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Actin and Myosin in Transcriptional and Post-Transcriptional Control of Gene Expression

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To Eva and Nela.

ABSTRACT

Actin plays a key role in basal gene regulation. In the past decade actin has been found to be a component of chromatin remodeling complexes, ribonucleoprotein particles and to associate with all eukaryotic RNA polymerases. Based on the above discoveries the main objective of this thesis has been to elucidate some of the molecular mechanisms through which nuclear actin controls synthesis and processing of RNA transcripts.

In mammals, elongation of pre-mRNA transcripts is regulated by the interaction between actin and the heterogeneous nuclear ribonucleoprotein hnRNP U. **In Paper I** we investigated the molecular mechanisms underlying their cooperative function. We discovered that actin and hnRNP U interact with the phosphorylated RNA polymerase II carboxy-terminal domain to recruit the histone acetyl transferase PCAF to active genes. This mechanism is required to establish permissive chromatin for efficient transcription elongation.

There is emerging evidence that changes in the polymerization state of nuclear actin are important for gene regulation. Along these lines, **in Paper II** we found that nuclear actin dynamics is necessary for RNA polymerase II mediated transcription. We show that the F-actin severing protein cofilin-1 maintains the pool of monomeric actin to be fed into growing actin polymers and this mechanism is specifically required for elongation of pre-mRNA. Altogether these findings suggest actin polymerization occurs to facilitate migration of elongating RNA polymerase II along active genes.

Evidence that the interaction between actin and nuclear myosin 1 (NM1) is important for RNA polymerase I transcription elongation led us to investigate their potential synergy in post-transcriptional control of rRNA biogenesis. **In Paper III** we found that in nucleoli NM1 associates with rRNA, NM1 becomes incorporated into newly synthesized ribosomal subunits and cooperates with actin for their maturation. We also found that rRNA-associated NM1 interacts with the export receptor CRM1 and the RNA binding nucleoporin Nup153 at the basket of the nuclear pore complex (NPC). We propose that NM1 accompanies newly assembled export-competent ribosomal subunits from nucleolus to NPC, thus modulating both their maturation and export.

LIST OF PUBLICATIONS

I. **Obrdlik Ales**, Kukalev Alexander, Louvet Emilie, Östlund Farrants Ann-Kristin, Caputo Luca and Percipalle Piergiorgio.
The histone acetyltransferase PCAF associates with actin and hnRNP U for RNA polymerase II transcription. *Mol Cell Biol* (2008) **28**, 6342-6357.

II. **Obrdlik Ales** & Percipalle Piergiorgio.
Cofilin-1 controls a nuclear pool of monomeric actin required for RNA polymerase II transcription elongation
Submitted manuscript.

III. **Obrdlik Ales**, Louvet Emilie, Kukalev Alexander, Naschekin Dimitri, Kiseleva Elena, Fahrenkrog Birthe and Percipalle Piergiorgio.
Nuclear myosin 1 is in complex with mature rRNA transcripts and associates with the nuclear pore basket. *FASEB J* (2010) **24**, 146-157.

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Other Publications

Obrdlik Ales, Kukalev Alexander & Percipalle Piergiorgio.
The function of actin in gene transcription. *Histol Histopathol* (2007) **22**, 1051-1055.

Percipalle Piergiorgio and **Obrdlik Ales.**
Analysis of nascent RNA transcripts by chromatin RNA immunoprecipitation. *Methods Mol Biol* (2009)**567**, 215-235.

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LIST OF ABBREVIATIONS

Arp	Actin related protein
ATP	Adenosine triphosphate
ATPase	ATP hydrolase
CBC	cap binding complex
CTD	Carboxy terminal domain
DNA	Deoxyribonucleic acid
EJC	Exon-junction-complex
F-actin	Filamentous actin
G-actin	Globular actin
HAT	Histone acetyl transferase
hnRNPs	Heterogeneous nuclear ribonucleoproteins
kb	Kilobase
kDa	Kilodalton
mRNA	Messenger RNA
NES	Nuclear export signal
NLS	Nuclear localization signal
NPC	Nuclear pore complex
Nup	Nucleoporin
ORF	Open reading frame
Pre	premature
RBD	RNA binding domain
RNA	Ribonucleic acid
RNAP	DNA dependent RNA polymerase
rRNA	Ribosomal RNA
rRNP	Ribosomal ribonucleoprotein particle
S	Svedberg
tRNA	Transport RNA
UTR	Untranslated region
WASP	Wiskott Aldrich Syndrome protein
WSTF	Williams syndrome transcription factor

1 INTRODUCTION

In eukaryotic cells, actin and myosin are highly conserved proteins which are present in cytoplasm and nucleus.

In the cytoplasm actin readily polymerizes into dynamic filamentous structures and synergizes with several myosin species to perform essential cellular functions. These include maintenance of cellular structure, cell motility, vesicle transport and signal transduction. The presence of actin and myosin in the cell nucleus has been quite controversial for many years. Only recently, a growing wealth of evidence has finally overruled the general skepticism around their presence in the nucleus. There is now evidence that actin is a component of chromatin remodeling factors, ribonucleoprotein particles (RNPs) and associates with all three eukaryotic RNA polymerases. These observations and the discovery of nuclear forms of certain myosin species implicated in gene transcription point towards the presence of nuclear actin-based myosin motors. At this stage we still have limited mechanistic insights. In either case these observations open several interesting questions on how actin and myosin cooperate with the different machineries involved in RNA biogenesis.

The main objective of the present thesis has been to elucidate some of the molecular mechanisms through which nuclear actin and myosin control synthesis and processing of RNA transcripts throughout the gene expression pathway.

2 GENE EXPRESSION

The gene expression pathway is a universal process which is highly conserved from prokaryotic to eukaryotic organisms. It proceeds through a number of critical steps during which the information encoded in the DNA molecule is transcribed into either non-coding RNA molecules or into message-containing mRNA transcripts which are then used as templates to make proteins.

In eukaryotic cells, due to the presence of a more complex genome and the physical division between nucleus and cytoplasm, gene expression is considerably more complex than in prokaryotes. There are multiple checkpoints that ensure a virtually faultless process. The most important checkpoint is at the transcriptional level but it is known that once the transcript has been correctly synthesized it is processed, assembled into transported particles and further exported into the cytoplasm where it serves as template for translation.

In the next chapters I discuss the mechanisms that control expression of genes at both transcriptional and post-transcriptional level and emphasize the importance of the interplay among these machines for efficient gene expression in eukaryotic cells.

2.1 EUKARYOTIC DNA DEPENDENT RNA POLYMERASES

Beside genes coding for proteins, there is an ever-growing family of genes encoding functional RNA molecules. Among these, the best known are rRNAs, tRNAs, which are found in prokaryotes and eukaryotes, being required for translation and protein synthesis. Eukaryotic small nucleolar (snoRNA) and small nuclear (snRNA) RNAs were shown to be involved in rRNA and mRNA processing as well as RNA export. In recent years however small interfering (siRNAs) and micro (miRNAs) RNAs have gained much attention due to their involvement in post-transcriptional regulation of gene expression. The abundance of these different RNAs in the eukaryotic cell underlines the complexity of the field of RNA biogenesis.

In eukaryotic cells synthesis of the different RNA species that we have so far mentioned requires highly specialized DNA-dependent enzymes known as RNA polymerases I, II and III. RNA Polymerase II (RNAP II) and RNA polymerase I have

been extensively studied over the past decades. RNAP II transcribes protein-coding genes into mRNA; it is also involved in transcription of miRNAs, snoRNAs and snRNAs. RNA polymerase I (RNAP I) specializes in transcription of rDNA genes into rRNA transcripts which incorporate into ribosomal subunits. RNA polymerase III (RNAP III) transcribes tRNA and 5S rRNA genes and it is by far the least well characterized.

RNAP I, II and III are multi protein complexes consisting of 14, 12 and 17 subunits respectively. The core enzyme of the RNAP II comprises 10 (Cramer et al. 2001) subunits from which five subunits (Rpb1, Rpb2, Rpb3, Rpb11 and 9) share sequence and structural similarities not only with RNAP I, RNAP III, but also with bacterial RNA polymerases (Zhang et al. 1999; Vassylyev et al. 2002) and the archeal RNA polymerase (Kusser et al. 2008) Interestingly the remaining five subunits (Rpb5, Rpb6, Rpb8, Rpb10 and 12) are shared by all three eukaryotic polymerases. In this scenario the two largest subunits Rpb1 and 2 and their paralogues in RNAP I and III shape the active site cleft in the enzymes where RNA polymerization takes place and function as polymerase specific interaction hubs for the remaining subunits and for RNAP specific general transcription factors (GTFs) (Cramer et al. 2008; Carter and Drouin 2009).

2.2 RNA POLYMERASE II

In eukaryotic cells protein coding genes are transcribed by RNAP II. Transcription can be subdivided into three major phases, namely initiation, elongation and termination. Despite principle similarities among the three RNAPs, the RNAP II C-terminal domain consists of tandem arranged $Y_1S_2P_3T_4S_5P_6S_7$ heptapeptide repeats and is commonly referred as CTD. The CTD in yeast encompasses 26, in C. elegans 32 and in mammals 52 repeats. Thus the number of heptapeptide repeats increases with genomic complexity. During the RNAP II transcription cycle, the CTD is subjected to specific phosphorylation and de-phosphorylation cycles on its serine residues in position 2 (Ser_2) and in position 5 (Ser_5). These specific phosphorylations serve as recognition signals for CTD binding factors. Up to date two kinases (CDK7 and CDK9) and three phosphatases (FCP1, Ssu72 and Rtr1) have been shown to target the CTD during each transcription cycle. The interplay between kinases and phosphatases contributes to determining the transcription state in which RNAP II is engaged i.e.

transcription initiation, elongation or termination phase (Meinhart et al. 2005). In either case the different CTD phopshorylation states function as a recruitment-code for specific factors which are recruited during the different phases of transcription to facilitate RNAP II activity (Maniatis and Reed 2002; Sims et al. 2004; Buratowski 2009).

