hoechst reagent (blue). Non-transfected HeLa cells stained with α-Mybbp1a recognizing the endogenous Mybbp1a are shown on the right. The postion of the nucleoli are indicated by arrows. Scale bar: 10 µm.

4.2.2 Mybbp1a interacts with the Pol I complex in human cells

Subsequently, the actual physical association of human Mybbp1a with the RNA Pol I complex was assessed by co-immunoprecipitation experiments using the Flag-Mybbp1a construct. Nuclear extracts from HEK293T cells transiently transfected with either empty vector or the Flag-Mybbp1a expression construct were incubated with α-Flag agarose. The Flag-agarose precipitates were thoroughly washed and directly resuspended in Laemmli buffer for further analysis. Load, flow-through and agarose bead fractions were separated by SDS-PAGE and analysed by Western Blotting with α-Mybbp1a and α-PAF53. Both proteins, Mybbp1a and the Pol I subunit PAF53, were enriched by the affinity tag purification from the extract containing Flag-Mybbp1a (lane 6) compared to the control (lane 3) (Figure 15). Snf2h has been previously described as an interacting protein of Mybbp1a (Cavellan *et al.* 2006) and was used as a positive control. The data thereby confirm a specific interaction of Mybbp1a and the Pol I complex also in the human system.

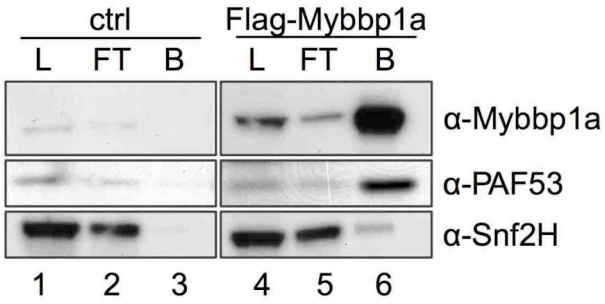


Figure 15: Immunoprecipitation with human Flag-Mybbp1a.
Nuclear extracts of HEK239T cells either transfected with Flag-Mybbp1a expression plasmid (lanes 4 to 6) or control DNA (ctrl; lanes 1 to 3) were incubated with α-Flag agarose. For the detection with α-Mybbp1a and α-Snf2h load (L, 5%), flow-through (FT, 5%), bead bound (B) and eluted (E) proteins were analyzed by Western blotting. For the detection with α-PAF53 0.3% of the load and flowthrough were loaded on the gel.

4.2.3 Mybbp1a acts as a repressor of Pol I transcription

As outlined before Mybbp1a has been described as a regulator of Pol II transcription in earlier studies. Considering the protein's association with the Pol I complex a potential role of Mybbp1a in the regulation of rRNA gene transcription was tested accordingly.

4.2.3.1 Mybbp1a represses the transcription from a Pol I reporter construct

To visualise RNA Pol I activity, the human rRNA minigene construct pHrD-Ires-Luc (Ghoshal *et al.* 2004; Nemeth *et al.* 2004) that harbours the rRNA gene promoter (-410 bp to +314 bp relative to the transcription start site) followed by an internal ribosome entry site (IRES) and the coding sequence of the firefly luciferase protein was employed (Figure 16A). The IRES sequence allows translation of the rRNA Pol I transcripts and relative quantification of the minigene RNA levels via the luciferase activity. The possibility of protein expression driven by Pol I was firstly introduced by McStay and colleagues and the respective specificity controls have convincingly demonstrated that expression from such construct is with great probability exclusively dependent on the Pol I but not the Pol II enzyme (Palmer *et al.* 1993). A later study with mouse constructs comparable to the human construct utilised in this study proofed their Pol I specificity by employing α-amanitin, which strongly inhibits Pol II but not Pol I, (Nemeth *et al.* 2008). RNA Pol I transcription activity from the minigene construct was calculated relative to a co-transfected RNA Pol II-dependent construct expressing the Renilla luciferase (pRL-TK; Promega) to normalise for potential differences in transfection efficiency (Figure 16A). For the assessment of Mybbp1a's impact on Pol I transcription activity from the rRNA minigene HeLa cells were transiently transfected with empty vector or different amounts of Flag-Mybbp1a expression construct together with the Pol I reporter pHrD-Ires-Luc and the Renilla control pRL-TK. As example for a known modulator of Pol I transcription different amounts of a Tip5 expression construct were similarly transfected. Tip5 is the large

subunit of the nucleolar remodelling complex, NoRC, that serves to silence rRNA genes by changing their chromatin structure and initiating heterochromatinisation of rDNA (Strohner *et al.* 2001; Santoro *et al.* 2002; Li *et al.* 2006). 48 hours after transfection luciferase activity was measured by the use of the Dual Luciferase Reporter Assay System (#E1960; Promega) in the luminometer Lumat LB 9501 (Berthold), and protein levels were determined by Western Blotting. When compared to control transfections overexpression of human Mybbp1a in HeLa cells resulted in a dose-dependent transcriptional repression of the rRNA minigene expression, comparable to the repressive effect of Tip5 (Figure 16B, Figure 17). Thus, Mybbp1a acts on Pol I transcription by a repressive mechanism characterising a novel role of the protein in the nucleolus.

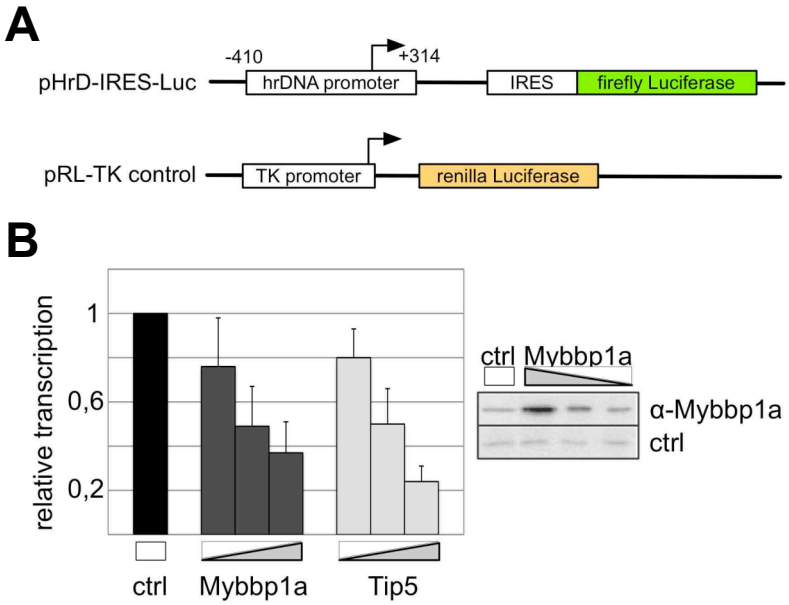


Figure 16: Transcription activity from a Pol I reporter after overexpression of Mybbp1a.
(A) Schematic representation of luciferase reporter constructs. The pHrD-IRES-Luc (Ghoshal *et al.* 2004) construct contains the human rDNA (hrDNA) promoter from -410 to + 314 (relative to the transcription start site) followed by an internal ribosome entry site (IRES) fused to the firefly luciferase coding region. The renilla control construct carrying the renilla luciferase gene under the control of a TK promoter (pRL-TK); transcribed by Pol II) was used to normalize for transfection efficiency. (B) HeLa cells were co-transfected with both luciferase reporter constructs illustrated in (B) and increasing amounts of either Mybbp1a or Tip5 expression plasmids (188 ng, 375 ng, 750 ng). 48 h after transfection luciferase activity was measured. Firefly luciferase counts (RNA Pol I reporter) were normalized to renilla luciferase counts and compared to the control transfection reaction (ctrl). Average and standard deviation values of 3 biological replicates (each in technical duplicate) are shown. Mybbp1a protein levels were analyzed by Western blotting with α-Mybbp1a antibody. An unspecific band served as loading control (ctrl).

To see if mouse Mybbp1a is able to function as the human protein due to their high conservation the impact of mouse Flag-mMybbp1a or the C-terminally truncated form Flag-mp67^{MBP}*NLS (see section 4.2.1, p. 57) on the human Pol I reporter was analysed (Figure 17). While the overexpression of mouse full-length protein led to a dose-dependent repression of transcriptional activity similarly to the human protein, transient expression of even higher protein amounts of mp67^{MBP}*NLS had no effect on Pol I-dependent transcription

(Figure 17). These results suggest that mouse Mybbp1a is capable of functionally complementing the human protein. Moreover the C-terminus of the protein seems to be required for efficient transcriptional repression.

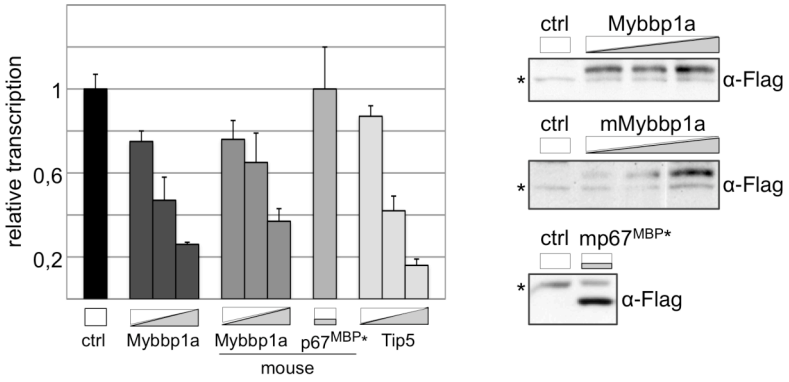


Figure 17: Transcription activity from a Pol I reporter after overexpression of human or mouse Mybbp1a constructs.
HeLa cells were co-transfected with both luciferase reporter constructs, pHrD-IRES-Luc and renilla control, and increasing amounts (188 ng, 375 ng, 750 ng) of either human Mybbp1a, mouse Mybbp1a or Tip5 expression plasmids. The mouse p67^{MBP}*NLS (p67^{MBP}*) expression plasmid was transfected with an amount of 188 ng. 48 h after transfection luciferase activity was measured and firefly luciferase counts (RNA Pol I reporter) were normalized to renilla luciferase counts and compared to the control transfection reaction (ctrl). Average and standard deviation values of two technical replicates are shown. Protein levels were analyzed by Western blotting with α-Flag antibody on the same membrane allowing a direct comparision of the protein levels expressed from the different constructs. An unspecific band (*) served as loading control.

The Pol I reporter experiments have determined novel functional properties of Mybbp1a within the nucleolar compartment. However, the mechanism, which leads to Mybbp1a-mediated repression of Pol I transcriptional activity remains unclear. A recent study by Spiegelman and colleagues (Fan *et al.* 2004) has shown mouse Mybbp1a to bind to PPAR gamma coactivator 1α (PGC-1α), a key regulator of metabolic processes, and thereby repress its transcriptional coactivator function. The authors attribute its suppressive activity at least in part to a histone deacetylase (HDAC)-dependent mechanism as reporter assays conducted in the presence of the HDAC inhibitor Trichostatin A (TSA) revealed a decreased repressive activity of Mybbp1a. Accordingly, the Pol I reporter assay was repeated as before but with cells treated with two different TSA concentrations (30 and 100 nM) or solvent prior harvesting to monitor for a potential HDAC-dependent process in Mybbp1a-mediated Pol I transcriptional repression. Tip 5 overexpression was again employed as a control as this protein is known to repress Pol I transcription in an HDAC-dependent manner (Santoro *et al.* 2002; Zhou *et al.* 2002). This is mirrored by the release of transcriptional repression by Tip5 with increasing amounts of TSA and was repeatedly observed (Figure 18). Similarly, an alleviation of Pol I transcriptional repression by Mybbp1a upon TSA treatment was observed (Figure 18) in initial experiments indicating a HDAC-dependent mechanism for Mybbp1a transcriptional repression. However, available experimental data is not significant yet and subsequent analysis is required for final confirmation.

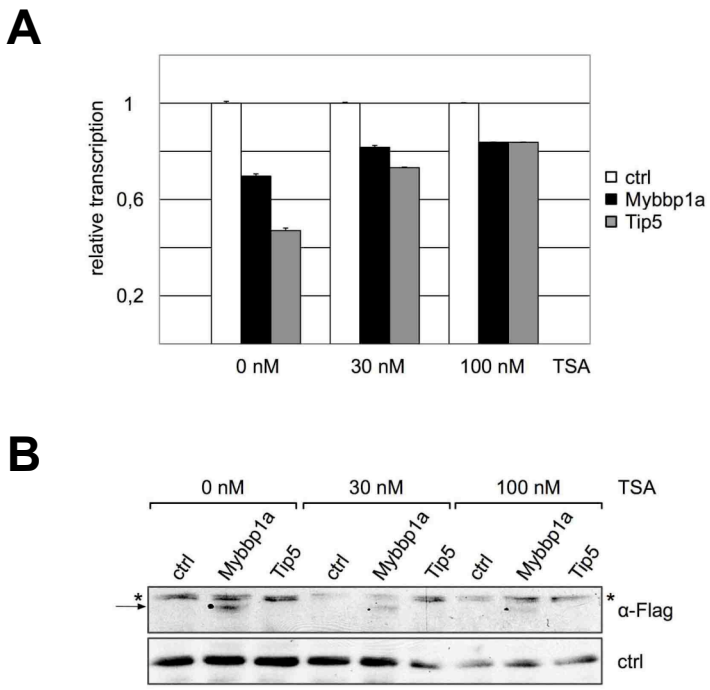


Figure 18: Transcription activity from a Pol I reporter after overexpression of Mybbp1a in presence of Trichostatin A.
(A) HeLa cells were co-transfected with both luciferase reporter constructs, pHrD-IRES-Luc and renilla control, and 750 ng of either human Mybbp1a or Tip5 expression plasmids. The cells were maintained in presence of 30 or 100 nM Trichostatin A or vehicle (0 nM) for 12 h prior harvesting. 48 h after transfection luciferase activity was measured and firefly luciferase counts (RNA Pol I reporter) were normalized to renilla luciferase counts and compared to the control transfection reaction (ctrl). Average and standard deviation values of two technical replicates are shown. (B) Protein levels were analyzed by Western blotting with α-Flag antibody. An unspecific band served as loading control (ctrl). *: cross-reacting band.

4.2.3.2 Mybbp1a overexpression leads to decreased Pol I occupancy at the rRNA gene promoter

To obtain more insight into the action of Mybbp1a at the rDNA promoter chromatin immunoprecipitation (ChIP) experiments were performed. Nuclear and nucleolar extracts of control cells and cells overexpressing the Flag-tagged human Mybbp1a were incubated with antibodies against the Flag-tag and hMybbp1a. Antibodies recognising the Pol I subunit RPA116 and the Pol I-specific transcription factor TTF-I were used as positive controls (Figure 19). Subsequently the immunoprecipitated chromatin was purified and the extracted DNA was investigated for presence of promoter and intergenic spacer sequences of the rDNA repeat by quantitative real-time PCR. As expected DNA precipitated with α-TTF-I contained sequences from the rDNA promoter but not from the intergenic spacer. However, neither the α-Flag nor the α-Mybbp1a antibodies were able to co-precipitate rDNA fragments under the applied conditions, which suggests that Mybbp1a function is not DNA mediated. Interestingly, a decrease of Pol I and TTF-I occupancy at the rDNA promoter was detected upon overexpression of Flag-Mybbp1a. This observation correlates with a Mybbp1a-dependent repression of Pol I transcription upon Mybbp1a overexpression

suggesting a repressive mechanism through its binding to the RNA Pol I complex but not to rDNA itself.