Eukaryotic DNA is packed into chromatin. Chromatin is a condensed structure that reduces severely the DNA accessibility to the transcription machinery. Nevertheless a set of genes has to be transcribed permanently to maintain cell homeostasis. Thus the chromatin structure in the proximity of these "housekeeping" genes is less compact and more accessible. On the other hand genes, which are transcribed in response to stimulation by metabolites, cell-cell contacts, hormones or other signals, are called inducible genes. When not induced the chromatin is condensed and needs to be unpacked and remodeled in order to enable binding of transcription factors and RNAP II. Therefore transcription initiation at inducible genes is accompanied by major chromatin rearrangements at gene promoters. However, both inducible and housekeeping genes need a set of general transcription factors, chromatin remodelers and co-activators to be activated and maintained in an "open" configuration.

2.2.1 Formation of the pre-initiation complex (PIC)

The transcription cycle starts with the assembly of the pre-initiation complex (PIC) at the gene promoter. This complex is established in a step wise manner during which general transcription factors (GTFs) such as TFIID, TFIIB, TFIIF, TFIIE, TFIIH and RNAP II itself are sequentially recruited. The first step during PIC assembly is the binding of TFIID via its TATA- box binding protein (TBP) subunit to the RNAP II promoter sites. TBP is a single polypeptide with two very similar domains which specifically recognize and bind to the minor groove of the DNA double helix at TA-rich sequence elements also referred to as TATA-boxes. These sequences are usually located 25 nucleotides upstream the transcription start site. TBP binding induces a bending of the DNA helix and an opening of the minor groove (Nikolov et al. 1996). This first step is essential for subsequent recruitment of TFIIB, TFIIF, RNAP II, TFIIE and TFIIH and for proper PIC positioning.

PIC formation is achieved not only through binding of the GTFs, but also through recruitment of gene specific transcription factors, activators or repressors to the

promoter and other regulatory sequences such as enhancer and silencer sites. PIC assembly is facilitated by the mediator complex, a large multiprotein complex that appears to be essential for subsequent transcription engagement (Takagi and Kornberg 2006). There is evidence that the mediator complex interacts with non-phosphorylated RNAP II CTD (Myers et al.1998) as well as activator and repressor proteins bound to their regulatory sequences (Lee et al. 2000; Kuras et al. 2003; Mo et al. 2004). Based on these observations the Mediator has been suggested to bridge activator or repressor proteins with the PIC complex to mediate transcription activation (Kornberg 2005).

2.2.2. Initiation

TFIIH plays a central role in transcription initiation. TFIIH consists of nine subunits including three essential enzymatic functions for successful initiation: DNA-dependent ATPase, ATP-dependent DNA helicase and a cyclin-dependent kinase (Thomas and Chiang 2006). While ATPase and helicase are important to create an open complex and to unwind the DNA double helix at the promoter site, the cyclin-dependent kinase CDK7, targets the RNAP II CTD. As mentioned above the eukaryotic mediator complex bridges DNA-bound activators with the unphosphorylated RNAP II in the PIC. Once the mediator is recruited to the RNAP II it strongly stimulates the CTD kinase activity of CDK7 in TFIIH. CDK7 phosphorylates Ser5 residues within the CTD heptapeptide repeats, which in turn results in disruption of the mediator binding to RNAP II. Thus, once the mediator has accomplished its mission in recruiting and stabilizing the RNAP II at the promoter site, it catalyzes its own release from the polymerase, remaining at its position for subsequent re-initiations (Yudkovsky et al. 2000; Max et al. 2007). The phospho-Ser5 modification at the CTD is a recruitment signal for mRNA capping enzymes to the CTD closely located near the mRNA exit pore in the RNAP II complex. The capping enzyme catalyzes formation of a protective 7-methyl-guanosine-cap at the 5'end of newly synthesized RNA (Fabrega et al. 2003). However this reaction is only feasible once the pre-mRNA 5'end emerges from the RNAP II. This moment in transcription is the final step in initiation and results in promoter clearance. Following the capping reaction, formation of stable elongation complex takes place only when the newly synthesized pre-mRNA/DNA hybrid in the RNAP II complex has reached a length of 20-30 nucleotides (Shatkin and Manley 2000; Sims et al. 2004).

2.2.3 Elongation

Transition from initiation to elongation is a multi-step process that is currently under investigation. Briefly, CTD phospho-Ser5 modification in the initiation phase triggers recruitment of mRNA-capping enzymes to the CTD. It also stimulates recruitment of the negative elongation factor NELF and positive elongation factor DSIF to RNAP II. DSIF consists of two subunits Spt4 and Spt5 that are believed to stabilize NELF at the polymerase. The presence of NELF at the polymerase stalls the transcription complex in close proximity, downstream the promoter site. These RNAP II complexes are often referred to as paused or poised RNAP II. After completion of 5' mRNA capping a third modifying factor is recruited to the complex, the positive transcription elongation factor p-TEFb. Interestingly, its recruitment to the CTD is mediated by the capping enzyme 7-methyl guanosine transferase which stays associated to the RNP II throughout the elongating phase. p-TEFb contains the kinase CDK9, which phosphorylates Spt5 in DSIF and the Ser2 residues at the CTD of RNAP II, thereby alleviating the inhibitory effect of NELF and providing the signal for the recruitment of elongation factors to the RNAP II (Peterlin and Price 2006).

As mentioned above the CTD phosphorylation state changes as the transcribing RNAP II complex travels along the gene cassette. These changes are mediated by the activities of kinases and phosphatase at promoter, proximal and distal regions of the transcribed protein coding gene. More specifically, during transcription the enzymatic activity of CDK7 and CDK9 is antagonized by at least three phosphatases, Fcp1, Ssu72 and Rtr1 (Buratowski 2009). The phosphatase Fcp1 de-phosphorylates phospho-Ser2 and acts at the promoter and coding regions. Fcp1 is considered as the antagonist of CDK9. However at promoter proximal regions Fcp1 is stimulated in its activity through TFIIF resulting in low levels of phospho-Ser2 signals at the 5'end of coding regions (Cho et al. 2001; Kamada et al. 2003; Buratowski 2009). Interestingly, Ser5 dephosphorylation has been an issue of investigations for the last decade. The originally described Ser5-phosphatase Ssu72 as subunit of the cleavage and polyadenylation complex could only be linked to CTD-dephosphorylation at the 3' end of gene coding regions and did not seem to have any effect on CTD phosphorylation during elongation (Krishnamurthy et al. 2004). Until recently the postulated Ser5-phosphatase (Sims et al. 2004) which may act during elongation

remained unknown. Rtr1, an evolutionary conserved RNAP II binding protein, seems to retain phosphatase activity, targeting phospho-Ser5 residues throughout the entire transcriptional elongation (Mosley et al. 2009). The shift between phospho-Ser5 to phospho-Ser2 via phospho-Ser2/5 (hyperphosporylated state) within the RNAP II CTD is an essential mechanism for transcriptional termination.

2.2.4 Termination

RNAP II transcription termination consists of two tightly linked events: cleavage and polyadenylation at the mRNA polyA site (PAS) and rapid degradation of downstream RNA which is still attached to the RNAP II. The elevated levels of phospho-Ser2 CTD towards the 3' end of transcribed genes play herein a central role. Two proteins that preferentially bind phopsho-Ser2 CTD are involved in this coupling. One of them is Pcf11, a component of the cleavage and polyadenylation factor complex. The other protein is Rtt103 which is known to mediate recruitment of a specific exonuclease that degrades RNA synthesized downstream the PAS (Barilla et al. 2001; Kim et al. 2004; Richard and Manley 2009). Pcf11 as a component of the cleavage/polyadenylation complex bridges this complex to the CTD of the elongating RNAP II. Once the polymerase has passed the polyadenylation consensus site (PAS), the RNA is cut 20 nt downstream the PAS. This reaction liberates the mRNA itself from the transcription complex. The free 3' OH group, serves the poly(A) polymerase (PAP) to add poly(A) tail to the nascent mRNA which is recognized and bound by the nuclear poly(A) binding (PABP). The novel 5' end of the remaining RNA is immediately targeted for fast degradation. The rapid RNA degradation is mediated by Rat1/Xrn2 5' to 3' exonuclease which is recruited via the CTD-associated Rtt103 to the elongating RNAP II. Once the RNA from the continuing RNAP II is digested the transcription complex becomes destabilized and disassembles from the DNA, an event which is considered as the RNAP II termination step. Since the exonuclease activity literally catches up with the polymerase, this mode of termination has been described as the “torpedo” model (West et al. 2004).

2.3 RNA POLYMERASE I

The human haploid genome contains around 200 copies of tandemly repeated ribosomal RNA (rRNA) genes which are transcribed by RNAP I. The rRNA gene repeats are arranged in long arrays and are termed nucleolar organization regions (NORs) which are located on acrocentric chromosomes. During early interphase NORs further assemble into nucleoli, the site where RNAP I transcription occurs.

The rRNA gene cassette is approximately 30kb long and encompasses a coding region of approximately 14kb. This coding region encodes a 47S rRNA precursor which is processed into 18S, 5.8S and 28S mature rRNAs. These rRNA products, together with the RNAP III-transcribed 5S rRNA are assembled into ribosomal subunits. RNAP I transcription is tightly regulated through cell growth and proliferation (Grummt 2003; Boisvert et al. 2007). Interestingly no more than 50% of the 200 rRNA gene copies are transcribed by RNAP I at a given time and therefore it is believed that chromatin organization plays an important role in the regulation of RNAP I mediated transcription (Lawrence and Pikaard 2004; McStay and Grummt 2008). In either case similarly to RNAP II, RNAP I requires a set of auxiliary factors that mediate promoter recognition, promote transcription elongation and facilitate transcription termination.

2.3.1 RNA polymerase I initiation and elongation

The rDNA promoter displays a modular organization with a transcription start site proximal core promoter (CP) and an upstream control element (UCE). In mammals RNAP I transcription begins with recruitment of the upstream binding factor (UBF) and promoter selectivity factor SL1/TIF1B to the promoter site. SL1 is a multiprotein complex composed of TBP and TBP-associated factors (TAFIs). UBF contains several high mobility (HMG) boxes. It was suggested that a UBF dimer binds UCE and CP elements through its tandemly arranged HMG boxes looping the intervening DNA into a nucleosome-like structure. These rearrangements bring UCE and CP in close proximity, leading to the formation of a stable UBF/SL1 complex that serves as platform for RNAP I recruitment to initiate transcription (Stefanovsky et al. 2001; Sanij and Hannan 2009). The transcription competent RNAP I complex contains numerous additional components which have direct regulatory or modulation effects on

the RNAP I transcription: TIF-1A, casein kinase II (CK2), chromatin modifiers like PCAF and G9a and components of the DNA repair machinery like PCNA, TFIIH and Topo2 (Drygin et al. 2010).

In this complex, TIF-1A is essential for transcription initiation and serves as a bridge between the UBF/SL1 complex and the RNAP I. It is unclear whether RNAP I undergoes a transition from a closed pre-initiation complex to an open complex (Grummt 2003; Drygin et al. 2010). In either case *in vitro* studies performed in the group of Joost Zomerdijk revealed, that UBF functions not only in the formation of stable recruitment platform for RNAP I at rRNA gene promoter but seems to be also engaged in promoter clearance (Panov et al. 2006). Additionally, TIF-1A was recently reported to be phosphorylated by CK2, a modification that seems to induce its release after transcription initiation as a pre-requisite for RNAP I elongation (Bierhoff et al. 2008).

UBF also seems to have a role in RNAP I transcription elongation. There is evidence that UBF occupies rRNA gene coding region (O'Sullivan et al. 2002). Insights into UBF function along rRNA gene coding region came from the observation that ERK-phosphorylated UBF functions as an activator of RNAP I transcription elongation by maintaining an open chromatin state in the rDNA (Moss et al. 2006; Stefanovsky et al. 2006). A conclusive mechanistic aspect derived from a recent *in vivo* study showed that UBF binding out competes the linker histone H1 from rDNA, inducing chromatin decondensation (Sanij et al. 2008).

2.3.2 RNA Polymerase I termination

RNAP I transcription termination is triggered by specific sequences located downstream the rDNA transcription unit in the so-called intergenic spacer (IGS). In the mouse IGS there are ten 18nt long termination elements. The first element, the primary terminator, is referred to as Sal-box. An additional important sequence for RNAP I termination is found upstream the Sal box. It represents a poly T stretch which functions as transcript release element. The Sal-box is recognized and bound by the transcription termination factor TTF-1 which causes sterical hindrance and induces RNAP I pausing. The RNA-DNA hybrid at the upstream T-stretch consists of an A-U heteroduplex which seems to destabilize the paused RNAP I (Lang and Reeder 1995). rRNA 3' end formation and release of paused RNAP I requires the

transcript release factor (PTRF) which is likely to be recruited to the poly U at the rRNA 3' (Mason et al. 1997; Jansa et al. 1998). Recent evidence in yeast demonstrated that deletion of Rat1, Xrn1 and Rai1 leads to a read-through of RNAP I elongation complex through the termination elements T1 and T2 (El Hage et al. 2008; Kawauchi et al. 2008). indicating that RNAP I termination may share common features with the "torpedo-model" suggested for the RNAP II termination pathway (Richard and Manley 2009).

2.4 CHROMATIN DYNAMICS AND TRANSCRIPTION

As mentioned previously the eukaryotic genome is compartmented into the nucleus. Since eukaryotic genomes are mostly composed of long stretches of non-coding sequences and their genes are monocistronically organized, eukaryotic genomes are larger than bacterial genomes. Consequently, eukaryotic cells developed during their evolution a way to condense their DNA into a higher order structure with a high packaging rate, which is referred to as chromatin. The nucleosome is the smallest packaging unit of chromatin. The core structure of a nucleosome is provided by a histone-octamer, built from two sets of histones, namely H2A/H2B heterodimers, one H3/H4 heterotetramer. The DNA double-helix is then wrapped through 1.67 left-handed superhelical turns on a stretch of approximately 146 bp around the octamer. Adjacent nucleosomes are joined through a stretch of linker DNA which can vary from 10 to 80 bp, a configuration that seems to be both species- and tissue-dependent. In higher eukaryotes a linker histone (most commonly H1) joins adjacent nucleosomes and it is usually found in chromatin regions with very high compaction (Kornberg and Thomas 1974; Felsenfeld and Groudine 2003). Histones are basic proteins which are evolutionary conserved. They comprise a structurally defined globular domain with a conserved histone fold and flexible N- and short C-tails that protrude outward from the nucleosome. Specific residues at these tails are subjected to post-translational modifications (PTMs), such as acetylation, methylation, phosphorylation ADP-ribosylation and ubiquitination. Collectively, these histone modifications have been proposed to serve as a "code" for factors, which subsequently bind the modified histone octameres and regulate chromatin-associated events like transcription, DNA repair and DNA replication (Jenuwein and Allis 2001). The dynamic changes in the compaction of chromatin, which are mediated by histone modifying enzymes in combination with

chromatin remodeling factors, regulate the accessibility for transcription factors and RNAPs to coding regions in DNA and are therefore essential for efficient gene expression regulation.

Two types of PTMs have been extensively characterized in the past years and are considered to play the most subtle role in regulating transcription, histone acetylation and histone methylation. Proteins that mediate histone acetylation are termed histone acetyl transferases (HATs). Methylations of histones are mediated by methyl transferases (HMTs). Together with histone deacetylases (HDACs) and demethylases that catalyze removal of acetyl and methyl groups, HATs and HMTs contribute to define highly dynamic actylation-methylation chromatin states (Selth et al. 2010).

2.4.1 Histone acetylation and histone methylation – marks for transcription

Acetylation of lysine residues within histone tails neutralizes their basic charge and results in an overall decrease in the number of positive charges. This modification is thus considered to weaken the high affinity of the nucleosome octamer towards the DNA, thereby loosening compaction of the corresponding chromatin. Acetylation facilitates RNAP progression through chromatin and supports ATP-dependent chromatin remodeling factors which alter nucleosomes arrays in active or inactive chromatin sites. On the other hand the effect of histone methylation on arginine and lysine residues is less understood. Methylation of positively charged amino-acids does not change the charge on these residues and thus it does not affect the net-charge of the nucleosome. It is believed that histone methylation rather functions as a mark for subsequent recruitment of factors which either loosen or tighten chromatin structure thereby regulating transcription or higher order chromatin packaging (Jenuwein and Allis 2001; Sims et al. 2004; Selth et al. 2010).

In RNAP II mediated transcription, some of the best characterized HATs are CBP, p300, PCAF and GCN5L, which are known to function as co-activators during transcription initiation (Marmorstein 2004; Thomas and Chiang 2006). Consistently, a recent genome wide analysis in yeast, revealed hyperacetylation at promoters of actively transcribed genes (Pokholok et al. 2005). In contrast gene coding regions showed only marginal acetylation levels (Hebbes et al. 1992; Clayton et al. 1993; Pokholok et al. 2005; Selth et al. 2010).

Insights into how acetylation may be impaired in gene coding regions were provided by the discovery of the histone methyltransferase Set2 in yeast and humans. Set2 is recruited via phospho-Ser2 CTD to the elongating RNAP II. It targets lysine 36 on histone H3 (H3K36) and catalyzes its tri-methylation (Krogan et al. 2003; Li et al. 2003; Schaft et al. 2003). The tri-methylation mark on H3K36 is found in coding regions of nearly all transcriptionally active genes in yeast as well as in humans (Pokholok et al. 2005; Barski et al. 2007). H3K36 methylation shows an opposite pattern in comparison to H3K9, K14 and H4K16 acetylation which are predominatly found at promoter sites. An insight into the link between the inverse pattern of acetylation at promoter and H3K36 methylation at gene coding regions came from studies in yeast. In this model system, the HDAC Rpd3S was shown to be recruited to this H3K36 tri-methylation via the chromodomain containing subunit Eaf3 and the PhD finger containing subunit Rco1. The presence of an HDAC in gene coding regions, as a result of co-transcriptional H3K36 methylation, leads to hypoacetylated chromatin when compared to gene promoter sites. At the beginning these findings were unclear. However two independent studies revealed that an impairment of the H3K36 methylation in yeast mutants not only resulted in high acetylation levels in gene coding regions but also in increased cryptic transcriptional initiations at certain gene coding sites (Carrozza et al. 2005; Joshi and Struhl 2005). Based on these findings it was concluded that co-transcriptional H3K36 methylation in chromatin is crucial for re-establishment of the chromatin state which otherwise becomes altered and accessible through the movement of the elongating RNAP II.

The above considerations imply the need for co-transcriptional recruitment of HATs to facilitate migration of elongating RNAP II through hypoacetylated gene coding regions. Indeed, there is evidence that several HATs target histones in gene coding regions. Among these were the human and yeast elongator HAT complex, the Gcn5 containing SAGA complex in yeast and p2D10 a homologue of TFIIIC 220 in *Chironomus tentans* (Kristjuhan et al. 2002; Sjölinder et al. 2005; Close et al. 2006). p2D10 is of special interest since its recruitment to the elongating RNAP II was shown to be dependent on actin and will be discussed later in this thesis.

2.4.2 ATP dependent chromatin remodelers and histone chaperones

ATP dependent chromatin remodeling complexes and histone chaperones are also recruited to active genes through histone modifications. Chromatin remodelers use the free energy of ATP hydrolysis to alter chromatin structure and can be subdivided into four main families: SWI/SNF, ISWI, CHD and Ino80. The role of chromatin remodelers in transcription regulation has been traditionally investigated at gene promoter and cis-acting regulatory elements. Examples for early involvement of chromatin remodelers in transcriptional activation can be found for the human SWI/SNF complex, recruited to promoter sites of glucocortisol-inducible genes via the GR receptor (Kwon et al. 1994). Human WSTF (Williams syndrome transcription factor), a subunit of the WINAC complex, is recruited to Vitamin D regulated genes (Kitagawa et al. 2003). There is also evidence that chromatin remodelers are coupled to all transcription stages (Clapier and Cairns 2009). Chd1 for example has been linked to transcriptional elongation in drosophila and yeast and was shown to interact physically with transcription elongation factors and elongating RNAP II (Simic et al. 2003; Marfella and Imbalzano 2007; Murawska et al. 2008). Others like human SWI/SNF have been shown to promote transcription elongation of hsp70 gene (Brown et al. 1996). Chromatin remodeling does not necessarily lead to activation of gene transcription: it can also have a repressive effect. In general repressive chromatin remodeling complexes reorganize chromatin into tight nucleosome arrays. One remarkable example of repressive chromatin remodeling is provided by the NoRC complex (Clapier and Cairns 2009). NoRC is recruited to rRNA gene promoter via TTF-1 and relocates the promoter bound nucleosomes to a position unfavorable for transcription initiation (Strohner et al. 2004; Li et al. 2006).