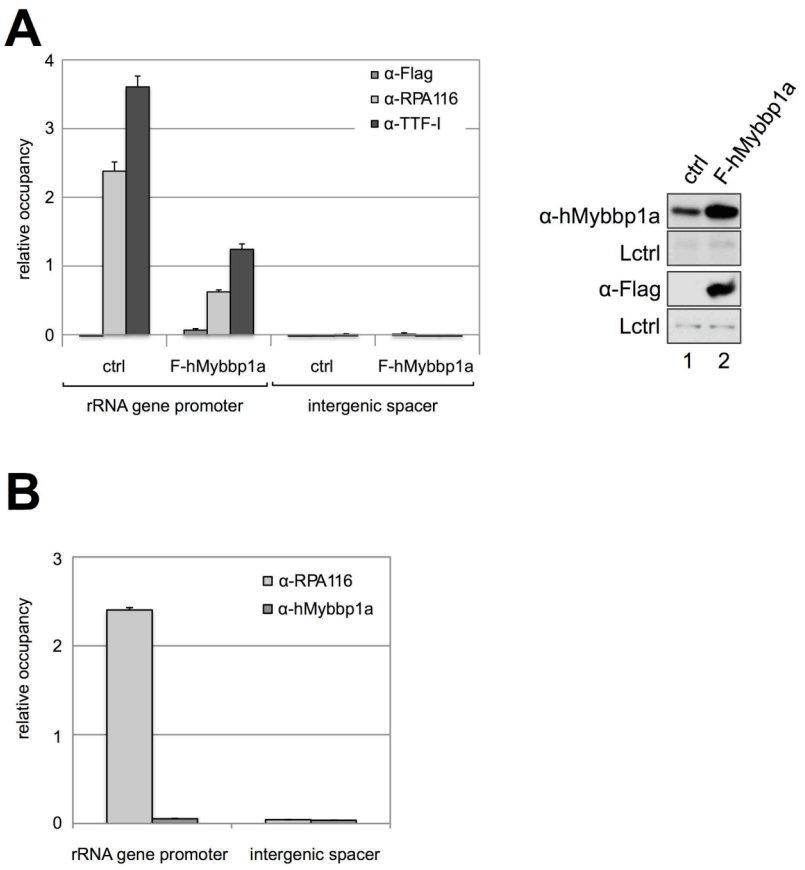


Figure 19: Chromatin immunoprecipitation (ChIP) analysis of Flag-hMybbp1a occupancy at the rDNA repeat.
ChIP experiments were performed with chromatin derived from (A) purified nucleoli or (B) whole cell extracts from cells either transfected with human Flag-Mybbp1a expression plasmid (F-hMybbp1a) or control DNA (ctrl). For immuno-precipitation of Flag-hMybbp1a either monoclonal α-Flag or polyclonal α-hMybbp1a were used as indicated within the graphs. Polyclonal antibodies against the Pol I subunit RPA116 and the Pol I-specific transcription factor TTF-I served as positive controls. Polyclonal α-IgG antibody was used for background estimation. Relative occupancies of the factors (% input after correction of non-specific binding) at the promoter region or intergenic spacer (IGS) of the rDNA repeat were quantified by real-time quantitative PCR as described in Materials and Methods. Average and standard deviation values of 2 technical replicates are shown. Flag-hMybbp1a protein levels in in the applied extracts derived from cells either transfected with control (ctrl, lane 1) or Flag-hMybbp1a (lane 2) plasmid were analyzed by Western blotting with α-Mybbp1a and α-Flag antibodies as indicated. An unspecific band served as loading control (Lctrl).

4.2.3.3 siRNA-mediated depletion of Mybbp1a protein levels leads to increasing amounts of 47S rRNA precursor

To study Mybbp1a's role in endogenous rRNA gene transcription and thereby verify the data from the Pol I reporter experiments, knockdown of the Mybbp1a mRNA was performed in HeLa cells using short interfering RNA (siRNA). Knockdown experiments were performed with GL3-siRNA (a control siRNA directed against the luciferase gene) and two different amounts of Mybbp1a-specific siRNA and followed over 4 days. Mybbp1a protein levels were monitored by Western Blotting on day 2, 3 and 4 after siRNA transfection and additionally by immunostaining on day 4. Western Blotting with α-Mybbp1a revealed a minor reduction of

Mybbp1a levels already on day 2 and efficient depletion on day 3 and 4 after transfection (Figure 20A). Immunostaining with α-Mybbp1a confirmed the effective protein depletion when comparing wildetype cells and cells transfected with either GL3- or Mybbp1a-specific siRNAs (Figure 20B). To monitor the effect of protein depletion on rRNA gene transcription, the amounts of newly synthesised 47S rRNA precursor (pre-rRNA) were measured by quantitative real time PCR. Therefore total cellular RNA was isolated and reverse transcribed, and the level of the 5' external transcribed spacer (5'ETS) was quantified by an oligo hybridising to the 5'ETS upstream of the first processing site A' (Kass *et al.* 1987). This terminal part of the 5'ETS is instantly degraded after cleavage and taking into account the rapidity of the pre-rRNA cleavage processes (Warner 2001) such designed oligo is therefore appropriate to visualise actual rRNA transcription activity. Figure 20C shows the rRNA gene transcription levels 2, 3 and 4 days after transfection with Mybbp1a-specific siRNA normalised to an internal control, β-actin mRNA, which were expressed relative to the transfection with GL3 control. In agreement with the rRNA minigene expression studies, depletion of Mybbp1a resulted in an increase of pre-rRNA levels in the cell, again suggesting a role ofMybbp1a in transcriptional repression. Additionally it was observed that transfection of double amounts of Mybbp1a-specific siRNA led to a stronger impact on the relative transcription level arguing for a dose-dependent effect. However this could not be resolved on the protein level. Finally, the elevation of rRNA gene transcription activity (Figure 20C) correlated with decreasing Mybbp1a protein levels (Figure 20A), which further emphasises their direct dependency.

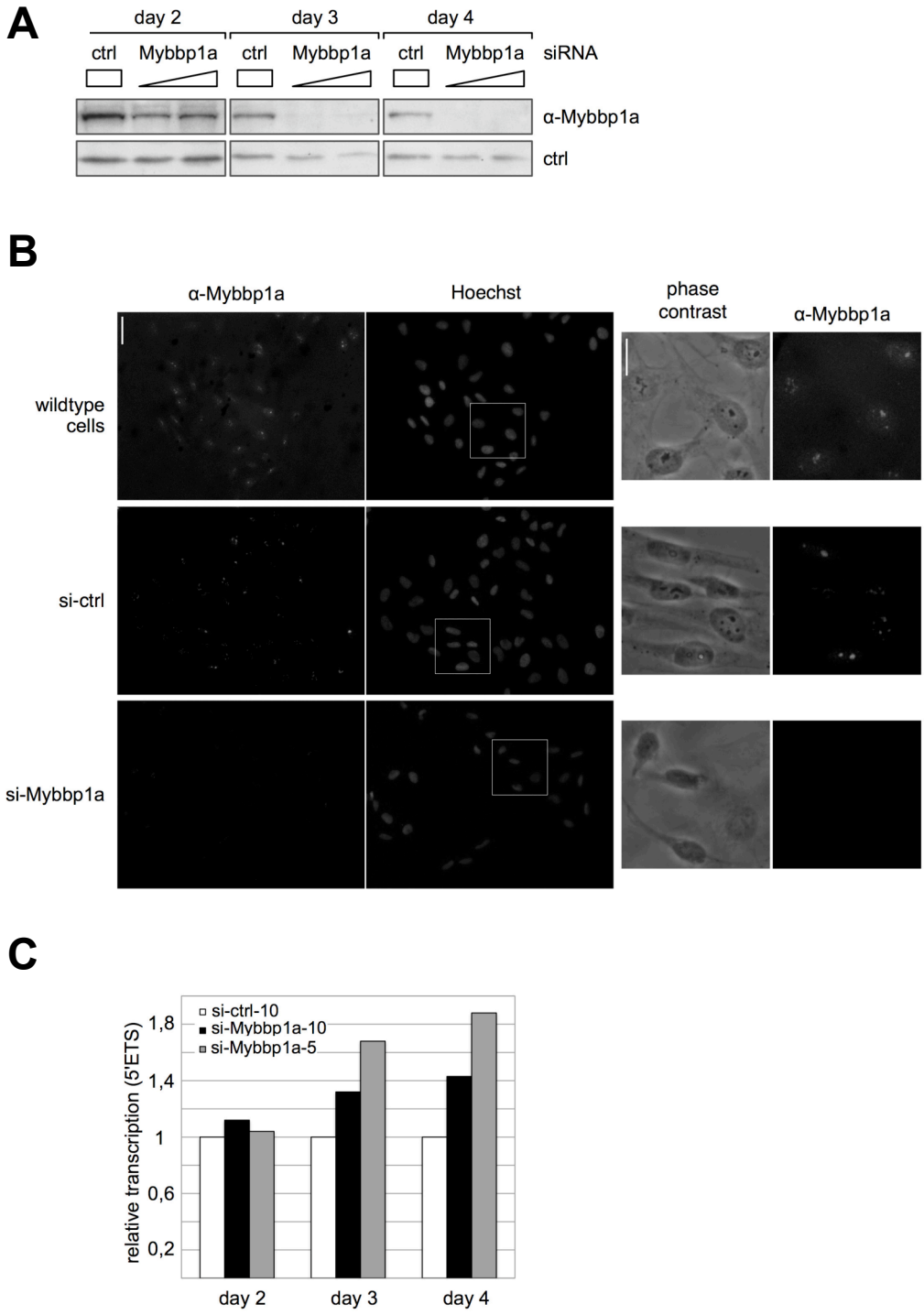


Figure 20: siRNA-mediated knockdown of Mybbp1a leads to increasing amounts of 47S rRNA precursor. (A) Western Blot of lysates from HeLa cells treated with different amounts (5 or 10 µl) of either a siRNA targeting Mybbp1a (Mybbp1a) or a control siRNA (ctrl). HeLA cells were harvested two, three or four days after siRNA transfection and lysates were separated by SDS-PAGE and analysed with α-Mybbp1a. An unspecific band served as loading control (ctrl). (B) Immunofluorescence and phase contrast microscopy of HeLa cells treated with either siRNA targeting Mybbp1a (si-Mybbp1a) or a control siRNA (si-ctrl) and wildtype cells. Four days after siRNA transfection cells were fixed with paraformaldehyde and stained with α-Mybbp1a. Nuclei were stained by Hoechst reagent. (C) Relative Pol I transcription activity corresponding to (A). HeLa cells were harvested two, three or four days after transfection with 5 or 10 µl siRNA targeting Mybbp1a (si-Mybbp1a-5 (black bar) and -10 (grey bar), respectively) or 10 µl of a control siRNA (si-ctrl-10; white bar). Ongoing Pol I transcription activity was measured by RT-qPCR with oligos detecting the region preceding the first cleavage site in the 5'ETS of the 47S rRNA precursor and normalized to an internal control (β-actin). The transcription levels were expressed relative to ctrl. Mybbp1a protein levels are shown in (A). Scale bars: 20 µm (left column) and 10 µm (right column).

To exclude any off-target effects of the Mybbp1a-specific siRNA several siRNAs targeting different sites of the Mybbp1a mRNA were tested for their effect on rRNA gene transcription activity (Figure 21). Transfection of each of the three analysed siRNAs led to efficient depletion of Mybbp1a in HeLa cells as revealed by Western Blotting with α-Mybbp1a and showed an increase in relative pre-rRNA levels in approximately the same dimension. These results thereby convincingly rule out any off-target effects.

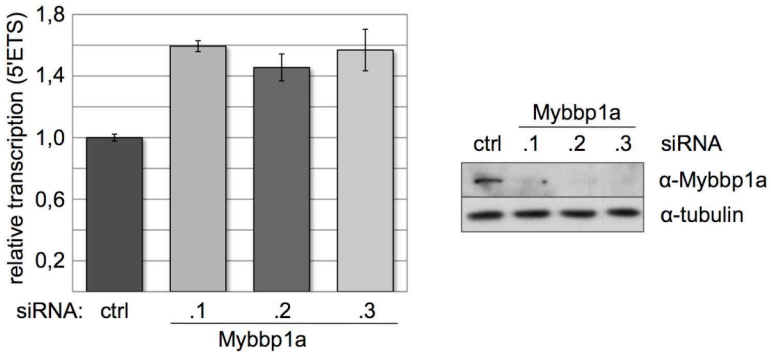


Figure 21: Different siRNAs targeting Mybbp1a have a similar effect on rDNA transcription measured by RT-qPCR.
HeLa cells were transfected with three different siRNAs each targeting Mybbp1a (Mybbp1a.1, .2 and .3) or GL3 control (ctrl) siRNA. Two days after transfection ongoing RNA Pol I transcription activity was measured by RT-qPCR with oligos detecting the region preceding the first cleavage site in the 5'ETS of the 47S rRNA precursor and normalized to an internal control (β-actin). The transcription levels after si-Mybbp1a.1 (grey bar), .2 (dark grey bar) and .3 (light grey bar) transfection were expressed relative to ctrl (black bar). Average and standard deviation values were calculated from 2 technical replicates. Mybbp1a protein levels were analyzed by Western blotting. The α-tubulin staining of the membrane served as a loading control.

Taken together, increased protein levels of Mybbp1a has been shown to repress rDNA transcription from a reporter while siRNA-mediated depletion of Mybbp1a lead to an augmentation of endogenous pre-rRNA levels thereby convincingly proofing a negative regulatory function of Mybbp1a on rRNA gene transcription.

4.2.4 Depletion of Mybbp1a influences growth characteristics of HeLa cells

Surprisingly, initial experiments employing siRNA-mediated knockdown indicated that Mybbp1a-depleted cells exhibit proliferation retardation or even stop. To investigate this observation in more detail the proliferation behaviour of cells transfected with either control, TIF-IA- or Mybbp1a-specific siRNAs was monitored over a period of several days. TIF-IA is a Pol I–specific transcription initiation factor (Buttgereit *et al.* 1985; Schnapp *et al.* 1990) and was used as a control. Depletion of Mybbp1a and TIF-IA was monitored at the protein level and followed for up to twelve days by Western Blotting (Figure 22A). siRNA-mediated knockdown of Mybbp1a efficiently reduced protein levels from day 2 through to day 7, followed by partial (day 9) and full (day 12) recovery of Mybbp1a protein levels. siRNA-

mediated knock down of TIF-IA was not as efficient as Mybbp1a depletion, but a visible reduction at the protein level was detected from day 2 to day 7.

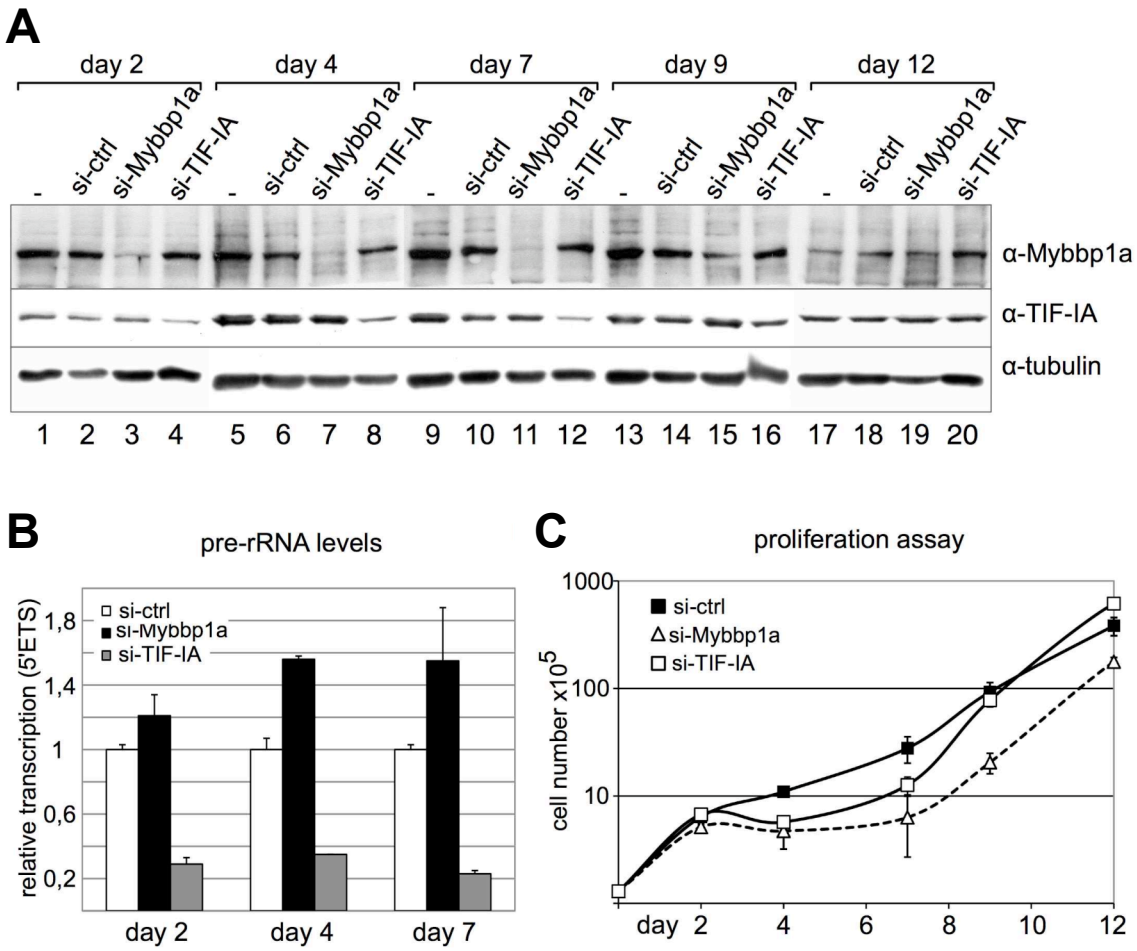


Figure 22: siRNA-mediated depletion of Mybbp1a augments rDNA transcription activity but delays cellular proliferation.
(A) HeLa cells were transfected with siRNA targeting Mybbp1a (Mybbp1a) or TIF-IA or with the GL3 control siRNA (si-ctrl). Protein levels were analyzed by Western blotting 2, 4, 7, 9 and 12 days after transfection, as indicated. The α-tubulin staining of the membrane served as loading control. (B) Ongoing RNA Pol I transcription activity was measured by RT-qPCR with oligos detecting the region preceding the first cleavage site in the 5'ETS of the 47S rRNA precursor and normalisation to β-actin. The transcription levels after si-Mybbp1a (black bar) and si-TIF-IA (grey bar) transfection are shown relative to the control reaction (si-ctrl, white bar). (C) Cellular proliferation upon siRNA treatment of the cells, using si-Mybbp1a (white triangle), si-TIF-IA (white square) or si-ctrl (black square), was followed from days 0 to 12 by counting the number of living cells. Cell numbers are plotted in log10 scale. The average and standard deviation values were calculated from three technical replicates. One of two independent data sets is shown.