Histone chaperones are also important to mediate dynamic chromatin changes. In contrast to histone modifying enzymes or chromatin remodelers histone chaperones directly target the nucelosome complex assembly and thus they are involved in intracellular histone dynamics. FACT and Spt6 both considered as essential elongation factors have been reported to have intrinsic chaperone activity. FACT removes the H2A/H2B dimers during elongation thereby facilitating migration of elongating RNAP II through nucleosome arrays (Belotserkovskaya et al. 2003). The elongation factor Spt6 which has a H3/H4 chaperone activity (Bortvin and Winston

1996) was found to be necessary for re-establishment of the nucleosome array after passage of an elongating RNAP II complex in yeast (Selth et al. 2010).

Altogether the combination of histone modifications, chromatin remodeling and histone chaperone activity defines the state of chromatin in living cells, playing a central role in the regulation of chromatin structure for gene expression.

3 RNA PROCESSING AND RNP ASSEMBLY

As mentioned in the previous chapter, eukaryotic gene expression is controlled by multiple mechanisms at nearly all stages of RNA biogenesis. Of special importance in the cell is the transmission of the DNA encoded message to the cytoplasm. Thus the assembly of an export competent RNP which can be translocated through the nuclear pore complex also represents a critical checkpoint in RNA biogenesis. Interestingly, non-coding RNAs, which function as ribozymes in the nucleus, often shuttle between nucleus and cytoplasm during their lifetime for maturation and/or recycling purposes. However, all RNAs which are exported to the cytoplasm have to be correctly processed and packed into RNP in the nucleus. Since the present thesis is centered on RNAP II and RNAP I-mediated transcription the following chapters briefly summarize nuclear events that lead to mRNA and rRNA maturation.

3.1 PRE-mRNA PROCESSING

The three main processing events that occur during the formation of a mature mRNA transcript are mRNA capping, splicing and 3' end processing (cleavage and polyadenylation). All these events take place on nascent pre-mRNA emerging from the transcribing RNAP II complex.

mRNA capping leads to the formation of a 7-methyl-guanosine cap at the 5' end of mRNA. It is essential for the establishment of a stable transcription complex at the gene and facilitates transcriptional elongation. The 7-methyl guanosine cap is immediately added to the 5’ end of the nascent mRNA through the synergy of two proteins CBP20 and CBP80. The 3' end processing, which can be subdivided into cleavage and polyadenylation, results in the release of mRNA from the transcription site and is essential for correct transcription termination. Both 5’ capping and 3’ end processing are essential for mRNA stability and nuclear export.

Splicing is a nuclear co-transcriptional process through which transcribed non-coding introns are excised from pre-mRNAs whereas flanking coding exons are cut and pasted together to form a coherent ORF in the mature RNA (Neugebauer 2002). In most genes non-coding introns are more abundant in comparison to coding

exons; therefore the discrepancy between gene size and coding ORF can be remarkable. The consequence is that splicing has considerable impact on the kinetics of RNAP II mediated transcription and simultaneously is affected by the processivity of elongating RNAP II (Batsche et al. 2006; Listerman et al. 2006).

Introns in pre-mRNA molecules are removed by a macromolecular complex termed spliceosome. In contrast to mRNA capping or 3' end processing, the catalytic activity of splicing is provided by short small nuclear (sn)RNAs (less than 200 nt in size) rather than by proteins which complete them to snRNPs. At least five of these snRNPs and a large number of protein splicing factors are found in the spliceosome (Kramer 1996). They are termed U1, U2, U4, U5 and U6 snRNPs. Many other components have been purified by mass spectrometry: at least 300 putative spliceosomal proteins have been revealed (Jurica and Moore 2003). Assembly of an active spliceosome is a sequential process which is induced by the binding of U1 snRNP at the 5' intron splice site that emerges at the exit pore of the elongating RNAP II complex. Subsequent loading of the remaining factors to the intron branch point and the 3' splice site leads to intron excision and joining of the two flanking exon sequences. snRNPs recruitment and positioning is mediated through the small nuclear RNAs which are the functional components of the snRNPs. The 5' splice site in a pre-mRNA is often marked by a GU and the 3' splice site by an AG nucleotide duplet (also termed GU-AG rule). Interestingly, the strength of a splice site is defined by the splicing enhancer and silencer elements which are bound by a number of RNA binding proteins belonging to the group of SR proteins and hnRNPs. Intron and exon localized enhancers (ISEs and ESEs) promote splicing while corresponding silencers (ISSs and ESSs) inhibit splicing. The balance between these elements and their associated RNA binding proteins defines the strength of a given splice site through facilitation or inhibition of spliceosome assembly (Singh and Valcárcel 2005). However these sites show a low conservation and thus can be missed by the spliceosome. Thus splicing has a high probability to lead to false splice-products. One way to control the quality of splicing is through the recruitment of the exon-junction-complex (EJC). The EJC complex is loaded 20 nt upstream exon-exon junctions during splicing and ensures that only properly spliced mRNAs finally become translated into proteins.

3.1.2 hnRNPs and Pre-mRNA packaging

The largest family of RNA binding proteins (RBDs) that associates with the nascent pre-mRNA are collectively called heterogeneous nuclear ribonucleoproteins (hnRNPs). The term heterogeneous refers to the fact that hnRNPs are originally recruited to pre-mRNA, a heterogeneous transcript due to its exon-intron organization. hnRNPs, together with other RBDs such as snRNPs, SR-proteins, EJC components, 5'cap-binding-complex components and the 3'poly(A) binding protein, assemble with corresponding pre-mRNAs into mature mRNP complexes.

The composition of hnRNPs in pre- and mRNP particles is highly dynamic and changes throughout the entire mRNA biogenesis pathway (Figure 1). Some hnRNPs harbor nuclear retention signals and are removed from the mRNP prior nuclear export, whereas others stay associated with the mRNP all the way from gene to polysomes to be shuttled back to the nucleus (Dreyfuss et al. 2002). In mammals at least 20 major hnRNPs have been identified and, designated from hnRNPA1 to hnRNPU (Dreyfuss et al. 1993). Most if not all hnRNP proteins contain one or more RNA binding domains termed RBDs and an auxiliary arginine-glycine rich (RG-) domain, which is believed to be involved in protein-protein interactions. These domains can be also found in non “traditional” pre- and mRNP components like in EJC subunits, SR-proteins, and poly(A) binding proteins. Thus the traditional separation of mRNP complex components in different classes is nowadays only applied due to the historical context through which they were described (Dreyfuss et al. 2002).

Correct mRNA processing, packaging and subsequent mRNP remodeling, is crucial for efficient mRNA export. As mentioned above splicing in eucaryotes is accompanied by the deposition of the EJC complex at RNA splice sites. One of the EJC components which is termed REF (Yra1 in yeast) was shown to interact directly with TAP (Mex67 in yeast). The TAP/p15-complex functions in vertebrates as a general mRNA export receptor, which mediates transport of mRNPs through the FG-nucleoporin meshwork in the channel of the nuclear pore complex (Gruter et al. 1998). Transport through the NPC will be discussed below, however it is important to mention at this stage that TAP/p15 mediated mRNA export is independent from the classical karyopherin RanGTP export pathway. On the mRNA transcript, REF is found to be in physical contact with another export adaptor protein called UAP56 which is a DEAD box RNA helicase. Together with the THO complex these proteins assemble into the

so-called transcription coupled export complex (TREX). In vertebrates TREX assembly on the mRNA was first shown to be coupled to splicing, what initially made sense since REF was one of the first described EJC components (Masuda et al. 2005). However, evidence was later provided indicating that TREX assembly is more dependent on 5' CAP formation than on splicing itself showing a clear difference to EJC (Cheng et al. 2006) and confirming the suggested mutual dependence of capping and mRNA export (Hamm and Mattaj 1990).

A set of factors which belong to the SR protein family, which are loaded to pre-mRNA for efficient splicing, have been reported to serve as mRNP binding sites for the general mRNA export factor TAP (Huang et al. 2003). SR proteins are recruited to pre-mRNA in their hyperphosphorylated state and become partially de-phosphorylated as splicing proceeds. Some of the SR proteins (including ASF/SF2, Srp20 and 9G8) stay associated in their de-phosphorylated form with the mRNA and accompany the transcript to the cytoplam where they are released and re-imported into the nucleus in a phosphorylation-dependent manner (Huang and Steitz 2005). Thus, the phosphorylation state of mRNP associated SR proteins has been suggested to serve as the signal for fully spliced export competent mRNP (Kohler and Hurt 2007; Moore and Proudfoot 2009).

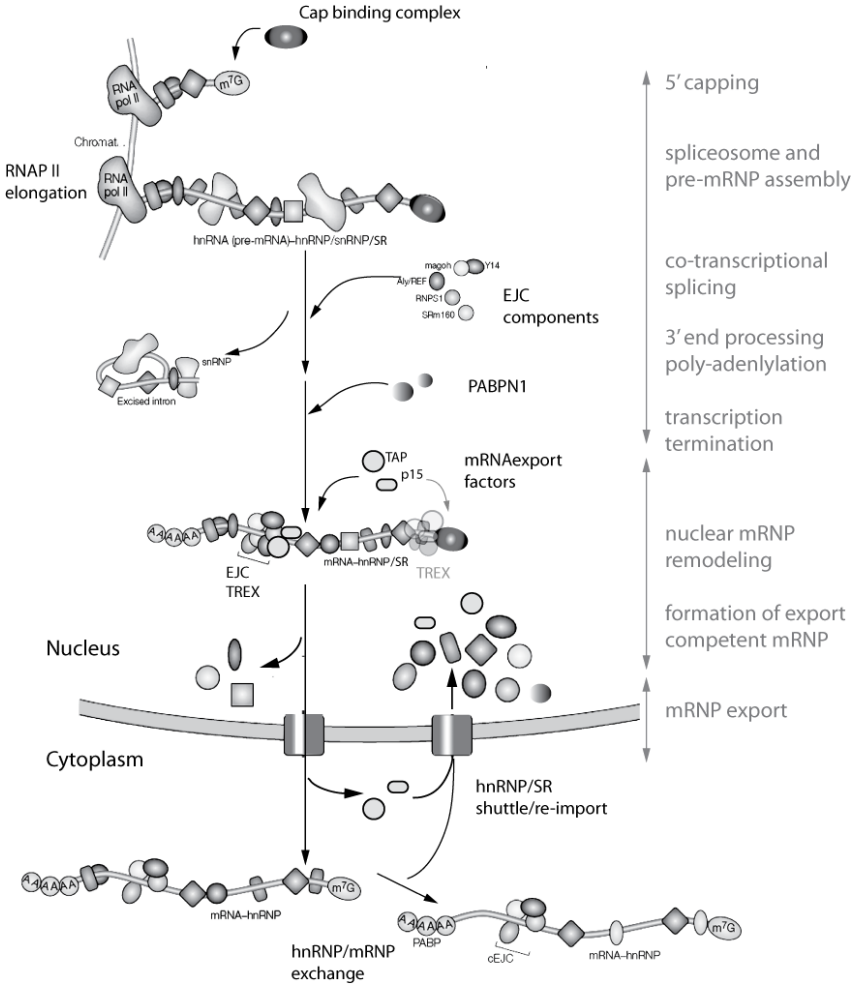

Figure 1. **Overview of pre-mRNP/mRNP assembly**. The protein composition of pre- and mRNPs is highly dynamic and changes during transcription, processing and nucleocytoplasmic export. EJC, exon-exon junction complex, hnRNP, heterogeneous nuclear ribonucleoproteins; mRNP, mRNA-protein complex; PABP, poly(A)-binding protein; PABPN1, nuclear poly(A)-binding protein; SR, SR protein; m7G, 5'7-methylguanosine cap; TREX, transcription coupled export complex; TAP/p15, mRNA export receptor (adapted from Dreyfuss et al. 2002).

3.2 PRE-rRNA PROCESSING AND PRE-RIBOSOMAL ASSEMBLY

Assembly of ribosomal subunits occurs in the nucleolus. The large 60S subunit contains 28S and 5.8S which are transcribed by RNAP I and 5S rRNA which is transcribed by RNAP III. The 40S subunit exclusively contains 18S rRNA. 60S and 40S subunits are independently exported to the cytoplasm where they assemble to reconstitute an active 80Ss ribosome on the specific mRNA templates for translation.

As mentioned earlier 28S, 5.8S and 18S rRNA derive from one long 47S pre-rRNA transcript. The 47S transcript is cleaved in its external and internal transcribed regions (ETSs and ITSs) and post-transcriptionally modified through its interaction with small nucleolar ribonucleoproteins (snoRNPs) and protein processing factors into mature 18S, 5.8S and 28S rRNA. This part of the ribosomal biogenesis pathway is referred to as rRNA processing. It is completed while the transcripts are still located in the dense fibrillar component of nucleolus, a nucleolar compartment which is located in close proximity to the RNAP I transcription site. Assembly of nascent ribosomal subunits starts during rRNA processing. Assembly is completed when the mature ribosomal subunits are recruited to the nuclear pore complex for nuclear export (Tschochner and Hurt 2003).

Early studies demonstrated that nascent rRNA transcripts are decorated co-transcriptionally with 5' terminal knobs (Miller and Beatty 1969). Furthermore the nuclear 90S pre-ribosome containing the 35S pre-rRNA and its subsequent processing and remodeling products 60S and 40S pre-ribosomal subunits have been described in yeast (Trapman et al. 1975). However it took about 30 years until a comprehensive analysis of the protein content in pre-ribosomal particles became available. This was a major step forward because it could be clarified that a majority of the 90S pre-ribosome associated factors are components of the small subunit (SSU) processosome and factors required for the synthesis of the 18S rRNA (Tschochner and Hurt 2003; Granneman and Baserga 2004; Kressler et al. 2009). The yeast SSU processosome is a relatively large complex with an approximate size of 2 MDa. It contains U3 snoRNA, U3 binding and non-ribosomal proteins termed Utps. Depletion of SSU processosome subunits such as U3 snoRNA and Utp7 resulted surprisingly in the release of the 5' terminal knobs which were known to be recruited co-transcriptionally to the nascent yeast 35S rRNA precursor (Osheim et al. 2004). The above observations led to the current view:

nucleolar rRNA processing similarly to mRNA splicing is initiated co-transcriptionally through assembly of the SSU processosome and 40S-associated factors to nascent pre-rRNA leading to formation of the 90S pre-rRNP particle. pre-rRNA is next modified and cleaved at the 5' and 3' ETS. Recruitment of additional 40S factors is considered to be the point at which the ITS1 site within the pre-rRNA is cleaved resulting in the separation of 40S and 60S pre-ribosomal assembly pathways (Tschochner and Hurt 2003).

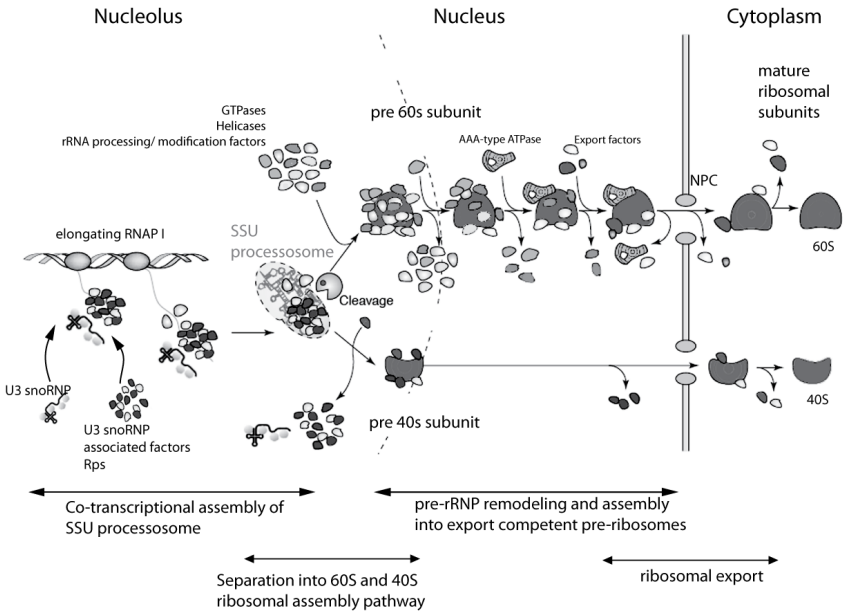

Figure 2. **Ribosomal biogenesis.** Pre-ribosomal assembly starts co-transcriptionally in the nucleolus with the formation of the SSU processosome. Subsequent cleavage of the precursor-rRNA results in the separation of 40S and 60S pre-ribosomal assembly pathways. Pre-ribosomal assembly and remodeling, leads to formation of export-competent pre-ribosomal subunits. Horizontal arrows indicate the different phases of ribosomal biogenesis. rRNP, rRNA-protein complex; Rps, ribosomal proteins of the small subunit; NPC, nuclear pore complex (adapted from Tschochner and Hurt 2003).

Nucleolar and nuclear assembly and remodeling of the 60s and 40s pre-ribosomal subunits is a highly dynamic process, which is not yet really understood (Kressler et al. 2009). Nucleolar assembly and remodeling of 60S and 40S pre-ribosomal subunits is a highly dynamic process which is not yet really understood (Kressler et al 2009). ATP dependent enzymes that induce structural changes at RNA

and protein level are found at all stages of ribosomal biogenesis. For example DExD/H-Box ATPases which function as RNA helicases are predicted to play an essential role in early rRNA processing steps. In contrast, AAA-type ATPases and GTPases which are necessary for release of certain non-ribosomal proteins and for structural remodeling of pre- rRNP complex are mostly recruited after separation of the pathway into 60S and 40S pre-ribosomes. Interestingly all pre-ribosome associated GTPases and all but one AAA-type ATPases are found to be associated to the nuclear 60S pre-ribosomes suggesting that 40S pre-ribosomal subunits undergo fewer structural rearrangements during their nuclear assembly than 60S pre-ribosomes (Tschochner and Hurt 2003; Kressler et al. 2009). Pre-ribosomal assembly is finalized once export-competent 60S and 40S subunits are formed. Nuclear export of pre-ribosomal subunits will be discussed in the subseqeunt chapter. However it is important to point out here that in contrast to mRNP export of pre-ribosomal subunits (or generally speaking, rRNPs) is dependent on exportin 1 and the RanGTP gradient (Hurt et al. 1999; Moy and Silver 1999).

4 THE NUCLEAR PORE COMPLEX

The nuclear pore complex (NPC) is a large proteinacious macromolecular assembly embedded in the nuclear envelope double membrane layer with a diameter of 90-120 nm. The NPC is the main gateway for transport of ions, proteins, RNAs and RNPs between cytoplasm and nucleus.

The NPC comprises three main parts. There is an internal core which is embedded in the double-layered nuclear envelope. It is characterized by a ring domain with an eight-fold rotational symmetry, known as spoke domain (Akey and Radermacher 1993; Elad et al. 2009). The peripheral components of the NPC at the cytoplasmic and nucleoplasmic face are considerably different. The cytoplasmic portion is characterized by short and flexible fibrills. The nucleoplasmic fraction is also characterized by filamentous structures which are assembled into a symmetrical fish trap like structure known as nuclear basket.

The spoke domain delineates the central cavity of the NPC through which nucleocytoplasmic trafficking takes place. The cavity is believed to be filled with a meshwork of the FG-rich repeats found in the majority of nucleoporins. FG-repeats are thought to form a semipermeable environment which inhibits diffusion of large proteins (>50kDa) through the NPC unless their transport is facilitated through nuclear transport receptor proteins. Approximately 30 nucleoporins have been identified to constitute the NPC (Rout et al. 2000; Cronshaw et al. 2002). Within the NPC nucleoporins are organized into distinct subcomplexes. The Nup62 complex is the major constituent of the spoke domain. The NUP107-160 complex, exhibits a Y-shape and it is referred to as the Y complex (Kampmann and Blobel 2009). In vertebrates the Y complex consists of 10 nucleoporins and is considered to form an octagonal ring of 8 complexes in the cytoplasmic and nuclear moiety of the NPC, sandwiching the less defined, 5 nucleoporins containing NUP155 subcomplex (Nic96 in yeast). The NUP155 complex is considered to tether the NUP62 subcomplex, composed of the nucleoporins Nup62, 58 and 54. Interestingly another Nup62 is found together with Nup214 and Nup88 to form a subcomplex in the cytoplasmic moiety of the NPC. Additionally the NUP155 subcomplex seems to be associated to the NDC1 complex which consist of the nucleoporins Ndc1, Gp210 and Pom121 that anchors the NPC to the envelope (Lim and Fahrenkrog 2006; Brohawn et al. 2009). On the nuclear face of the NPC, the octagonally arranged Y complexes provide the binding site for the FG-repeat

containing Nup153 which in turn anchors Tpr to the nuclear basket (Hase and Cordes 2003). The NPC is a highly dynamic multiprotein assembly. FRAP experiments with GFP-tagged nucleoporins revealed that different components of the NPC display different residence times. While structural NPC components such as the Y- or NUP155-subcomplex are stably associated, FG-repeats containing nucleoporins are very dynamic (Rabut et al. 2004).