To monitor the effect of protein depletion on rDNA transcription, the amounts of newly synthesised pre-rRNA were again measured by quantifying the level of 5'ETS and normalising it to β-actin mRNA by quantitative real time PCR from day 2 to 7. Confirming previous results depletion of Mybbp1a resulted in an increase in pre-rRNA levels negatively correlating with the Mybbp1a protein levels in the cell, once more emphasising its role in transcriptional repression (Figure 22B). As expected, reduced TIF-IA levels resulted in a strong decrease of the endogenous rRNA gene product, as this protein is an essential transcription initiation factor. In parallel to the evaluation of protein levels and rRNA gene

transcription activity the number of living cells was assessed every two or alternatively three days over a period of 12 days to thoroughly record the proliferation behaviour of the differently treated cells (Figure 22C). As initially observed cells depleted of Mybbp1a indeed ceased proliferation and thereby behaved similarly to cells depleted of the transcriptional activator TIF-IA. Furthermore, quantification of cellular proliferation revealed a correlation between the time frame of decreased protein levels and proliferation arrest between day 2 and 7 and recovery from day 9 onwards. Interestingly, visual inspection of the Mybbp1a knock down cells by phase contrast microscopy showed an irregular, flattened and enlarged morphology by day 4 (Figure 23).

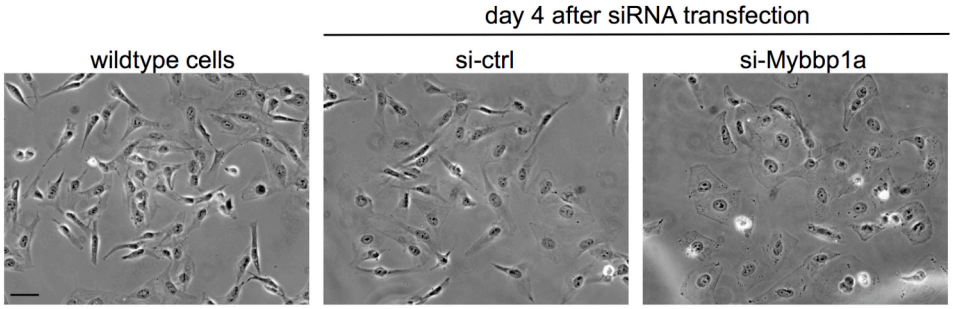
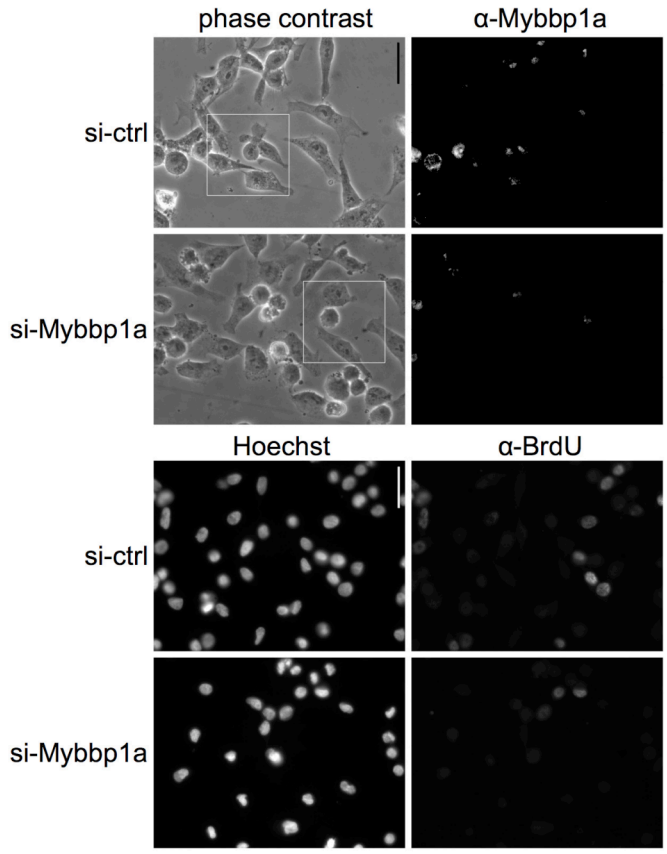


Figure 23: Mybbp1a-depleted cells show a flattened phenotype.
Phase contrast microscopy of wildtype HeLa cells and HeLa cells 4 days after transfection with siRNA targeting Mybbp1a (si-Mybbp1a) or a Gl3 control siRNA (si-ctrl). Mybbp1a protein depletion seems to influence cell cycle progression. Scale bar: 20 µm.

To investigate whether the proliferation stop might depend on any cell cycle-related parameters HeLa cells were analysed with respect to cell cycle progression after siRNA-mediated depletion of Mybbp1a. Therefore 2 days after siRNA transfection cells were incubated with the desoxythymidine analogue Bromodesoxyuridine (BrdU), which incorporates into newly synthesising DNA and thereby allows visualisation of cells actually passing S-phase by immunostaining. Cells were subsequently fixed and Mybbp1a depletion and BrdU-positive cells were determined with α-Mybbp1a and α-BrdU antibodies, respectively, by immunofluorescence microscopy. An initial analysis of Mybbp1a-depleted cells revealed a reduced number of cells in S-phase compared to cells treated with GL3 control (Figure 24) pointing to a disturbance of the proper cell cycle progression and proliferation defect.



siRNA	total cells	BrdU pos	BrdU pos [%]
ctrl	237	62	26,2
$p160^{MBP}$	245	34	13,9

Figure 24: Depletion of Mybbp1a influences cell cycle progression.
Phase contrast and immunofluorescence microscopy of HeLa cells 2 days after transfection with siRNA targeting Mybbp1a (si-Mybbp1a) or a GL3 control siRNA (si-ctrl) as indicated on the left. Cells were BrdU-labelled, fixed and stained with the antibodies indicated on top of each panel. The lower right panels represent a magnification of image sections (white frames) of the left panels. Nuclei were stained with Hoecshst. The table shows the number of cells positive for α-BrdU staining (BrdU pos) and their percentage (BrdU pos [%]) of the total cell number counted (total cells). Scale bars: 10 µm (upper right panel), 20 µm (upper left panel) and 40 µm (lower left panel).

Given the fact that Mybbp1a has been convincingly shown to be a repressor of rDNA transcription before increased proliferation rates after Mybbp1a protein depletion would have been expected. The observed contrary effect appears to be absolutely counterintuitive as rDNA transcription activity and rRNA levels are the major determinants of cellular growth and proliferation. Further supported by the detected defects in cell cycle progression an additional role for Mybbp1a in growth-related processes other than rDNA transcription must be taken into account.

4.2.5 Mybbp1a is important for regular processing of the rRNA precursor

In a recent study, Greenblatt and colleagues analysed the composition of yeast pre-rRNA-processing complexes and found that Pol5p, the suggested yeast homologue of Mybbp1a, is associated with the UtpA complex (Krogan *et al.* 2004). UtpA is likely to be the equivalent of the t-Utp sub-complex of the SSU processome, which has been identified in an independent study and shown to be equally required for rDNA transcription and pre-rRNA processing

(Gallagher *et al.* 2004). In consideration of the proliferation behaviour of Mybbp1a-depleted cells and the yeast data a potential role of Mybp1a in pre-rRNA processing was studied. Pre-rRNA processing involves a number of ordered and consecutive endo- and exonucleolytic cleavages of the 47S precursor rRNA to produce the 18S, 5.8S and 28S rRNAs and is comprehensively described in the introduction (chapter 2.3, p. 16). A short overview of the major mammalian rRNA processing pathway is again depicted in Figure 25A.

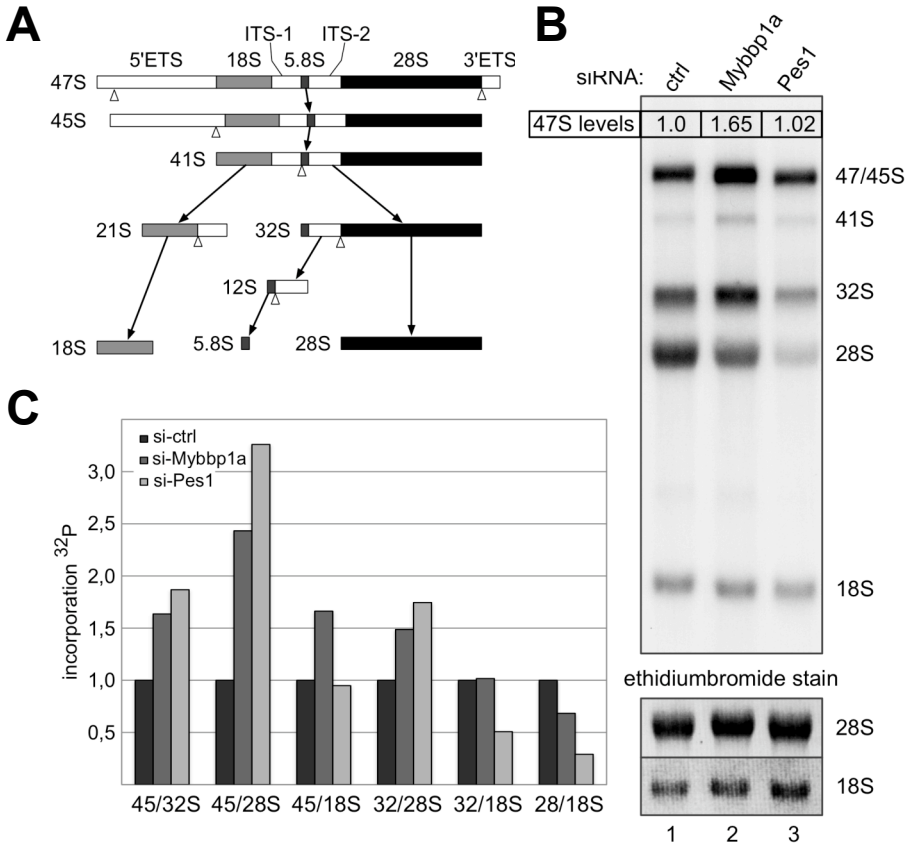


Figure 25: siRNA-mediated depletion of Mybbp1a affects pre-rRNA processing.
(A) Schematic representation of the pre-rRNA processing pathway. Cleavage events in pre-rRNAs are indicated by triangles. Intermediates and final processing products are shown. ETS: external transcribed spacer; ITS: internal transcribed spacer. (B) Metabolic labeling of nascent rRNA transcripts. 4 days after transfection with siRNAs targeting Mybbp1a (Mybbp1a) or Pes1 HeLa cells were incubated for 6 h with ^{32}P-orthophosphate, total cellular RNA was isolated and separated on an agarose gel. The incorporated radioactivity was quantified with a PhosphoImager. 47S pre-rRNA levels were normalized to the total rRNA load (ethidiumbromide stain of the 18S rRNA) and expressed relative to the GL3 control reaction (ctrl). The individual rRNA species are indicated on the right. One of four independent experiments is shown. Protein levels were analyzed by Western blotting (see Figure 26B siRNA Mybbp1a.2) (C) Quantification of the rRNA species shown in (B). ^{32}P incorporation was quantified with a PhosphoImager and the values of the rRNA species were compared to each other as indicated. Ratios were normalized to the control reaction. Metabolic labelling experiments were conducted in collaboration with M. Hölzel and D. Eick, Helmholtz Center Munich.

To follow and quantify pre-rRNA processing after Mybbp1a depletion, newly synthesised RNA was metabolically labelled with ^{32}P-orthophosphate 4 days after transfection of HeLa cells with Mybbp1a-specific siRNA (Figure 25B). Total cell RNA was extracted, resolved by gel electrophoresis, and radioactively labelled transcripts were visualised on a Phosphoimager. As expected the depletion of Mybbp1a led to an increase of the 47S pre-

rRNA species as compared with the control due to the de-repression of rRNA gene transcription, confirming the quantitative PCR data. Furthermore, the depletion of Mybbp1a resulted in pre-rRNA processing defects that correlated with a relative decrease in the 18S, 32S and 28S pre-rRNA cleavage products when compared to the 47S pre-rRNA levels. A quantitatively similar, but qualitatively different, processing defect was observed in control cells depleted of Pes1, a factor involved in the processing of the 32S precursor into 28S and 5.8S (Lapik *et al.* 2004; Grimm *et al.* 2006). As expected, the levels of 32 S and 28S were strongly decreased, whereas 47S and 18S did not change as compared with the control. The quantification of the distinct pre-rRNA species is depicted in Figure 25 and Figure 26, for which pre-/rRNA levels were either expressed as ratio between the different rRNA species of one experiment (Figure 25C) or as absolute numbers by normalising to the total cell RNA (total 18S rRNA, ethidium bromide stain) load and expressing them relative to the GL3 control transfection (Figure 25B and Figure 26). Figure 26A shows the quantification of rRNA levels of analogous experiments with the average and standard deviation from 4 independent experiments. Confirming the previous analysis the data show an increase of 47S precursor in combination with processing defects to a comparable extent. The application of several siRNAs targeting distinct sites in the Mybbp1a mRNA all led to an accumulation of 47S precursor after Mybbp1a protein depletion to a similar extent, again ruling out any siRNA off-target effects (Figure 26B). All metabolic labelling experiments were conducted in collaboration with Michael Hölzel and Dirk Eick (Department of Molecular Epigenetics, Helmholtz Center Munich). The results obtained so far suggest that Mybbp1a plays a dual role in rDNA metabolism: firstly, regulating rDNA transcription initiation and second, being essential for the correct processing of the pre-rRNA. As such, Mybbp1a appears to have an impact on processing steps important for the synthesis of both the small and large ribosomal subunit rRNAs, which consequently leads to retardations within the production of the intermediate and mature rRNAs upon Mybbp1a depletion. However, to what extent the increase of 47S rRNA precursor level is effectively due to a derepression of rDNA transcription upon Mybbp1a protein depletion or rather reflects the consequence of disturbed pre-rRNA processing accompanied by an accumulation of non-processed precursor can not be definitely answered by the applied experimental approaches. This issue will be addressed in more detail within the discussion section (chapter 5.2.1, p. 86).

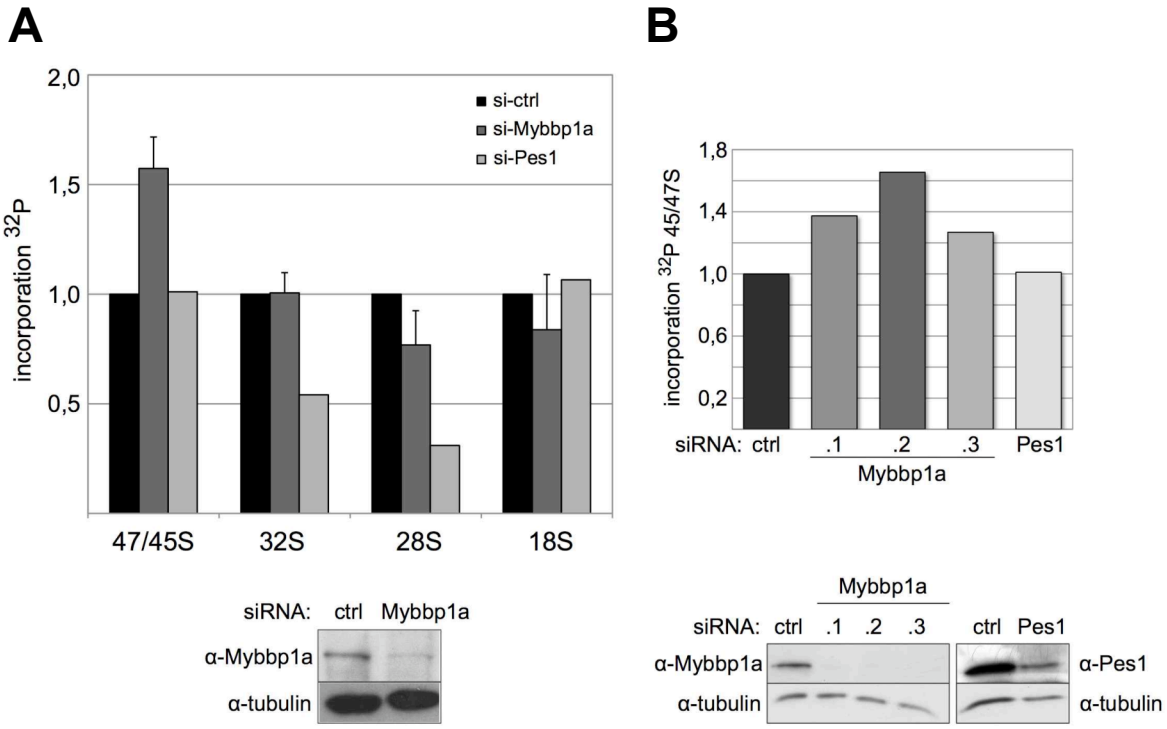


Figure 26: Different siRNAs targeting Mybbp1a have a similar effect on rDNA transcription measured by metabolic labeling.
(A) Quantification of metabolic labeling of nascent rRNA transcripts. HeLa cells were transfected with Mybbp1a (si-Mybbp1a) or GL3 control (si-ctrl) siRNA and prepared as described above. ^{32}P incorporation was quantified with a PhosphoImager and the values of the rRNA species were compared to each other as indicated. Average and standard devation values were calculated from four biological replicates. Protein levels were analyzed by Western blotting with the indicated antibodies (one example is shown). The α-tubulin staining of the membrane served as a loading control. (B) HeLa cells were transfected with three different siRNAs each targeting Mybbp1a (Mybbp1a.1, .2 and .3), Pes1 or GL3 control (ctrl) siRNAs. Four days after transfection HeLa cells were incubated for 6 h with ^{32}P-orthophosphate, total cell RNA was isolated and separated on an agarose gel. The incorporated radioactivity was detected with a PhosphoImager. (A)The level of 47S pre-rRNA after Mybbp1a.1 (grey bar), .2 (dark grey bar), .3 (light grey bar), Pes1 (white bar) and GL3 ctrl (black bar) siRNA transfection was normalized to the total rRNA load (ethidiumbromide stain of the 18S rRNA) and expressed relative to the ctrl. Metabolic labelling experiments were conducted in collaboration with M. Hölzel and D. Eick, Helmholtz Center Munich.

4.3 Purification and characterisation of Mybbp1a-associated factors

The functional characterisation of Mybbp1a has revealed the protein's involvement in the regulation of rRNA gene transcription potentially through direct interaction with the Pol I complex. Furthermore it was shown that Mybbp1a is required for a correct processing of the rRNA precursor to obtain mature rRNAs for subsequent ribosome biogenesis. To gain more insights into Mybbp1a-dependent complex compositions and thereby further obtain a better understanding of the functional mechanisms underlying the responsibilities of Mybbp1a in the nucleolus Mybbp1a-associated factors were purified by immunoprecipitation and analysed by a mass spectrometry-based approach. At the time of the following experiments no complex had so far been characterised by using Mybbp1a as bait for purification.

4.3.1 Mybbp1a localisation to the nucleolus is RNA-dependent

Several large RNA-containing complexes are involved in ribosome synthesis and implement pre-rRNA processing and pre-ribosome assembly in accordance with rDNA transcription (reviewed in (Granneman & Baserga 2005)). To test whether Mybbp1a is associated with RNA and the nucleolar ribosome biogenesis machinery in mammals, its cellular localisation after RNase A treatment of HeLa cells was studied by immunofluorescence microscopy firstly (Figure 27). Therefore HeLa cells were permeabilized and incubated with Ribonuclease A (RNase A) prior fixation. Immunodetection of the endogenous protein with α-Mybbp1a revealed a re-localisation of Mybbp1a from the nucleoli to the nucleoplasm upon RNase A treatment, whereas the Pol I-specific and rDNA-binding transcription factor TTF-I remained in the nucleoli. Further, control treatment with DNase I, an enzyme that does not hydrolyse RNA, did not visibly change the localisation of Mybbp1a. These findings suggest that nucleolar localisation of Mybbp1a depends on the interaction with an RNase A-sensitive complex, such as the ribosome biosynthesis machinery.

4.3.2 Mybb1a interacts with Fibrillarin, a rRNA processing factor

Next it was investigated whether Mybbp1a is indeed part of an RNA-containing complex, as suggested by the immunofluorescence experiments. Therefore, HeLa nuclear extract was fractionated by FPLC chromatography on a Superose 6 sizing column, and the fractions were assayed for the presence of Mybbp1a by Western Blotting (Figure 28A). Mybbp1a predominantly eluted with an apparent molecular mass in the MDa range (fraction 12, 14), showing that it is part of a large multi-subunit complex. In addition, comparatively lower amounts eluted in later fractions (fraction 22, 24) indicating a complex size of approximately 700 kDa. The observed elution pattern of Mybbp1a in extracts derived from human HeLa cells thereby qualitatively reflects the findings obtained on mouse Mybbp1a again

emphasising their conserved functional properties (see size fractionation of mouse MB III extracts depicted in Figure 13).

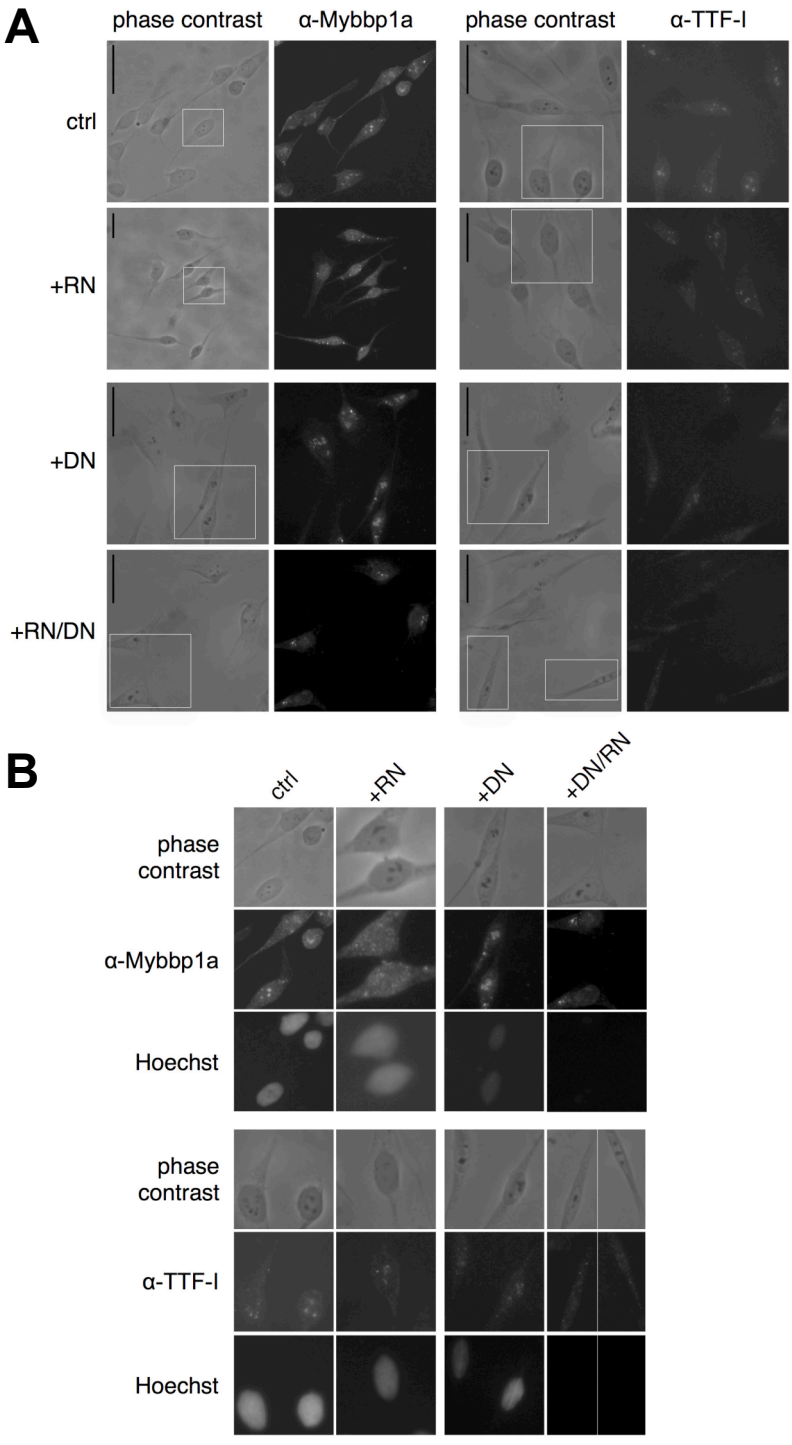


Figure 27: Localization of Mybbp1a is RNA-dependent.
(A) HeLa cells were either incubated with RNase A (+RN), DNase I (+DN), RNase A and DNase I (+RN/DN) or mock-treated (ctrl) prior to fixation and immunofluorescence staining with the indicated antibodies. pc: phase contrast image. Scale bars: 20 µm. (B) Magnifications of (A). Magnified image sections of (A) are marked by white frames. Phase contrast, Hoechst stain and antibodies for immunofluorescence images are indicated on the left and right. The respective treatment of cells is marked on top.

Furthermore, the protein was found to co-migrate with Fibrillarin, a protein involved in almost all major steps of ribosome biogenesis (Tollervey *et al.* 1993). Mybbp1a, and in part Fibrillarin, were shifted to fractions corresponding to lower molecular masses (fraction 22-26) when the extract was incubated with RNase A prior size-exclusion chromatography. The Pol I-specific transcription factor TTF-I and the Pol I core subunit PAF53, however, did not change their running behaviour RNase-dependently. Thus, Mybbp1a is very likely to be a part of a complex, of which its integrity is dependent on a RNA component. To confirm that Fibrillarin and Mybbp1a are present together in one complex, HeLa cells were transfected with the human Flag-Mybbp1a expression construct and co-immunoprecipitation experiments from nuclear extracts were performed with Flag-agarose (Figure 28B). Flag-Mybbp1a co-precipitated Fibrillarin (lane 7), and Fibrillarin also specifically eluted with Mybbp1a from the affinity matrix (lane 8) when compared to the control (lane 3 and 4, respectively). The data thereby confirm earlier proteomics data by Takahashi and colleagues (Yanagida *et al.* 2004) and identify Mybbp1a as an interactor of Fibrillarin.

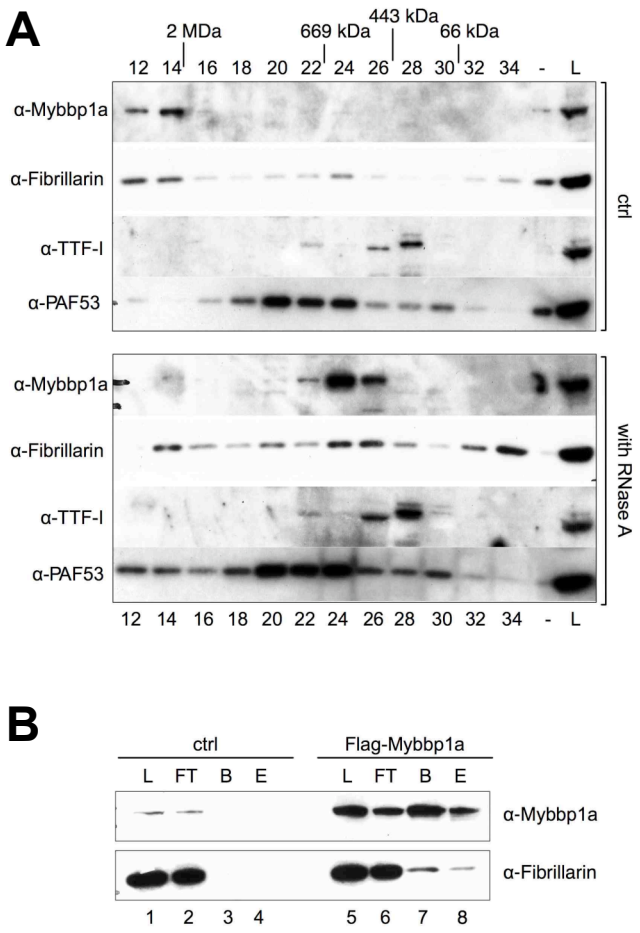


Figure 28: Mybbp1a is part of a RNA-sensitive protein complex and interacts with Fibrillarin.
(A) RNase A-treated and mock-treated (ctrl) HeLa nuclear extracts were separated on a Superose 6 gelfitration column. Load (8,7%) and collected fractions (every second fraction from 12 to 34) were analyzed by Western blotting with the antibodies indicated on the left. (B) Nuclear extracts of HeLa cells either transfected with Flag-Mybbp1a expression plasmid (lane 5 - 8) or control DNA (ctrl; lane 1 - 4) were incubated with α-Flag agarose. Load (L; 5%), flowthrough (FT; 5%), bead-bound (B) and eluted proteins (E) were analyzed by Western blotting with the indicated antibodies.

4.3.3 Mybbp1a interacts with the ribosome biogenesis machinery

To reveal additional binding partners of Mybbp1a, the Flag-tagged protein was transiently overexpressed in HeLa cells, and associated proteins were purified by Flag-agarose affinity chromatography, separated by gel electrophoresis and analysed by MALDI-TOF mass spectrometry. Several proteins were found enriched in the Flag-Mybbp1a-containing extract compared to extract from cells transfected with empty vector (Figure 29A).

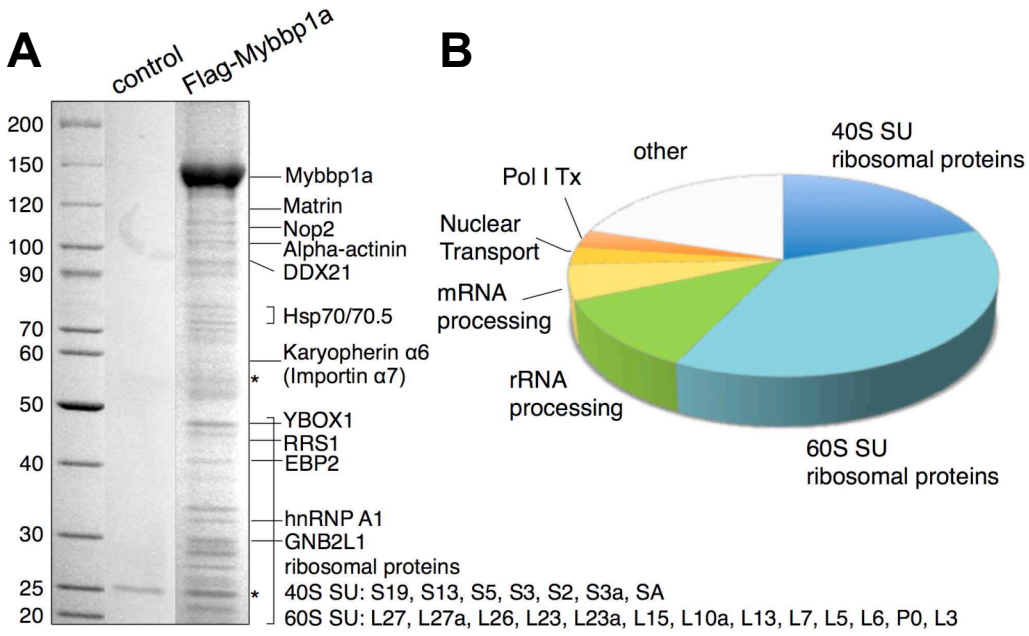


Figure 29: Purification of Mybbp1a-interacting proteins.
(A) Nuclear extracts of HeLa cells either transfected with Flag-Mybbp1a expression plasmid or control DNA (ctrl) were used for immunoprecipitation with α-Flag agarose. Eluted protein fractions were separated by SDS-PAGE, silver stained, protein bands were cut and subjected to MALDI-TOF mass spectrometry. Proteins identified are indicated on the right. (B) Schematic representation of gene ontology terms of Mybbp1a-associated proteins shown in (A).