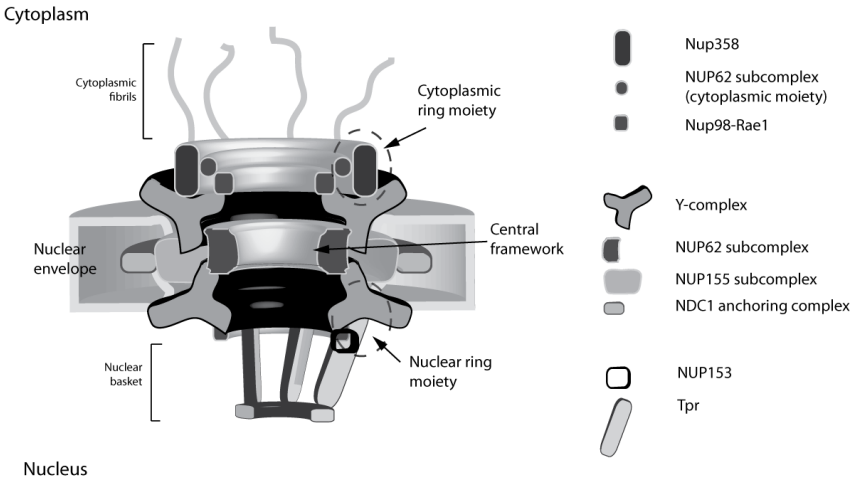

Figure 3. **Simplified view of the NPC.** This 3D cartoon of the NPC shows the major subcomplexes of the cytoplasmic and nuclear moiety and those present in the central framework embedded in the nuclear envelope. Structure, composition and function of specific subcomplexes are described above and are not presented in scale.

4.1 THE KARYOPHERIN-RanGTP SYSTEM AND RNP EXPORT

Large proteins and protein-RNA complexes are actively transported through the NPC. Nucleoporins specific to either nucleoplasmic or cytoplasmic face of the NPC cooperate with soluble transport receptors known as karyopherins and the the GTPase Ran for efficient transport in and out from the nucleus. Karyopherins are classified into importins, if mediating nuclear import, and exportins, if mediating nuclear export. During nucleocytoplasmic transport all karyopherins bind RanGTP and interact transiently during NPC transport with the nucleoporin FG repeat meshwork. The RanGTP/GDP gradient between nucleus and cytoplasm is maintained by two

spatially separated reactions. In the nucleus Ran is kept in a GTP-bound form by the GTP exchange factor (RCC1).

Proteins which are destined for nuclear import or nuclear export carry signal sequences on their primary structures known as nuclear localization signals (NLSs) and nuclear export signals (NESs), respectively (Vasu and Forbes 2001). NLS signals are recognized by a dimeric receptor comprising importin-alpha which binds the NLS and acts as an adaptor for importin-beta which mediates the translocation of the cargo through the pore. Once the cargo reaches the nuclear moiety of the NPC, RanGTP binds the importin-beta and displaces it from the cargo-receptor-complex. This leads to destabilization of the importin-alpha/NLS interaction and release of the cargo into the nucleoplasm. Importin-beta which is still bound to RanGTP is exported back to the cytoplasm. Importin-alpha is then recycled back into the cytoplasm through the exportin CAS (cellular apoptosis susceptibility protein). RanGAP which in mammals is found associated with the cytoplasmic NPC fibrils induces hydrolysis of the Ran-bound GTP and mediates disassembly of Ran-bound exportins and their cargos. RanGDP diffuses back to the nucleus where it is made available for the next cycle (Vasu and Forbes 2001; Fried and Kutay 2003). Nuclear import of ribosomal proteins, mRNA binding proteins such as hnRNPs or SR proteins is importin-alpha independent being exclusively mediated by importin-beta.

Nuclear tRNAs, microRNAs, snRNAs and rRNPs have their own set of export receptors and adaptor proteins (Fried and Kutay 2003). An interesting example herein is the exportin1 (CRM1), which binds in presence of RanGTP to leucine rich NES directly (Fornerod et al. 1997). Interestingly CRM1 is also mediating the export of ribosomal RNPs, U snRNAs, and HIV RNA. Since CRM1 does not directly bind RNA this export is mediated via NES containing adaptor proteins (Fried and Kutay 2003).
When it comes to export of ribosomal subunits, while there is limited information for the 40S subunit, there is evidence that CRM1 is important for export of the 60S subunit (Thomas and Kutay 2003; Hedges et al. 2005; West et al. 2005). CRM1 and the adaptor protein Nmd3 are loaded into nascent 60S ribosomal subunit. During export the rRNP-Nmd3-CRM1-RanGTP complex is translocated to the cytoplasm where CRM1 is released from Nmd3 upon hydrolysis of Ran-bound GTP. Nmd3 is next released from the 60S subunit via the cytoplasmic GTPase Lsg1 (Gadal et al. 2001; Thomas and Kutay 2003; West et al. 2005). Export of the 60S subunit seems to require another export receptor which works in a RanGTP independent manner, the Mex67-Mtr2 (human TAP/p15) dimer. Even though the precise mechanisms are still unclear, it is

believed that the Nmd3 and Mex67 pathways are believed to cooperate for efficient nuclear export of the 60S subunit. In support of this view, the Mex67-Mtr2 dimer is able to bind nucleoporins and it seems to be also implicated in export of the 5S rRNA, a transcript that is considered to be an anchor in the 60s pre-ribosome (Yao et al. 2007). Even though the nuclear export of both pre-ribosomal subunits has been shown to be dependent on the RanGTP and CRM1, and Nmd3 was shown to be the adaptor for CRM1 in 60s pre-ribosomes protein, it still remains unclear which CRM1 adaptor may be found in 40s pre-ribosomes.

The mechanisms underlying the mRNA export are less clear. Even though independent from the RanGTP cycle, mRNP export is also directional. It has been hypothesized that directionality could be achieved through changes in the phopshorylation state of mRNP-associated SR proteins prior and after translocation through the NPC. The human DEAD box helicase DDX19 (Dbp5 in yeast) was shown to be essential for mRNA export in human and yeast and seems to be associated with the NPC cytoplasmic fibrils (Tseng et al. 1998; Schmitt et al. 1999). Furthermore the helicase activity of DDX19/Dbp5 seems to mediate through dissociation of the mRNP export receptor Mex67 (TAP in human) at the cytoplasmic site of the NPC thereby inhibiting re-import into the NPC (Lund and Guthrie 2005; Tran et al. 2007).

5 ACTIN AND MYOSIN: THE MULTI-FUNCTIONAL PROTEIN MOTOR IN THE CELL

Actin is a 43 kDa highly conserved protein that comes in three isoforms. It was initially discovered as component of the actomyosin complex in the 1860s and later on purified as an individual protein in the laboratory of Szent-Gyorgyi in Hungary (Hofmann 2009). The cytoplasmic actin is a major component of the cytoskeleton and it is engaged in multiple cellular processes such as cell shape, cell migration, cytokinesis and cytoplasmic trafficking (Pollard and Cooper 2009). ATP-bound actin monomers have the tendency to polymerize at a critical concentration, thus cytoplasmic actin co-exists in two forms, monomeric G-actin and polymeric F-actin, which forms the microfilaments. In contrast to thin filaments in myocytes, cytoplasmic microfilaments in non-muscle cells represent a highly dynamic structure, which can rapidly rearrange and acquire different shapes. Thus, the cytoplasmic actin functions are intrinsically coupled to the dynamics of actin polymerization, which responds to extracellular signals (Papakonstanti and Stournaras 2008).

Cytoplasmic actin filaments are known to associate with multiple myosin species to act as molecular motors. In humans over 40 ORFs are known to code for individual myosin proteins (Berg et al. 2001). All myosins share a tripartite structural organization divided into a N-terminal actin binding and ATP binding motor domain, a calmodulin binding neck domain characterized by a number of IQ motifs and a divergent C-terminal tail which is either involved in protein interaction or in self-dimerization (Alberts 2008). ATP hydrolysis in the myosin motor domain induces directional movement of myosin along actin filaments. In general movement occurs towards the plus end. MyoVI is the exception to the rule given that it moves towards the minus ends of actin filaments (Wells et al. 1999).

The following two chapters summarize our present knowledge on the function(s) of actin and myosin in the cell nucleus.

5.1 DENIAL AND RE-DISCOVERY OF NUCLEAR ACTIN AND MYOSIN

Evidence that actin is an abundant nuclear protein was already reported in the 1060s (Ohnishi et al. 1963; Ohnishi et al. 1964). Later on two milestone studies suggested that nuclear actin could be engaged in expression of protein coding genes (Egly et al. 1984; Scheer et al. 1984). These findings were met with great skepticism. Biochemists could not rule out whether the presence of actin in the nucleus was simply due to cytoplasmic contamination. Furthermore, phalloidin, a fungi derived drug which exclusively stains actin filaments, could only visualize cytoplasmic actin filaments and no nuclear signals as detected by microscopy methods (Pederson and Aebi 2002), further supporting the ever growing family of doubters of nuclear actin. However, during the past 12 years evidence has been provided which confirmed the presence of actin in the nucleus and uncovered its engagement in fundamental nuclear processes. Nowadays actin is a well defined component of certain chromatin remodeling complexes (Zhao et al. 1998), of nascent pre- and mature mRNP particles (Percipalle et al. 2001), DNA dependent RNA polymerases (Fomproix and Percipalle 2004; Hofmann et al. 2004; Hu et al. 2004; Philimonenko et al. 2004; Kukalev et al. 2005) and was recently implicated in long range chromosomal movements in the nucleus (Chuang et al. 2006; Dundr et al. 2007; Hu et al. 2008).