As suggested by the previous experiments, Mybbp1a interacts with proteins belonging to the ribosome biogenesis pathway and the small and large ribosomal proteins that are assembled during rRNA transcription. Figure 29B gives an overview on the functional nature (gene ontology terms) of the purified proteins. Additionally, proteins, which have been directly connected to ribosome biogenesis before, are listed in Table 1 with their detailed function described in this pathway.

factor	pre-40S	pre-60S	export	citation
Nop2		+		y (Hong *et al.* 1997; Hong *et al.* 2001)
DDX21	+	+		m, x (Henning *et al.* 2003; Yang *et al.* 2005)
Karyopherin α6 (Importin α7)			potential function	y (Moy & Silver 1999)
RRS1	+	+		y (Tsuno *et al.* 2000; Zhang *et al.* 2007)
EBP2		+		y (Huber *et al.* 2000; Tsujii *et al.* 2000; Lebreton *et al.* 2008)
hnRNP A1				mRNA processing (Michael *et al.* 1995)
ribosomal proteins	+	+	+	m (Michael & Dreyfuss 1996)
Fibrillarin	+	+	+	y (Tollervey *et al.* 1991; Tollervey *et al.* 1993)

Table 1: Overview on Mybbp1a-associated factors with a function in ribosome biogenesis.
This table lists Mybbp1a-associated factors recorded in Figure 29, which have been shown to be involved in ribosome biogenesis, with their respective function in this pathway. The particular publications describing the proteins' functions are cited in the last column together with the respective model system. (y):yeast; (x): Xenopus laevis; (m): mammals.

4.3.4 Initial characterisation of Mybbp1a-associated proteins

Within the timely scope of the thesis work only an initial analysis of some of the proteins identified by mass spectrometry was possible to confirm their interaction with Mybbp1a. Additionally, the purified Mybbp1a-containing complex or complexes were further characterised with respect to protein and potential RNA composition.

4.3.4.1 Co-immunoprecipitation of Mybbp1a and associated factors

To confirm the interaction of Mybbp1a with the co-purifying proteins identified by mass spectrometry nuclear extract from HeLa cells was incubated with antibodies against the respective proteins, and immunoprecipitates were subsequently purified with Protein G-coated sepharose 4 Fast Flow (Amersham). Load, flow-through and precipitated fractions were analysed by Western Blotting with the according antibodies and α-Mybbp1a to monitor precipitation efficiency and co-purification of Mybbp1a, respectively (Figure 30). In parallel, Protein G sepharose was incubated with extract to visualise potential background binding. The data confirm the interaction of Mybbp1a with hnRNP1A when comparing α-Mybbp1a staining in α-hnRNP A1-precipitated fractions (lane 6) with the IgG control precipitation (lane 3). The results for EBP2 and Nol1 were not equally clear due to a relatively high background in the IgG control precipitations when stained with α-EBP2 and α-Nol1, respectively (lane 3). However, when comparing the Mybbp1a staining in the bound fraction to the corresponding load fraction of the IgG control lane (lane 3 to lane 1) and the specific antibody precipitation

(lane 6 to lane 4), a relative enrichment of Mybbp1a in both precipitations with the specific antibody seems to be indicated. Yet, this needs to be confirmed by further experiments.

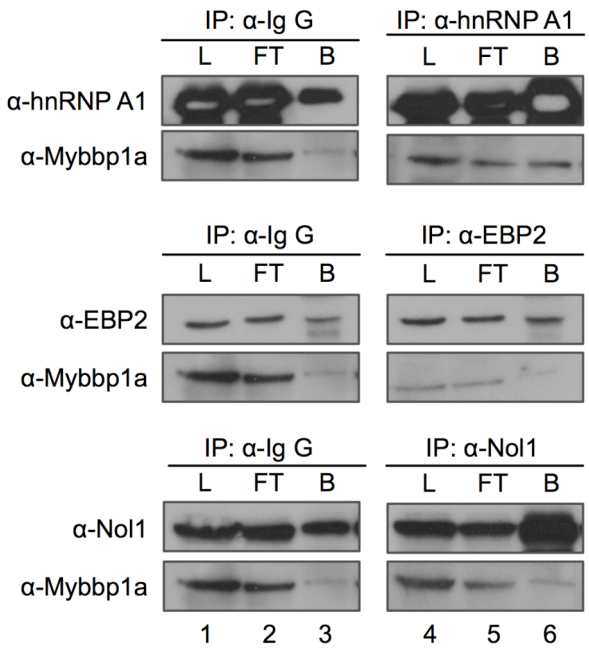


Figure 30: Co-immunoprecipitation of Mybbp1a and associated factors.
(A) Western Blot of immunoprecipitations from HeLa nuclear extract with antibodies against the newly identified, Mybbp1a-associated factors. Antibodies against the proteins indicated on top of each panel were incubated with nuclear extract and subsequently precipitated with Protein G-Sepharose. α-Ig G was used as background control. Immunopurified protein fractions were analysed with the antibodies indicated on the left.

4.3.4.2 Mybbp1a and its associated factors form a RNase-sensitive complex

To characterise the composition of the purified Mybbp1a-containing complexes several of the identified proteins were tested for their running behaviour on a Superose 6 size exclusion column in the presence or absence of RNA. Nuclear extracts were therefore treated with RNase A or with the vehicle, FPLC chromatography was conducted and the collected factions were analysed by Western Blotting with the according antibodies. Interestingly, all of the proteins assayed co-migrated with Mybbp1a in a large complex in the MDa range (fraction 12, 14) but were present in distinct smaller complexes when HeLa nuclear extracts were treated with RNase A prior chromatography (Figure 31). Thus, the results further support the presence of these factors and Mybbp1a in one RNase-sensitive complex. However, the exact composition of the smaller RNase-resistent modules still remains to be answered.

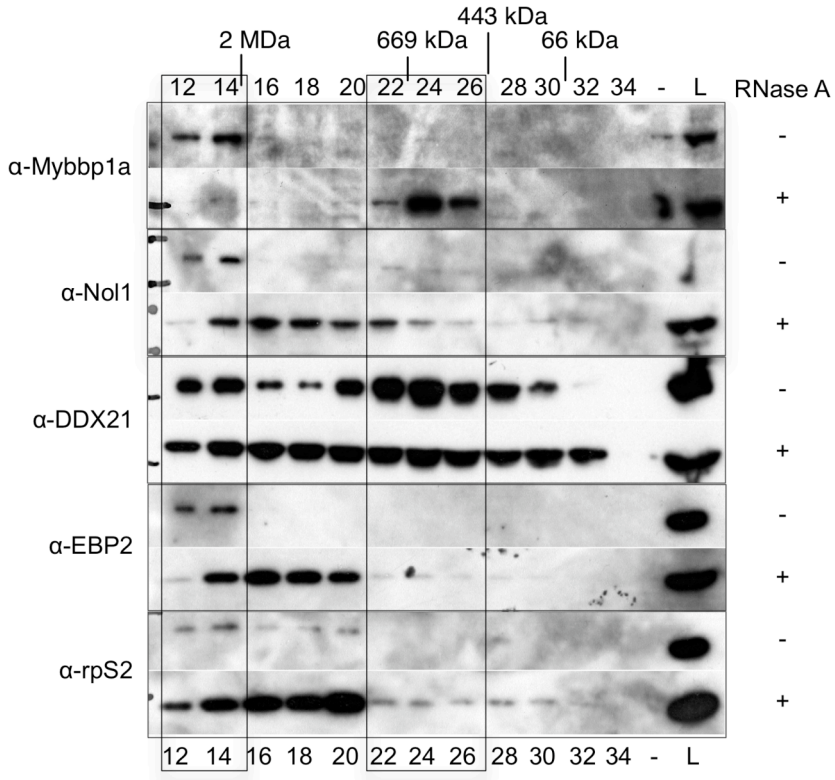


Figure 31: Mybbp1a and associated factors form a RNA-sensitive complex.
RNase A-treated (+) and mock-treated (-) HeLa nuclear extracts were separated on a Superose 6 gelfitration column. Load (8,7%) and collected fractions (every second fraction from 12 to 34) were analyzed by Western blotting with the indicated antibodies.

Overall, the data obtained during this thesis work demonstrate that Mybbp1a plays a direct role in the regulation of rRNA gene transcription, very likely through the interaction with the Pol I enzyme, and is a functional and structural component of the ribosome biogenesis machinery at the same time, placing the protein at the interface of transcription and processing. Thereby the so far unknown function of Mybbp1a in the nucleolus, the site of its predominant localisation, could be determined. In the following section the results will be precisely analysed and discussed in the context of currently available knowledge, particularly with respect to potential mechanisms underlying Mybbp1a's function and its interconnection with other cellular pathways. Next steps to further investigate the Mybbp1a protein and its cellular role will be evaluated.

5 Discussion

Despite its predominant localisation to the nucleolus a function of Mybbp1a in this nuclear compartment remained unknown. The data obtained during this thesis work determined two novel functions of Mybbp1a in the nucleolus. Firstly, the protein was shown to associate with the Pol I complex and serve as a negative regulator of rRNA gene transcription. At the same time it is a structural component of the ribosome biogenesis machinery and needed for efficient maturation of the single rRNAs and cellular proliferation, placing the protein at the interface of rRNA gene transcription and processing. In the following the experimental outcome will be discussed with regard to contents and technical particularities in the light of current knowledge. A major focus will be laid on the potential functional mechanisms underlying Mybbp1a's distinct responsibilities in rRNA gene transcription and rRNA processing. Furthermore, potential insights on Mybbp1a's contribution to the co-ordination of both processes as well as to regulatory events on cellular basis will be elaborated. Finally, prospective approaches for further characterisation of the Mybbp1a protein and its nucleolar and cellular role are evaluated.

5.1 Mybbp1a as a regulator of rRNA gene transcription: the mechanism behind

The activity of rRNA gene transcription in a given cell is the major determinant of cell proliferation and cell growth, and transcription is tightly regulated to match ribosome amounts with the physiological needs of the cell. The data achieved during the first part of this doctoral thesis revealed the ability of mammalian Mybbp1a to regulate rRNA gene transcriptional activity by a repressive mechanism. Transfection of a Pol I-specific reporter plasmid with parallel overexpression of different amounts of Mybbp1a disclosed a dose-dependent repression of Pol I-dependent transcription. The protein's repressive effect was further confirmed by siRNA-mediated depletion of Mybbp1a, which led to an augmentation of endogenous pre-rRNA levels. Originally Mybbp1a had been described as an interacting partner of the proto-oncogene c-Myb (Favier & Gonda 1994) and, since then, it has been shown to interact with, and to modulate the activity of, several other regulators of Pol II-dependent transcription. In this context, Mybbp1a was described to negatively modulate the activity of transcriptional regulators such as PGC-1α (Fan *et al.* 2004), NFκB (Owen *et al.* 2007) or the homeobox protein Prep1 (Diaz *et al.* 2007), thereby modifying many different cellular pathways. Thus, it appears as Mybbp1a might play an important role as a central regulator of proliferation, cell cycle progression and differentiation in the mammalian system, an idea that specially fits with its role in the regulation of rDNA transcription described in this study. However, the actual regulatory mechanism of Mybbp1a with respect to rRNA gene transcription is not yet fully resolved. Given the absence of any enzymatic domains as

revealed by the Conserved Domain algorithm and the presence of several potential protein-protein or protein-nucleic acid interaction motifs (Tavner *et al.* 1998; Keough *et al.* 1999; Keough *et al.* 2003; Yang *et al.* 2003) a rather non-enzymatic function of Mybbp1a for example as scaffold protein is indicated. Integrating the data from previous Mybbp1a studies and the results of this thesis work support several interesting conclusions on the potential events during Mybbp1a-mediated transcriptional repression.

5.1.1 Mybbp1a represses rRNA synthesis at early steps of transcription

Findings from distinct experiments presented here strongly suggest that Mybbp1a-mediated repression is targeting Pol I transcription already at the step of transcription initiation. Thus, the employed Pol I reporter construct contains solely the rDNA promoter sequence spanning the base pairs from -410 to +314 with respect to the transcription start site (Ghoshal *et al.* 2004). Therefore, Mybbp1a is ought to interrupt Pol I transcription either during the initiation process or at early steps of elongation. Importantly, transient overexpression of Mybbp1a in HeLa cells resulted in reduced binding of Pol I and the Pol I-specific transcription factor TTF-I at the rRNA gene promoter as monitored by ChIP experiments. Taken together these findings strongly suggest a repressive mechanism through a Mybbp1a-mediated perturbation of pre-initiation complex formation, most probably by directly interacting with the Pol I machinery leading to decreased rRNA synthesis. Interestingly, two different studies dealing with Mybbp1a-mediated repression of Pol II transcription report on a transcriptional impairment also at the stage of transcription initiation. Blasi and colleagues have shown by *in vitro* protein interaction and EMSA (electro mobility shift assay) experiments that Mybbp1a can directly disturb the interaction of Prep1 with its binding partner Pbx1, both of which are homeodomain-containing transcription factors and conjoint co-regulators of Hox-gene expression, and impede DNA-binding activity of the heterodimer to Hox-gene enhancer elements. Ectopic expression of Mybbp1a furthermore inhibited the activating role of Prep1-Pbx1 on Hox-gene expression *(Diaz et al. 2007)*. Similar indications for a regulatory role of Mybbp1a during transcription initiation came from the work of Hottiger and colleagues, which revealed a direct interaction of Mybbp1a with the activation domain of RelA/p65, a subunit of the transcription factor NF-κB, and co-repression of NF-κB-dependent transcription by Mybbp1a *in vitro*. Importantly, i*n vitro* transcription from DNA templates assembled into chromatin was only repressed when the template and RelA/p65 were pre-incubated with Mybbp1a prior to the addition of the transcriptional-active extract suggesting an intervention of Mybbp1a in a step even preceding the assembly of the pre-initiation complex. Still, also different mechanisms and time points of Mybbp1a-mediated transcriptional repression can be taken into account and will be briefly discussed in the following.

5.1.2 Is a chromatin-dependent process involved in Mybbp1a-mediated repression

The first hint to an involvement of chromatin-dependent processes in the repressive mechanism of Mybbp1a came from the work of Spiegelman and colleagues (Fan *et al.* 2004). When investigating the inhibitory activity of Mybbp1a on the transcriptional co-activator PGC-1α in C2C12 muscle cells overexpressing Mybbp1a they found cellular protein levels of PGC-1α and its promoter occupancy unchanged compared to the control, which is in contrast to the here presented data related to Pol I at the rDNA promoter. The authors have therefore suggested that Mybbp1a either has an intrinsic or is recruiting repressive activity and confirmed this assumption by tethering Mybbp1a alone to the promoter of a Pol II-dependent reporter gene. Their experiments further showed a dependency of its repressive activity on chromatin-related processes as Mybbp1a-mediated repression was relieved in the presence of the HDAC inhibitor TSA. Initial Pol I reporter experiments presented in this work also hinted to a chromatin-related mechanism for Mybbp1a repressor activity with respect to rRNA synthesis as incubation of cells with TSA led to a mild relieve of Mybbp1a-mediated repression compared to non-treated cells. Therefore Mybbp1a could be targeted to the Pol I promoter through the association with the Pol I machinery to subsequently recruit chromatin-dependent modifiers leading to the formation of a repressive chromatin structure. However, this initial observation needs to be thoroughly confirmed preferably by the help of a different experimental approach. The Pol I reporter assay uses a second reporter plasmid, which is Pol II-dependent and is co-transfected in order to adjust for potential differences in transfection efficiencies. As TSA also influences Pol II-dependent transcription it was difficult to gain reproducible results since too many parameters were affected at the same time and difficult to dissect. Interestingly, Hottiger and colleagues have added another and mechanistically different piece of evidence for chromatin-dependent transcriptional co-repression by Mybbp1a by showing a competition of Mybbp1a with the histone acetyl-transferase p300 for the binding to NFκB (Owen *et al.* 2007). Furthermore, Mybbp1a binding to the promoter of the *Per2* gene, which gets subsequently repressed, has been shown to correlate with di-methylation of the Lysine 9 residue of associated histone H3 (H3K9) (Hara *et al.* 2009), a histone modification tightly linked to transcriptional repression (Melcher *et al.* 2000; Rea *et al.* 2000). Particularly interesting, Ishida and colleagues subsequently demonstrated the capacity of Mybbp1a to bind to a H3 peptide di-methylated at K9 *in vitro,* which was two-fold higher compared to unmodified or acetylated peptide by peptide immunoprecipitation (Hara *et al.* 2009). Until now only few factors are identified to directly bind to histone modifications. One of the most characterised chromatin-binding proteins is HP1, which interacts specifically with di- and trimethylated lysine 9 of histone H3, and is mostly found in silenced chromatin (reviewed in (Fanti & Pimpinelli 2008)).

Taken together the information discussed above strongly indicates a chromatin-dependent process to be involved in the general regulatory mechanism of Mybbp1a. Still, it remains unclear if this is also true for the Mybbp1a-dependent regulation of Pol I transcription. An important issue to consider in this regard is the outcome and technical valuation of the performed ChIP experiments, which will be discussed in the subsequent chapter.

5.1.3 Conclusions from chromatin immunoprecipitation experiments

With respect to the data discussed above and given the interaction with the Pol I machinery a specific chromatin-binding pattern of Mybbp1a might be expected at the rRNA gene repeat, especially in or near the promoter region. Indeed, such binding has been identified for the potential Mybbp1a homologue Pol5p in budding and baker's yeast (Shimizu *et al.* 2002; Nadeem *et al.* 2006). Yet, none of several experimental approaches during the course of this thesis work revealed binding of Mybbp1a to rDNA associated chromatin in the mammalian system by chromatin immunoprecipitation. While binding of the Pol I subunit RPA116 and other factors related to rRNA gene transcription to the rDNA repeat was repeatedly identified, an association of Mybbp1a could not be detected despite the usage of different antibodies detecting endogenous or Flag-tagged Mybbp1a as well as qualitatively different chromatin preparations from HeLa cells either transiently overexpressing Flag-Mybbp1a or wildetype cells.

The negative outcome does certainly not rule out any existing direct or indirect chromatin interaction of Mybbp1a and therefore technical limits should be thoroughly considered. The conditions used for cross-linking (0,25% or 0,5% formaldehyde) prior chromatin preparation to covalently stabilise the protein-chromatin interactions were applied relatively mild. In the case that Mybbp1a does not directly bind to rDNA but via other factors such as the Pol I machinery association to r-chromatin might require higher concentration of or qualitatively different cross-linking agents, which particularly target protein-protein interactions and allow more sensitive detection of indirect chromatin association (Zeng *et al.* 2006). Furthermore, Mybbp1a was characterised as a repressor of rRNA gene transcription and it is therefore well possible that the chromatin association is additionally rather week in proliferating cells, which exhibit strong rRNA gene transcription activity. Re-evaluation of the immunoprecipitation experiment with extracts derived from proliferating mouse MB III cells stably expressing the Flag-tagged Pol I subunit RPA116 (presented in Figure 12, p. 54) led to a rough estimation of less than 1% of whole cell protein amounts of Mybbp1a to be associated with the Pol I complex. It might be therefore interesting to reassess a potential association of Mybbp1a to the Pol I complex and r-chromatin, respectively, after exposing cells to a repressive, stressful environment e.g. through nutrient starvation or growth factor deprivation. Finally, another

technical issue might be represented by an unsuitability of the employed antibodies or an inaccessible Flag-epitope under the selected conditions.
However, Mybbp1a function might actually not require association to r-chromatin, which is also indicated by the herein presented immunofluorescence studies of Mybbp1a localisation showing its RNA- but not DNA-dependent localisation to the nucleolus. Following this line of thought a hypothesis, in which Mybbp1a impairs pre-initiation complex formation by binding to the Pol I complex prior its recruitment to the rRNA gene promoter, is favourable. How this model could integrate chromatin-related processes to play a role in the repressive mechanism of Mybbp1a as indicated by the Pol I reporter assay in presence of TSA and the comprehensive data of other groups in the context of Pol II transcription remains to be investigated.

5.1.4 Insights from the potential yeast Mybbp1a-homologue Pol5p

Before finally concluding the discussion on the regulatory mechanism of Mybbp1a with respect to rRNA gene transcription, it is still important to briefly focus on the potential yeast homologue of Mybbp1a, Pol5p, which has been connected with an essential role in rRNA synthesis in earlier studies (Shimizu *et al.* 2002; Nadeem *et al.* 2006). *S. pombe* and *S. cerevisiae* Pol5p knockout strains have exhibited reduced amounts of newly synthesised rRNAs compared to wildetype in [^{3}H]-uridine pulse-labelling experiments, which seems, at first sight, to contradict our results of Mybbp1a being a transcriptional repressor in the mammalian system. However, the functional outcomes of Pol5p or Mybbp1a depletion are similar and can be explained by the second role of human Mybbp1a in ribosome biogenesis determined during this work. In the absence of Mybbp1a, the level of the rRNA precursor was increased as anticipated for a transcriptional repressor, but unexpectedly HeLa cells ceased proliferation. A detailed metabolic labelling analysis revealed that rRNA production was defective at the level of rRNA processing with decreased levels of newly matured rRNAs. While early yeast studies did not particularly look at rRNA precursor levels in Pol5p-deficient yeast strains the analysis by Hughes and colleagues delivered very detailed data sets on cellular levels of precursor and mature rRNAs by microarray analysis of whole cell RNA from numerous mutant strains. Their characterisation of a strain lacking Pol5p confirmed the decrease of mature rRNAs but also revealed the accumulation of the initial rRNA precursor (Peng *et al.* 2003), which nicely correlates with the here presented data from human cells depleted of Mybbp1a. Therefore it appears that the functional role of Mybbp1a and its potential yeast homologue, Pol5p, rather involves the coordination of transcription and processing than a stimulation of rRNA gene transcription. Remarkably, Pol5p was also found associated with the Pol I transcription machinery in yeast as the protein co-eluted with Rrn3, the yeast homologue of the rRNA transcription initiation factor TIF-Ia, after TAP-tag

purification (Robert Steinbauer, Herbert Tschochner, University of Regensburg, 2010; personal communication). This interaction remained intact during several biochemical purification steps with a final complex size of approximately 250 kDa corresponding to the size of a Pol5p-Rrn3-heterodimer. This association would certainly need to be confirmed in the mammalian system. Such assumed conserved interaction would further support that Mybbp1a represses rRNA synthesis prior transcription start through an impediment of TIF-IA co-activator function thereby antagonising the assembly of an initiation-competent Pol I complex. The following sections will now focus on Mybbp1a's function in rRNA processing and discuss it with respect to its role in Pol I transcription regulation.

5.2 Mybbp1a as part of a rRNA processing complex

Impaired proliferation in Mybbp1a-depleted cells early indicated an additional role for Mybbp1a in growth-related processes other than rDNA transcription and subsequently a defect at the stage of rRNA processing was confirmed by metabolic labelling of cellular RNA. Furthermore Mybbp1a was identified as a component of a pre-ribosomal complex associating with several rRNA processing factors as well as ribosomal proteins of the small and large ribosome subunits. Together, these results strongly argue for a direct role of Mybbp1a in the rRNA processing step of ribosome biogenesis. Even if the detailed functional mechanism of Mybbp1a and the exact processing step, in which the protein is involved, were not finally determined, the results of this thesis work allow certain implications on its purpose and indications for future work.

5.2.1 Accumulation of pre-rRNA upon Mybbp1a depletion is likely to be a combined consequence of transcriptional de-repression and impaired processing

As initially revealed by quantitative real-time PCR also metabolic labelling of nascent rRNA transcripts after siRNA-mediated depletion of Mybbp1a confirmed the accumulation of 47/45S precursor rRNA matching with Mybbp1a's role as a repressor of rRNA transcription. Importantly, the visualisation of not only the rRNA precursor but also its processed intermediates and final mature rRNAs has disclosed Mybbp1a's necessity for efficient and correct rRNA processing. Upon Mybbp1a depletion the mature rRNAs 18S and 28S as well as 32S, the rRNA intermediate preceding mature 28S, decreased below the levels of control cells when compared to the initial 47S rRNA amount. However, the employed assays systems cannot distinguish to which extent the accumulation of 47S pre-rRNA upon Mybbp1a depletion is caused by a transcription up-regulation due to de-repression of the rRNA gene or reflects the consequence of impaired rRNA processing. An effect similar to the latter was suggested for Pwp2 (Utp1), a component of the SSU processome (Bernstein *et al.* 2007), whose depletion led to pre-rRNA accumulation due to stalling rRNA processing.

Yet, it is possible to exclude an accumulation of 47/45S due to an overloading of the rRNA processing machinery after de-repression of rRNA gene transcription. In this case one would at least expect levels of newly synthesised intermediate and mature rRNA in Mybbp1a-depleted cells comparable to the amount in control-treated cells but no reduction. This is disproved by the results of the metabolic labelling experiments displayed earlier showing a clear decrease of the processed rRNAs (Figure 26, p. 72). A promising approach to distinguish between the transcription and processing reaction to analyse their respective effects after Mybbp1a depletion could include another set of Pol I reporter assays with different reporter plasmids. Such constructs could be composed of different sections and

lengths of the rDNA sequence, potentially also in association with mutated promoter sequences respectively processing sites, leading to different combinations of rRNA processing sites. By employing such strategy Huang and colleagues were able to investigate the stepwise recruitment of transcription and processing factors to specific rDNA sequences in dependency of Pol I transcription activity and the nature and length of rDNA sequence incorporated into Pol I reporter constructs (Kopp *et al.* 2007).

5.2.2 Mybbp1a is involved in early to middle but not in late steps of pre-rRNA processing

Depletion of Mybbp1a leads to a decrease of newly synthesised mature rRNAs needed for both, the SSU and the LSU of the ribosome. Unlike Pes1, which has been shown to be important for 28S maturation nicely confirmed by the metabolic labelling experiments, mybbp1a seems to be important for either several steps of rRNA processing or in very early processing steps, which would have an impact on both subsequent pathways. Notably, a comprehensive study by the Hughes lab using microarrays to measure the abundance of non-coding RNAs in several mutant yeast strains showed a qualitative similar pattern of pre-rRNA and newly processed mature rRNA levels in a strain lacking Pol5p as was observed for Mybbp1a-depleted mammalian cells. The authors subsequently classify this aberrant pattern as a processing defect related to the cleavage sites in the 5'ETS and A2 located in the intergenic spacer 1 (ITS1), which is similar to some of the mutants lacking components of the U3- (or SSU-)processome (Peng *et al.* 2003) and indicate a defect in very early processing steps. These indications from the potential yeast homologue overlap with the observations on mammalian Mybbp1a made during the work presented here or executed in collaboration with other laboratories.

Thus, the involvement of Mybbp1a in rather early processing steps is supported by the analysis of the sub-nucleolar distribution of Mybbp1a at sub-diffraction resolution, which was conjointly conducted by Dr. Attila Nemeth (Längst laboratory, Biochemie III, University of Regensburg, Germany) and Dr. Lothar Schermelleh (Department of Biology, LMU München, Germany). To distinguish the different functional parts of the nucleolus antibodies against the Pol I subunit RPA43, fibrillarin and B23/nucleophosmin were employed serving as markers for the fibrillar centre, dense fibrillar component and granular component, respectively. The images revealed that Mybbp1a localizes closer to, or overlaps in part with RNA Pol I and fibrillarin, but not with B23/nucleophosmin (Figure 32) suggesting a role in transcription and earlier but not in late processing events. Also nucleoplasmic and cytoplasmic staining of Mybbp1a was detected in agreement with previous studies, in which Mybbp1a was shown to shuttle between the nucleus and the cytoplasm (Keough *et al.* 2003; Yamauchi *et al.* 2008).

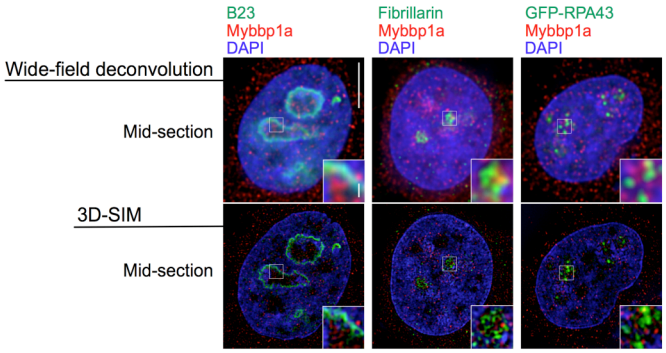


Figure 32: Subnuclear localization of MYBBP1A in relation to nucleolar marker proteins analyzed with super-resolution structured illumination microscopy (Schermelleh *et al.* 2008).
Co-immunofluorescence staining of HeLa cells with anti-MYBBP1A antibody vs anti-B23 antibody (granular component, top panel), anti-Fibrillarin antibody (dense fibrillar component, middle panel) and GFP-RPA43 (fibrillar center, bottom panel). DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI). Mid-section with conventional optical resolution are shown for comparison (left column). Scale bars: 5 µm and 0.5 µm (inset). Taken from (Hochstatter *et al.* unpublished).

Evidence that Mybbp1a is part of distinct rRNA processing complexes important for maturation of both the small and the large ribosomal subunits came from the analysis of Mybbp1a's sedimentation behaviour on a so-called polysome (sucrose) gradient, which visualises pre-ribosomal, ribosomal and polysomal particles by measuring the RNA absorbance and was performed in collaboration with Prof. Dr. Dirk Eick and Dr. Michaela Rohrmoser (Helmholtz Zentrum München, Germany) (Hochstatter *et al.* unpublished). The distribution of RNA and protein levels displayed in Figure 33 indicate a co-migration of Mybbp1a with the pre-40S and pre-60S particles but rather no integration into the 90S pre-ribosomal particle, which is responsible for the very early processing steps. The parallel control detection of Pes1 shows a different pattern and, as expected, the protein co-migrates predominantly with pre-ribosomal complexes important for the maturation of the large ribosomal subunit.

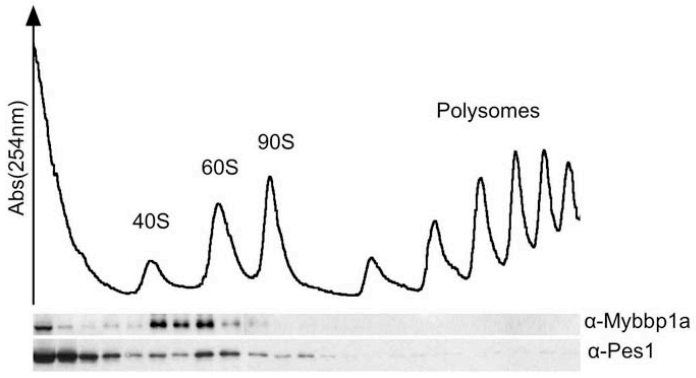


Figure 33: Sedimentation behaviour of Mybbp1a and (pre-)ribosomal particles on a sucrose gradient.
Polysome fractionation was performed on a sucrose gradient and Mybbp1a and Pes1 protein levels of the fractions were detected by immunoblotting. Pre-assembled ribosomal subunit, monosome and polysome fractions were detected by measuring UV absorbance at 254 nm as indicated on the graph. Taken from (Hochstatter *et al.* unpublished).

The involvement of Mybbp1a in maturation pathways of both ribosomal subunits is further supported by the characterisation of Mybbp1a-associated complexes presented in this thesis

work. Hence, Flag-affinity purification of Mybbp1a-associated factors from HeLa cells overexpressing Flag-Mybbp1a identified ribosomal proteins and rRNA processing factors required for the assembly of both the small and the large ribosomal subunit and the detailed composition of Mybbp1a-associated complexes and related functional implications will be subject of the following chapter.