Evidence for the presence of myosin in the cell nucleus came from studies that were performed already in the 1970s. These studies reported that myosin may be a component of non-histone protein fractions associated with eukaryotic heterochromatin (Douvas et al. 1975; Comings and Okada 1976). In the 1980s Berrios et al. provided additional evidence that myosin may be a control element for nucleocytoplasmic transport, being associated with the nuclear pore complex (Berrios and Fisher 1986; Berrios et al. 1991). These studies were left aside for many years. A revival of nuclear myosin came from the laboratory of Primal de Lanerolle, who could show together with his co-workers that a subpopulation of unconventional myosin 1 (M1) is present in cell-nuclei (Nowak et al. 1997). Further characterization revealed that this form of myosin 1 that was found to localize to the cell nucleus has an extra N-terminal stretch of 16 amino acid residues, which is essential for its presence in the nucleus. Thus the authors termed this novel protein nuclear myosin 1 (NM1) (Pestic-Dragovich et al. 2000). In the same study the authors provided *in vitro* evidence that NM1 could be involved in RNAP II transcription. Subsequent studies applying a

specific NM1 antibody in confocal and electron microscopy experiments demonstrated that NM1 is in nucleolar transcription foci. These foci were located in dense fibrillar component and fibrillar centres where RNAP I transcription is known to take place. Furthermore it was discovered that NM1 and actin associate with the largest RNAP I subunit and their interaction is required for RNAP I transcription (Fomproix and Percipalle 2004; Percipalle et al. 2006; Percipalle and Östlund Farrants 2006). Later on chromatin immunoprecipitation experiments supported the above conclusions since both actin and NM1 were found at promoter and coding region of rRNA genes (Philimonenko et al. 2004; Percipalle et al. 2006).

Since the discovery of the NM1, four other myosin species were found in the cell nucleus. Two myosins, myosin VI (MyoVI) and the conventional myosin II (MyoII) were shown to have a role in RNAP II mediated transcription. Myo VI was shown to be associated with RNAP II, to be important for active transcription in mammalian cells and to be present at promoter and intragenic regions of actively transcribed genes (Vreugde et al. 2006). In contrast, the involvement of MyoII in RNAP II transcription was deduced from a study on specialized colonic smooth muscle cells. In these cells MyoII was found to specifically associate with the core promoter regions of the ICAM-1 gene (Li and Sarna 2010), but its general function in transcription remains to be elucidated. The other myosin species which were found in the cell nucleus are Myo16b and MyoVa. MyoVa, in its phosphorylated form (Ser1650) was found to co-localize with the splicing factor SC35 in nuclear speckles. MyoVa was not found in any other nuclear sub-compartments. However, a transcriptional block by actinomycin D treatment induced a migration of MyoVa to nucleoli (Pranchevicius et al. 2008). Even though these results suggest a role for MyoVa in controlling nuclear events, molecular insights into its function have still to be provided. Myo16b has been investigated through a comprehensive microscopic approach using GFP-tagged wild type and deletion Myo16b constructs, monitoring their localization in nuclear sub-compartments. The authors found that Myo16b is targeted to the cell nucleus via a specific sequence located in its C-terminal domain. Interestingly this region directed its localization to a nuclear sub-compartment which apparently contains profilin and F-actin. In support of a causal relation between localization and nuclear function, over-expression of the full-length protein as well as the C-terminal region led to a significant delay in cellular proliferation (Cameron et al. 2007). However, biochemical insights into Myo16b nuclear function are still lagging behind.

5.1.1 Actin and NM1 in chromatin remodeling

The first real evidence on how actin may be involved in transcriptional regulation came from the Crabtree laboratory, where it was shown that actin is a component of the human SWI/SNF-like BAF chromatin-remodeling complex. The authors uncovered that actin is not only interacting with BRG1, the ATPase subunit of BAF, but also that actin is needed for the ATPase activity required for BAF association with chromatin (Zhao et al. 1998). Besides actin, a number of actin-related proteins (ARPs) have been shown to be associated with chromatin remodeling complexes and histone acetyl transferases. The fact that actin and ARPs often bind to ATPase subunits of chromatin-remodelers indicates that actin and ARPs may work as allosteric regulators of certain chromatin remodeling complexes (Olave et al. 2002; Blessing et al. 2004). Interestingly in a subsequent study, Crabtree and co-workers showed that BRG1 tends to bind to F-actin *in vitro* (Rando et al. 2002).

Initial findings suggested a role for actin and NM1 in RNAP I and RNAP II transcription activation (Fomproix and Percipalle 2004; Philimonenko et al. 2004; Hofmann et al. 2006). However the discovery that NM1 is a component of the novel chromatin remodeling complex B-WICH that occupies rRNA gene promoter and coding region changed this perspective (Percipalle et al. 2006). Percipalle and co-workers could reveal through a combination of size exclusion, ion exchange and affinity chromatography assays that NM1 is associated to the core components of the WSTF/SNF2h complex. Inhibition of NM1 and the core component WSTF blocked RNAP I transcription elongation in a chromatin dependent manner. It was proposed that NM1, as part of the chromatin remodeling complex B-WICH, facilitates migration of the elongating RNAP I transcription complex through the nucleosomal barrier of the gene associated chromatin (Percipalle et al. 2006; Louvet and Percipalle 2009). Interestingly a subsequent study on B-WICH showed that this complex may also associate with nascent 47S pre-rRNA and with factors needed for rRNA processing, including the RNA helicase DDX21 (Cavellán et al. 2006), suggesting that NM1 may be involved in ribosomal biogenesis at transcriptional and post-transcriptional levels.

5.1.2 Actin in RNP assembly

Already shortly after the "re-discovery" of nuclear actin as a component of the BAF chromatin remodeling complex, a study in the laboratory of Bertil Daneholt on the dipteran *Chironomus tentans* polytene chromosomes revealed that actin associates with the giant Balbiani ring (BR) transcriptional puffs and is a component of the BR pre-mRNP/mRNP particle. Overall this study demonstrated that actin accompanies RNPs from the site of transcription to polysomes. Furthermore, biochemical analysis provided evidence that the presence of actin in pre-RNPs and mRNPs is mediated through its interaction with the hnRNP A1-like protein hrp36 (Percipalle et al. 2001). A subsequent proteomic analysis in mammals revealed that this is a general scenario. Actin was identified in the 40S pre-mRNP/mRNP fraction isolated from rat liver extracts where actin was found to interact with a subset of A/B-type hnRNPs including the novel hnRNP CBF-A (Percipalle et al. 2002). Another study performed in the *Chironomus tentans* model system confirmed the presence of actin in RNPs and showed the role of specific actin-hnRNP interactions (Percipalle et al. 2003). The authors identified an isoform of the RNA binding protein hrp65 to bind with actin. hrp65 shares strong homology with the mammalian DBHS domain containing proteins PSF and p54nrb/NonO. It was found that the isoform hrp65-2 specifically binds actin within mRNPs. Interestingly the hrp65-2 isoform has, in contrast to the remaining isoforms hrp65-1 and hrp65-3, a unique C-terminal prolongation in its peptide sequence. *In vitro* and *in vivo* analysis demonstrated that this unique peptide found in hrp65-2 exhibits a novel actin binding motif. Nuclear microinjections of short peptides encompassing the actin binding site into salivary glands cell nuclei specifically disrupted the actin-hrp65-2 interaction and blocked RNAP II mediated transcription *in vivo* (Percipalle et al. 2003).

In silico screening for the hrp 65-2 actin-binding consensus in mammals led to the discovery of an actin binding motif located in the C-terminus of the RNA binding protein hnRNP U, in close proximity to the RNA binding domain (Kukalev et al. 2005). Overall hnRNP U shows only weak homology to hrp65-2 and belongs to the group of historical hnRNPs (Dreyfuss et al. 2002). Interestingly enough, when comparing the actin-binding motifs on hrp65-2 and hnRNP U it was found that the amino acid residues required for actin binding are highly conserved. *In vivo* disruption of the actin-hnRNP U interaction resulted in reduced transcription levels (Kukalev et al.

2005). Taken altogether these observations point towards a conserved mechanism whereby specific actin-hnRNP interactions control assembly of nascent RNPs while mediating RNAP II transcription.

5.1.3 Actin and myosin in RNA polymerase mediated transcription

In the 1980s, two important studies provided circumstantial evidence that actin is important in RNA biogenesis. Briefly Scheer and coworkers microinjected actin antibodies and actin-binding proteins into oocyte nuclei from *Pleurodeles waltlii*. Analysis by light and electron microscopy revealed that transcription of lampbrush chromosomes was reduced when actin was inhibited by antibodies or when F-actin severing proteins were injected. Interestingly the authors could not rule out any effects on formation of mature rRNA transcripts and also did not see any changes in miller spreads at active rDNA loci. Therefore they concluded that actin may be involved in transcription of protein coding genes (Scheer et al. 1984). Almost concomitantly with the above observations, Egly et al. set up a purification assay to analyze the protein content in fractions from HeLa cell cultures which were commonly utilized for *in vitro* transcription assays. In this study they found a novel transcription factor, which was enriched in a sub-fraction usually containing RNAP II initiation factors. This 43kDa factor showed stimulatory effects on transcription *in vitro*. It also revealed specific tendency to filament formation. Finally it was present in three isoforms (as shown by 2D polyacrylamide gel electrophoresis) and displayed strong affinity to DNase I. All this features indicated that this novel transcription factor essential for RNAP II initiation was nothing else than actin (Egly et al. 1984). As mentioned previously, these striking results have been criticized or simply ignored by most researchers in the molecular biology field. However, the discovery that actin is a component of the human BAF complex, its cotranscriptional recruitment into nascent mRNPs, its involvement in export of retroviral RNAs together with the discovery of NM1 has considerably decreased the ratio between skeptics and supporters of nuclear actin and myosin (Zhao et al. 1998; Kimura et al. 2000; Pestic-Dragovich et al. 2000; Percipalle et al. 2001; Percipalle et al. 2002)..

The next question is whether the synergy between actin and NM1 is conserved during transcription by all three eukaryotic RNA polymerases. NM1 was shown to be involved in RNAP II transcription *in vitro* (Pestic-Dragovich et al. 2000).