Taken together, Mybbp1a is supposed to be part of pre-ribosomal complexes important for the maturation of both ribosomal subunits, which are involved in early to middle but not in the late processing steps. However, to definitely determine the individual processing steps, in which Mybbp1a is required, different types of experiments would be needed such as extended pulse labelling experiments to follow the maturation of the different rRNA species over time and/or Northern Blot analyses to be able to distinguish the entireness of rRNA processing intermediates and potential aberrations.

5.2.3 Lessons from the composition of the Mybbp1a-associated proteins

To get further information on Mybbp1a's mode of action Mybbp1a-containing complexes were investigated in detail. Analysis of HeLa nuclear extracts by size-exclusion chromatography has revealed Mybbp1a to be present in large multi-protein complexes, in which RNA seems to be an integral part as it is required for complex integrity and the nucleolar targeting of Mybbp1a. Directed interaction studies disclosed the association of Mybbp1a with Fibrillarin, a 2'-O-methyltransferase and a known component of box C/D small nucleolar ribonucleoprotein (snoRNP) particles (Tollervey *et al.* 1993), supporting a direct role of Mybbp1a in the rRNA processing events. Furthermore, a mass spectrometry-based purification of Mybbp1a-associated proteins identified a large variety of ribosomal proteins of both the SSU and LSU, as well as several other factors such as DDX21, Rrs1, Ebp2 and Nol1 (yeast Nop2), to which a role in rRNA processing and ribosome assembly has been assigned by previous studies (Tollervey *et al.* 1993; Hong *et al.* 1997; Tsujii *et al.* 2000; Tsuno *et al.* 2000; Henning *et al.* 2003). Although initial confirmatory co-immunopurification experiments with subsequent immunodetection of the proteins again indicated the interaction of Mybbp1a with some of these factors the results still need to be more thoroughly assessed and quantified by further co-immunopurification experiments or *in vitro* binding assays. Yet, mass spectrometry data is further supported by experiments from other laboratories confirming interaction of Mybbp1a with some of the purified factors (Yamauchi *et al.* 2008; Kuroda *et al.* 2011). The identified factors Rrs1 and Ebp2 were shown to affect rRNA processing, interestingly mirroring the effects observed for Mybbp1a, an accumulation of rRNA precursor and decrease of mature rRNAs (Tsujii *et al.* 2000; Tsuno *et al.* 2000). Rrs1 is a ribosome assembly factor that recruits the 5S rRNA and the ribosomal proteins rpL5 and rpL11 into nascent ribosomes (Zhang *et al.* 2007). Intriguingly, Rrs1 seems to be involved in

rDNA transcription regulation, as a yeast rrs1 mutant has been shown to reduce transcriptional repression of the rRNA gene (Tsuno *et al.* 2000). Interestingly, both proteins, Mybbp1a and Rrs1 have recently been connected to a role in the progression of mitosis. Mybbp1a is phosphorylated by the mitotic kinase Aurora B, and it has been revealed that depletion of Mybbp1a or Rrs1 leads to a mitotic delay and abnormalities in spindle organisation (Gambe *et al.* 2009; Perrera *et al.* 2010). These findings are especially interesting in the light of the proliferation defects upon Mybbp1a depletion observed by the here presented study and will be revisited again in the following. Even though expected, this purification approach did not identify any factors belonging to the Pol I complex. This might be due to the fact that an interaction of Pol I and Mybbp1a might be rather rare in strongly proliferating cells and is only present in cells with impaired rRNA biogenesis (see also 5.1.3, p 83, 2nd paragraph).

According to the mass spectrometry results and the data from size-exclusion chromatography, Mybbp1a is part of a large pre-ribosomal complex including an RNA component. Upon hydrolysis of the RNA the large complex is degraded into distinct sub-complexes, suggesting that the RNA functionally links the different sub-complexes. However, the detailed composition of the Mybbp1a-containing sub-complex remains to be uncovered. The existence of these distinct RNA-depleted sub-complexes potentially reflects the existence of various modules that constitute the ribosome biogenesis machinery, as shown by Krogan and co-workers for the SSU processome in yeast. In their work, they were able to identify three sub-complexes, called UTP A, B and C. Interestingly, Pol5p associates with the UTP A complex, which additionally includes the t-Utps (transcriptional U three proteins) (Krogan *et al.* 2004). As shown by Baserga and colleagues, these factors are not only required for proper rRNA processing, but also for efficient rDNA transcription (Gallagher *et al.* 2004). However, purification of the SSU processome by the group of Baserga did not reveal any association of Pol5p (Gallagher *et al.* 2004). Still, it would be interesting to assess a potential interaction of Mybbp1a with the human t-Utp homologues, which have been recently characterised by McStay and colleagues (Prieto & McStay 2007), in future to learn more on Mybbp1a's mode of action.

To summarise, the data presented here clearly show that Mybbp1a is a component of pre-ribosomal complexes and is functionally and structurally involved in pre-rRNA processing. Furthermore there is convincing evidence that Mybbp1a is involved in several, rather early steps of rRNA processing contributing to the maturation of both, the LSU and SSU of the ribosome. Therefore it is tempting to speculate that Mybbp1a links and coordinates the maturation of the SSU and LSU. Due to the fact that the protein lacks enzymatic domains but harbours several protein-protein interaction domains, Mybbp1a might act as a kind of scaffold to recruit and connect enzymatic activities and other factors needed for proper pre-

rRNA processing. However, the exact functional mechanism of Mybbp1a's contribution to an efficient and correct rRNA maturation remains unknown. A final characterisation of the Mybbp1a-containing RNase-resistant sub-complex was started, but could not be concluded during this thesis work. Its outcome may be expected to contribute conclusive insights on Mybbp1a's functional mechanism. Besides, the results obtained thus far deliver promising aspects for further directions of analyses and interesting cross-references to other cellular pathways.

5.3 Nucleolar business of Mybbp1a - integrating transcription and rRNA processing?

Almost all processes leading to mature ribosomes are organised within one specialised cellular compartment, the nucleolus. Pol I transcription, rRNA processing and ribosome assembly are therefore, even if localised in 3 different morphological sub-domains, in close proximity (Schwarzacher & Mosgoeller 2000). Although anticipated for long time evidences for directed co-ordination of these different processes remained unknown. First hints on molecular basis came from Tschochner and colleagues when biochemically purifying the Pol I complex in yeast. Next to the expected core Pol I subunits and transcription initiation factors they have additionally found rRNA processing factors associated (Fath *et al.* 2000). Intriguingly, the large complex was not only transcriptionally active but also capable of modifying RNA *in vitro*. Further evidence was subsequently provided by the visualisation of chromatin spreads of the rDNA loci, also known as 'Christmas trees', which impressively display the SSU processome capping the nascent rRNA precursors (Dragon *et al.* 2002) nicely visualising co-transcriptional rRNA processing. Eight years later Tollervey and colleagues have put a number to these images by measuring kinetics of Pol I transcription coupled with the quantification of cleavage events in nascent precursors. Thereby they could show that approximately 70% of nascent transcripts are cleaved co-transcriptionally at the early processing site to generate the precursor to mature 18S rRNA (Kos & Tollervey 2010). However, at present still only few data is available enlightening the co-ordinative mechanisms behind and their respective factors. Experimental results of this work have identified Mybbp1a as an interactor of RNA Pol I and demonstrated that Mybbp1a serves both as a negative regulator of rRNA gene transcription and as a functional subunit of the ribosome biogenesis machinery. As such, Mybbp1a plays a dual role in rRNA metabolism and by this means might serve to coordinate rRNA gene transcription and processing. Different mechanisms by which Mybbp1a might execute this function are discussed in the following.

5.3.1 A potential Mybbp1a-mediated feedback mechanism from aberrant rRNA processing to Pol I and II transcriptional activity

Mybbp1a is part of a large multi-protein complex and experimental results of this thesis work have demonstrated RNA being an integral part of this complex as it is required for complex integrity and the nucleolar targeting of Mybbp1a. A potential regulatory mechanism to adapt transcription rates to the actual presence of effective processing complexes for efficient rRNA processing may involve an intact RNA, potentially the rRNA precursor itself, and tethering of Mybbp1a to the rRNA processing machinery. Upon defects in rRNA processing,

Mybbp1a may be released from the processing complex to repress rDNA transcription and thereby coordinate the activity of transcription and processing.

Interestingly, Yamauchi and colleagues have recently shown that Mybbp1a is processed upon ribosomal stress induction (Yamauchi *et al.* 2008). Treatment of cells with actinomycin D, cisplatin or UV, all of which inhibit ribosome biogenesis, lead to a partial proteolytical cleavage of Mybbp1a to generate C-terminally truncated $p140^{MBP}$ and $p67^{MBP}$ proteins and their partial translocation to the nucleoplasm (Yamauchi *et al.* 2008). These findings conform to an earlier proteomic study by Lamond, Mann and colleagues, in which the whole set of nucleolar proteins was determined and subsequently investigated for their presence after Actinomycin D treatment (Andersen *et al.* 2002; Andersen *et al.* 2005). After translocation to the nucleoplasm Mybbp1a might then be able to also adapt cellular pathways other than rRNA expression to the actual state of the cell. As previously reported Mybbp1a as well as its posttranslational cleavage product $p67^{MBP}$ are able to regulate several cellular key pathways by interacting with their respective transcriptional regulators such as NFκB, PGC-1α or c-Myb (Tavner *et al.* 1998; Fan *et al.* 2004; Owen *et al.* 2007). Furthermore, Yamauchi and colleagues found the full-length Mybbp1a and the processed forms to be present in distinct complexes possibly emphasising different responsibilities. While both complexes share nucleolin and nucleophosmin, the larger complex including full-length Mybbp1a contains as well many ribosomal proteins, topoisomerase I, nucleostemin and histone H1x and the smaller complex EBP1 (Erb3-binding protein) (Yamauchi *et al.* 2008). The Mybbp1a-containing ribosome assembly/processing machinery identified in this study is likely to be the equivalent to the large complex purified by Yamauchi and colleagues as it is of similar size and as well contains a large set of different ribosomal proteins. However, both studies did not succeed to identify the complete set of proteins purified and therefore might have missed several interaction partners, which could explain the remaining differences.

5.3.2 Is Mybbp1a a typical t-Utp?

As mentioned before a group of the U3-snoRNA-associated proteins, the t-Utps, has been found essential not only for proper rRNA processing but also for optimal rDNA transcription *in vivo* by Baserga and colleagues (Gallagher *et al.* 2004). Next to their capability of binding to the 5'ETS region of rDNA chromatin, the assembly of these factors revealed to be independent of the presence of U3 snoRNA or ongoing rDNA transcription. Their capacity to participate in both reactions, rDNA transcription and rRNA processing, make these proteins potential candidates for co-ordination of these processes. Shortly beforehand Greenblatt and colleagues had independently affinity-purified these factors as a sub-complex of the SSU processome when investigating the composition of yeast RNA-processing complexes and named it the UTP A complex (Krogan *et al.* 2004). Presumably reflecting the same

complexes the UTP A complex purification still slightly differed from the t-Utp sub-complex by lacking one of the t-Utps (t-Utp 5) but, interestingly, including the yeast homologue of Mybbp1a, Pol5p. The presence of Pol5p in this rRNA processing complex nicely supports our finding of Mybbp1a being important for proper rRNA processing in the mammalian system. However, the epitope-tagged purification of Mybbp1a-associated components of this thesis work did not identify any interaction with the human t-Utp homologues. Their requirement for both, efficient transcription and processing of the 47S pre-rRNA, could be confirmed (Prieto & McStay 2007) indicating that this coordinative mechanism is conserved throughout evolution. As an interaction between the human t-Utps and Mybbp1a was not explicitly assessed, a re-investigation might be of benefit to confirm their structural relationship in the mammalian system. However it is already evident now that Mybbp1a does not exhibit the mechanistic features of a typical t-Utp as Mybbp1a seems to functionally antagonise these factors. Even though t-Utps as well as Mybbp1a are both important for efficient rRNA processing t-Utps are needed to guarantee efficient rRNA gene transcription while Mybbp1a was shown to contrariwise repress rRNA gene transcription activity. Hence, Mybbp1a seems to play a distinct role in a feedback mechanism adapting transcription activity to later steps in ribosome biogenesis.

5.3.3 Nucleolin - lessons from a factor with similar functional features

Nucleolin, a more well-characterised factor present in both differently sized, Mybbp1a-containing complexes determined by Yamauchi and colleagues, is especially interesting since it displays several functional features, which have been also described for Mybbp1a. Thus, nucleolin is required for several steps in ribosome biogenesis such as efficient pre-rRNA maturation (Ginisty *et al.* 1998), ribosome assembly (Ginisty *et al.* 2000) and subsequent nuclear export (Borer *et al.* 1989; Schmidt-Zachmann *et al.* 1993) and, notably, has been also implicated in negative regulation of rDNA transcription (Roger *et al.* 2002, 2003). Furthermore nucleolin is not only a regulator of ribosome biogenesis but has been additionally connected to the modulation of Pol II-dependent transcription in several cases (nucleolin's multiple functions are reviewed in (Mongelard & Bouvet 2007)). Intriguingly, nucleolin was identified as a direct interaction partner of c-Myb and co-transfection of nucelolin reduced c-Myb regulated transcriptional activity (Ying *et al.* 2000), which is identical to the findings of Gonda and colleagues related to the N-terminal cleavage product of Mybbp1a, the $p67^{MBP}$ protein (Tavner *et al.* 1998). A very recent study by Bouvet and colleagues has assessed the capacities of nucleolin from a different point of view aiming to integrate the distinct aspects of nucleolin function (Angelov *et al.* 2006). According to their data nucleolin exhibits histone chaperone activity by promoting the destabilisation of the histone octamer and stimulating a SWI/SNF-mediated transfer of H2A-H2B dimers.

Furthermore the protein facilitates transcription through chromatin *in vitro* thereby acting as an elongation factor. Suggested by the authors its capability of stimulating different remodelling enzymes could thereby explain the different roles of the nucleolin factor. Interestingly, Mybbp1a has been also found in a complex containing the Snf2h remodeler, the B-WICH complex, for which a role in Pol III transcription has been suggested (Cavellan *et al.* 2006). Taken together nucleolin-related data might further underline a potential chromatin-related function of Mybbp1a and give a possible explanation of the various influences of Mybbp1a on different metabolic pathways in distinct cellular compartments. In this context it would remain to uncover if Mybbp1a acts by recruitment of other factors or harbours intrinsic chromatin-specific activity, which could be assessed in diverse experimental settings for example as its application as co-factor in the nucleosome mobility assay or its binding capacity to chromatin exhibiting different histone modifications. Furthermore Mybbp1a's ability to migrate between nuclear structures under specific conditions might further emphasize a role of the protein in the mutual adjustment of important cellular pathways. Here, the final chapter will focus on the impact of Mybbp1a on cell cycle regulation and cellular proliferation.

5.4 Mybbp1a - an interface for ribosome biogenesis and cell cycle regulation?

The experimental results of this thesis work have clearly shown an essential role of Mybbp1a in cellular proliferation, an effect that may relate either to its function in pre-rRNA processing or in the regulation of extra-nucleolar interaction partners. The presented experiments cannot discriminate between these scenarios. Notably, Mybbp1a-depleted cells exhibit a flattened and enlarged morphology that is clearly visible on day 4 after siRNA-mediated knockdown (Figure 23, p. 68). This phenotype mirrors the 'flat cell phenotype' of Retinoblastoma (Rb)-negative SAOS-2 sarcoma cells, when overexpression of Rb leads to a cell cycle arrest in late G_1 phase (Hinds *et al.* 1992). Interestingly, in yeast, the potential Mybbp1a homologue Pol5p has recently been described to interact with Cdc10p (Nadeem *et al.* 2006), a component of the cell cycle-regulating complex MluI Cell Cycle Box [MCB] Binding Factor (MBF), which is the functional equivalent of the mammalian E2F-DP (Aligianni *et al.* 2009). These factors participate in the specific gene expression wave during the G_1-S transition, named 'Start' in yeast and 'restriction point' in mammalian cells (Caligiuri & Beach 1993). A number of studies suggest a co-regulation of ribosome biogenesis and cell cycle progression in the yeast and mammalian systems. Regulation occurs at various steps during the cell cycle and employs several factors, including components of both the SSU and LSU processome, such as Utp 1-15, 17, 18 (Bernstein & Baserga 2004), human Utp 18 (Holzel *et al.* 2010) and Bop1 (Pestov *et al.* 2001), as well as ribosomal proteins (Lohrum *et al.* 2003; Zhang *et al.* 2003; Jin *et al.* 2004). These factors are crucial for proper cell cycle progression and target p53- and Whi5- (the yeast functional equivalent of Rb (Jorgensen *et al.* 2002; de Bruin *et al.* 2004)) mediated control mechanisms. A first hint on a direct interaction came from a very recent study, which has characterised a physical association of Mybbp1a with p53 in the nucleoplasm leading to a Mybbp1a-dependent stabilisation of a p53-p300 complex. As a consequence p53 gets acetylated and stabilised by the acetyl transferase and subsequent p53-dependent gene expression leads to cell cycle arrest (Kuroda *et al.* 2011). Thus, it would be interesting to further address a possible direct role of Mybbp1a in coordinating transcription regulation, pre-rRNA processing and cell cycle progression in future studies.

5.5 The working hypothesis and implications for a future characterisation of Mybbp1a

Based on the experimental outcome of this thesis work and in consideration of the available literature a future working hypothesis is introduced in the following. Suggestions for potential future experiments to further explore Mybbp1a function under different aspects have been already given throughout the discussion part. Still, this final chapter aims at integrating potential future approaches to provide a concluding comprehensive picture of the actual state of experiments. The transcription of rRNA genes, subsequent pre-RNA processing and ribosome assembly processes together constitute the major energy-consuming process in the cell and therefore the rate of ribosome biogenesis needs to be tightly linked to cellular proliferation. Currently available data suggest that in proliferating cells, Mybbp1a is mainly associated with the pre-ribosomal complexes where it acts as a scaffold for rRNA processing and assembly factors and is functionally required to drive efficient ribosome biogenesis. Reduced levels of ribosome biogenesis upon stress signals and/or reduced demands of ribosomes potentially result in the disassembly of pre-ribosomal particles, which involves the partial processing of Mybbp1a and its release to the nucleoplasm.

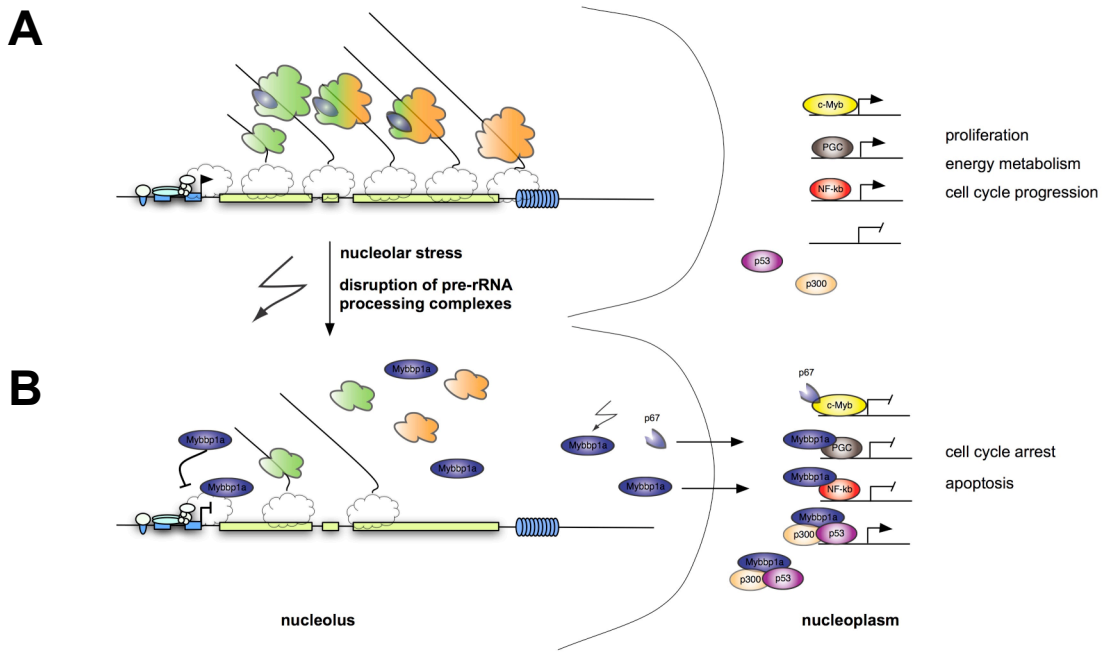


Figure 34: A future working hypothesis - Mybbp1a is part of a feed back mechanism to co-ordinate Pol I transcription and rRNA processing with the actual conditions of the cell.
(A) Under proliferative conditions Mybbp1a (blue ellipse) is part of the pre-rRNA processing complex and supports efficient ribosome biogenesis and cellular proliferation. (B) Nucleolar stress might lead to disruption of pre-ribosomal particles, partial processing of Mybbp1a and its release to the nucleoplasm. While full length Mybbp1a would repress rDNA transcription nucleoplasmic Mybbp1a would influence Pol II-dependent gene expression and p53 stabilisation leading to cell cycle arrest and eventually apoptosis (see also text).

While nucleolar full-length Mybbp1a would repress RNA Pol I transcription, potentially by disturbing pre-initiation complex formation or recruitment of chromatin-related factors to the

rDNA promoter, processed nuclear Mybbp1a would modulate the activity of transcription regulators, such as c-Myb, PGC-1α or NF-κB, to cease cell cycle progression, proliferation and energy production. It is well established that internal and external signalling pathways target Pol I-associated factors to translate the current demand of ribosomes in pre-initiation complex formation and subsequent rRNA synthesis rates. It will be interesting to assess such a role for the heavily phosphorylated Mybbp1a protein (Beausoleil *et al.* 2004; Beausoleil *et al.* 2006; Nousiainen *et al.* 2006; Olsen *et al.* 2006; Yu *et al.* 2007; Dephoure *et al.* 2008; Wang *et al.* 2008; Gauci *et al.* 2009).

The suggested hypothesis raises two major questions for future characterisation with respect to Mybbp1a function. On the one hand a definite mode of action or even different mechanisms underlying Mybbp1a function could not be finally determined during this thesis work. Here, three different aspects would be especially interesting for future assessment. First, the detailed characterisation of direct interaction partners of Mybbp1a could allow further insights on how Mybbp1a exerts its function as rDNA transcriptional repressor or component of the rRNA processing machinery. It would be interesting to see by the help of *in vitro* binding studies with recombinant proteins if Mybbp1a directly interacts with a subunit of the Pol I enzyme complex or rather with one of its associated factors such as TIF-IA. In addition, a final identification of the associated factors within the RNase-resistant complex or the assessment of existing interaction of Mybbp1a with the human t-Utps would be helpful to further shape the protein's role in rRNA processing. Second, taking into account the experimental results of this thesis work and others it seems prospective to investigate a potential involvement of chromatin-related processes in Mybbp1a's mode of action in more detail. Besides the reassessment of Mybbp1a binding to r-chromatin by qualitatively modified and extended ChIP experiments the analysis of potential interaction with specific chromatin modifications or modifiers such as remodelling machines or histone modifiers as well as its impact on chromatin-related processes such as nucleosome remodelling by functional assays would allow further conclusions on this issue. Finally, it would be important to distinguish between the impact of Mybbp1a on rDNA transcription and rRNA processing, which is rather difficult on the endogenous level. Therefore a dissection of these processes could be better realised by transient transfection of various Pol I reporter constructs including different lengths of rRNA coding sequence to analyse Pol I transcriptional output and eventually the recruitment of the rRNA processing machinery including Mybbp1a.

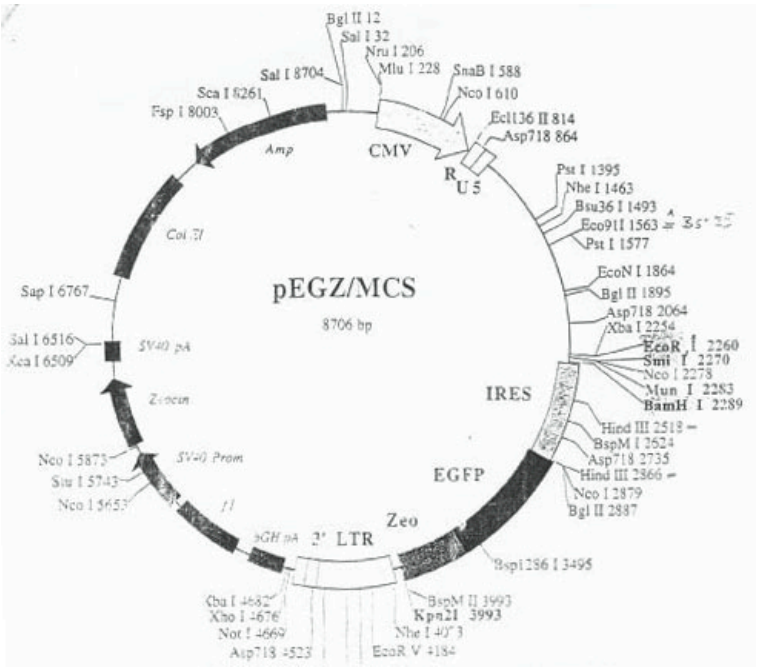
The second major issue for further investigation is the potential involvement of Mybbp1a in cell cycle regulation and/or other specific signalling pathways. Different data sets from the yeast and mammalian systems point in this direction. It will be especially interesting to test any physic interaction or functional impact of Mybbp1a with or on the Rb or the p53 pathway.

Intriguingly, very recently Yanagisawa and colleagues have determined a Mybbp1a-dependent stabilisation of p53-p300 interaction upon nucleolar stress to enhance p53-mediated transcription activation (Kuroda *et al.* 2011). Furthermore, it will be interesting to analyse, which signalling pathways target Mybbp1a by phosphorylation and in which way this might modulate its activity and function with the of help mutation analysis and the application of small molecule inhibitors directed against these pathways.
Importantly, several of the latest Mybbp1a-related publications also put a clinical relevance to the protein. For example, Mybbp1a was found to interact with the survival of motor neurons protein (SMN), of which reduced levels cause the inherited disorder spinal muscular atrophy (SMA). Mybbp1a partially co-localised with SMN in Cajal bodies in HeLa cell nucleoplasm and, like SMN, was reduced in cells from an SMA patient (Fuller *et al.* 2010). Most notably in this regard is the identification of Mybbp1a interaction with PGC-1α, a key regulator of energy metabolism, as well as Prep1, a homeodomain transcription factor (Fan *et al.* 2004; Diaz *et al.* 2007). The Prep1 factor has subsequently been shown to have a role in glucose homeostasis and insulin sensitivity in mouse models mediated, at least in part, by the interactor Mybbp1a (Oriente et al. 2008). These findings thereby contribute important implications for the understanding of energy balance and the development of diabetes.

Taken together Mybbp1a integrates many interesting functional features with respect to rRNA metabolism as revealed by this thesis work and various other important cellular pathways such as regulation of cellular proliferation, cell cycle and energy metabolism. Additionally, its further characterisation is not only important with regard to its cellular role(s) but most probably also to the understanding of mechanisms underlying fundamental cellular processes such as transcription regulation and co-ordination with subsequent RNA processing.

6 Appendix

6.1 Plasmid maps



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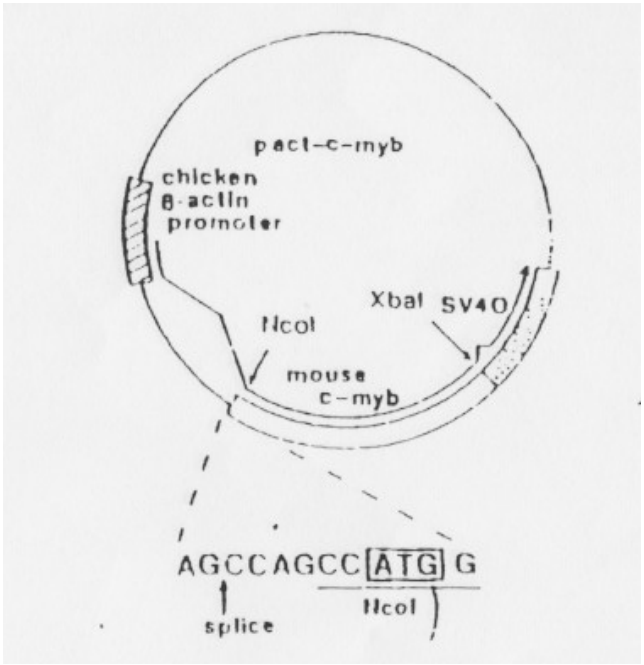

pact-c-myb

6.2 Abbreviations

3'/5'ETS	3'/5' external transcribed spacer
3C	chromosome conformation capture assay
aa	amino acid
ALP	acetyltransferase-like protein
ATP	adenosine-5'-triphosphate
AM-X	Tris based buffer containing X mM KCl
bp	base pairs
BSA	bovine serum albumine
CEA	chicken egg albumin
CHIP	chromatin Immunoprecipitation
Ci	Curie
CK2	casein kinase 2
CPE	core promoter element
CSB	Cockayne syndrome protein B
DMEM	Dulbecco's modified Earle's medium
DNA	deoxyribonucleic acid
DNase I	deoxyribonucleosidase I
DNMT	DNA methyl transferase
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinase
EX-X	Tris based buffer containing X mM KCl
FBS/FCS	foetal bovine/calf serum
FRAP	fluorescent recovery after photo bleaching
g	gram or relative centrifugal force
HDAC	histone deacetylase
HMG	high mobility group
HRP	horse radish peroxidase
h	hour
Ig	immunoglobulin
IGS	intergenic spacer
IRES	internal ribosome entry site
ITS1/2	internal transcribed spacers 1 and 2
JNK	c-Jun N-terminal kinase

kDa	kilo daltons
LSU	large subunit of the ribosome
M	molar
MBD	methyl-CpG-binding domain
MEM	modified Earle's medium
min	minute(s)
MNase	Micrococcus Nuclease
MOPS	3-(N-morpholino)propanesulfonic acid
mTOR	mammalian target of rapamycin
Mybbp1a	Myb-binding protein 1a
NAD	nucleolus-associated chromatin domains
ncRNA	non-coding RNA
NLS	nuclear localisation sequence
NOR	nucleolar organiser region
NoRC	nucleolar remodeling complex
NP-40	Nonidet P-40
Nt	nucelotide
N-terminal	amino-terminal
$p67^{MBP}$	N-terminal fragment of Mybbp1a (67 kDa)
$p67^{MBP*}$NLS	N-terminal fragment of Mybbp1a (67 kDa) containing the SV40 T-antigen NLS
$p140^{MBP}$	N-terminal fragment of Mybbp1a (140 kDa)
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate-buffered saline
PBS-T	Phophate buffered saline –Tween [0,1%]
PCR	polymerase chain reaction
PCV	packed cell volume
PEI	Polyethylenimine
Pen/Strep	Penicillin/Streptomycin
PIC	pre-initiation complex
Pol I, II, III	RNA polymerase I, II, III
pre-rRNA	precursor of ribosomal RNA
PTRF	polymerase I transcript release factor
r-chromatin	rRNA gene-containing chromatin
rDNA	ribosomal DNA
RNA	ribonucleic acid
RNP	ribonucleoprotein

rpm	rounds pro minute
r-proteins	ribosomal proteins
rRNA	ribosomal RNA
RT	room temperature
RT-qPCR	real-time quantitative PCR
S	Svedberg unit
SDS	sodium Dodecyl Sulphate
sec	second
siRNA	small interfering RNA
SL1	selectivity factor 1
Snf	sucrose non-fermenting
Snf2h	Snf2 homolog protein
snoRNA	small nucleolar RNA
SSU	small subunit of the ribosome
TAF	TBP-associated factor
TBE	Tris borate EDTA buffer
TBP	TATA-binding protein
TIF-IA / B / C	transcription initiation factors for RNA polymerase I
Tip5	TTF-I interacting protein 5
Tris	Tris(hydroxymethyl)-amino-methane
TSA	Trichostatin A
TTF-I	transcription termination factor for RNA polymerase I
TTFΔN185	N-terminal (aa 1 to 185) truncated form of TTF-I
t-Utp	transcriptional-Utps
Tween-20	polyoxyethylene-sorbitan monolaurate
UBF	upstream binding factor
UCE	upstream control element
Utp	U three proteins
V	Volt

References

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