Actin was found to be part of the PIC complex. Indeed affinity chromatography approaches proved that actin interacts with RNAP II and TBP while associating with interferon-inducible promoters upon transcription activation (Hofmann et al. 2004). While these findings suggested a role for actin in transcription activation, they did not address how actin interacts with RNAP II (Hofmann et al. 2004). Actin also associates with phosphorylated RNAP II both in human and insect cells and we have discussed earlier the suggested mechanisms in complex with specific hnRNPs (Percipalle et al. 2003; Kukalev et al. 2005). Mechanistic insights into actin function in transcription elongation came from the discoveries that actin interacts with hrp65-2 and hnRNP U (Percipalle et al. 2001; Percipalle et al. 2003; Kukalev et al. 2005; Sjölinder et al. 2005). The laboratory of Neus Visa showed that in *Chironomus tentans* the actin-hrp65-2 complex functions as recruitment platform for a histone acetyl transferase (HAT), a mechanism that is required for transcription elongation (Sjölinder et al. 2005). A similar role was discovered for the interaction between actin and hnRNP U (Kukalev et al. 2005). Both proteins were found associated with phosphorylated RNAP II and they are implicated in HAT recruitment (see results). In either case, it was proposed that the interaction between actin and specific hnRNPs is required for recruitment of transcriptional coactivator for efficient pre-mRNA elongation (Percipalle and Visa 2006; Louvet and Percipalle 2009). However evidence that throughout transcription initiation and elongation actin cooperates with NM1 still lags behind. The only evidence of a synergy in RNAP II transcription comes from a study by the laboratory of P Hozak where it was reported that actin and NM1 colocalize to nucleoplasmic transcription sites (Kysela et al. 2005).

When it comes to RNAP I transcription the group of Piergiorgio Percipalle provided herein the first cytological and biochemical evidence that actin interacts with NM1 and RNAP I at active nucleolar transcription sites in a transcription dependent manner. NM1 was found in physical contact with nascent rRNAs. Furthermore, inhibition of the actin-NM1 interaction using the drug butane dione monoxime (BDM) which blocks myosin ATPase activity and interferes with actin binding, considerably decreased nucleolar transcription. Thus the authors concluded that the actin-NM1 complex serves as intranuclear motor coupled to transcription of rRNA genes (Fomproix and Percipalle 2004). In support of this findings, the laboratory of Ingrid Grummt in collaboration with the groups of Primal de Lanerolle and Pavel Hozak showed that *in vitro* inhibition of actin and NM1 by specific antibodies strongly inhibits RNAP I transcription. The inhibition rates on chromatinized DNA templates

were significantly stronger than naked DNA indicating that the regulatory function of the actin-NM1 interaction may be dependent on rDNA chromatin. Philimonenko and co-workers further showed that the actin-NM1 complex is only established in transcriptionally active cells. *In vitro* actin inhibition did not affect formation of the initial RNAP I transcript but inhibited synthesis of longer transcripts. These observations together with evidence that actin and NM1 occupy both rRNA gene promoter and coding region indicated that they may cooperate for assembly of transcription competent RNAP I and for elongation of pre-rRNA transcripts (Philimonenko et al. 2004; Percipalle et al. 2006; Percipalle and Östlund Farrants 2006). Percipalle and co-workers found in a combination of gel filtration, ion exchange and affinity chromatography assays that NM1 is a component of a novel chromatin remodeling complex termed B-WICH. In this complex NM1 is a core subunit together with WSTF and the ATPase SNF2h. All subunits were found to occupy both rRNA gene promoter and coding region. Furthermore, *in vitro* transcription assays revealed that inhibition of the B-WICH core subunits only affected elongation of pre-rRNA in a chromatin dependent manner (Percipalle et al. 2006). It was proposed that the dynamic interaction between actin and NM1 is therefore required to recruit B-WICH to the gene coding region for efficient RNAP I transcription elongation (Percipalle and Östlund Farrants 2006).

Finally, the laboratory of Nouria Hernandez provided insights into how actin may be interacting with all eukaryotic RNA polymerases. In line with the work mentioned above, actin was found to associate with RNAP III, occupying RNAP III promoter sites of actively transcribed genes and inhibition of actin led to a block of RNAP III transcriptional initiation *in vitro* (Hu et al. 2004). The authors further revealed that actin interacts directly with three subunits of the RNAP III complex, of which two, the RPABC2 and RPABC3, are common to all three polymerases. While there is no information on the potential involvement of NM1 in RNAP III transcription, the main consequence of the above findings is that actin may have a conserved mode of binding to all three eukaryotic RNA polymerases, even though specific differences (like RNAP II CTD association) should be taken into consideration.

5.2 NUCLEAR ACTIN DYNAMICS

The discovery that actin is an abundant nuclear protein involved in multiple gene regulatory functions raise the question of its polymerization state (Gieni and Hendzel 2009; Percipalle 2009). Using GFP-tagged versions of beta-actin in combination with FRAP analysis, McDonald and coworkers showed, that actin in the nucleus can be found in a dynamic equilibrium between low mobile polymeric actin species and rapidly diffusing actin populations (McDonald et al. 2006). However, conical actin filaments, which are commonly seen in the cytoplasm and can be visualized by phalloidin are not convincingly detectable in native nuclei (Visa and Percipalle 2010). Therefore it was suggested that in the nucleus actin may be present as monomeric G-actin, short oligomers as well as polymeric F-actin which may not be assembled in a conventional filamentous structure (Obrdlik et al. 2007; Pederson 2008; Louvet and Percipalle 2009). The presence of monomeric and polymeric nuclear actin species was indirectly confirmed by the discovery that the cell nucleus hosts both G-actin and F-actin binding proteins such as cofilin, profilin, β-thymosin, gelsolin and gelsolin-like proteins such as Mhb1 (Prendergast and Ziff 1991; Pendleton et al. 2003; Skare et al. 2003; Huff et al. 2004; Percipalle, 2009). Furthermore, DNase I chromatography assays utilized in studies from our group, successfully precipitated RNAP II and I together with actin from nuclear lysates (see also results in the next section of the present thesis). DNase I binds to G-actin with high affinity and to F-actin with low affinity (Zechel 1980). Thus the results obtained by DNAse I affinity chromatography suggested that actin co-precipitating RNAP II and RNAP I is likely to be present in its monomeric/short oligomeric form (Fomproix and Percipalle 2004; Kukalev et al. 2005). Several F-actin biding proteins are known to be involved in nuclear function. In particular a study from the laboratory of Jun-Li Guan showed that the F-actin nucleation factor N-WASP associates together with PSF-NonO complex with the RNAP II and that N-WASP can be found at promoter sites of RNAP II transcribed genes. RNAi-mediated depletion of N-WASP resulted in decreased levels of transcription. In a follow up study the same authors showed that the ARP2/3 complex (which cooperates with N-WASP and branches actin filaments) is required for RNAP II transcription *in vivo* (Wu et al. 2006; Yoo et al. 2007). Other proteins that facilitate actin nucleation have been recently reported to be in the nucleus, too. Examples are the formin-like protein mDia (Miki et al. 2009) and the novel actin

nucleation factor JMY (Zuchero et al. 2009), which has been originally identified as a transcriptional co-activator of p53. However the engagement of these factors in transcriptional regulation has not yet been addressed.

If G- and F-actin biding proteins are present in the cell nucleus, it means that nuclear actin dynamics is important for nuclear function. Data from the laboratory of Richard Treisman suggested a potential involvement of actin dynamics in gene transcription. A study by Miralles and co-workers uncovered that the protein MAL which acts as a transcriptional co-activator, functions as a sensor for cellular concentrations of G-actin in serum starved cells and it is translocated to the nucleus upon serum induced actin polymerization. MAL in complex with the serum response factor SRF regulates transcription of SRF target genes. Interestingly MAL only binds to SRF in its G-actin unbound form (Miralles et al. 2003). The authors concluded that either cytoplasmic G-actin pools are retaining MAL in cytoplasm or the G-actin-MAL complex is permanently exported from the nucleus. A later study by the Treisman laboratory revealed that nuclear G-actin regulates both nucleocytoplasmic shuttling of MAL and activation of SRF target genes (Vartiainen et al. 2007). However, altogether these findings could not rule out whether MAL regulation by changes in actin polymerization is mediated in nucleus or in cytoplasm.

Two recent independent studies from the laboratories of Ingrid Grummt and Francesco Blasi further addressed the question of nuclear actin dynamics in gene transcription. Ye et al. showed that only polymerization-competent forms of actin can drive together with NM1 RNAP I transcription *in vitro*. In this study the authors made use of exogenously expressed actin mutants which could not polymerize and NM1 mutants with impaired ATPase activity, actin binding ability and calmodulin binding activity. Their results indicated that expression of actin constructs which can not polymerize does not support RNAP I transcription. Furthermore NM1 mutants that lack ATPase activity, actin and calmodulin-binding activity failed to associate with RNAP I and rDNA coding cassettes. Additionally, the authors showed by drug treatments, that a stabilization of filamentous actin did not affect transcription whereas drug mediated actin filament severing reduced rRNA expression levels. This results motivated the authors to propose a model in which F-actin and NM1 may serve as motor that helps the transcription machinery to slide along rDNA (Ye et al. 2008). However, Ye and co-workers did not address whether and how actin polymerization *per se* is regulated while the RNAP I moves along the gene. Ferrai and co-workers in the group of Francesco Blasi investigated the role of actin polymerization in transcriptional activation of the

RA inducible HoxB genes. The authors found that impairment of actin polymerization by drug treatments prevents HoxB gene induction. Interestingly inhibition resulted from impaired recruitment of the HoxB regulator Prep1, actin-nucleating factors N-Wasp and ARP2/3, RNAP II and accessory splicing factors p54Nrb and PSF to the enhancer element of HoxB (Ferrai et al. 2009). Since transcription of constitutively expressed genes was not affected in their experimental setup, the authors suggested that actin polymerization may represent a mechanism to orchestrate transcription of certain inducible genes and thereby fine tuning the transcriptome of mammalian cells (Ferrai et al. 2009).

Both studies thus provided evidence that actin polymerization is important for gene transcription, a mechanism that may be important for recruitment of transcription machineries to certain regulatory elements like enhancers. However, these studies left several open questions. Two of the most important questions partly addressed in this thesis are whether and why actin polymerization accompanies elongating RNAP complexes along gene coding regions and how actin polymerization is cotranscriptionally regulated.

6 AIM OF THE THESIS

The general objective of this thesis was to understand how nuclear actin and NM1 regulate transcription and post-transcriptional events during RNA biogenesis. We used the human RNAP II and RNAP I system to monitor and dissect these functions *in vitro* and *in vivo*. The specific aims were:

I To study the molecular mechanism through which actin and hnRNP U facilitate RNAP II mediated transcription

II To investigate nuclear actin-dynamics, their effects on the elongating RNAP II transcription complex and how these are regulated in the cell nucleus.

III To establish if NM1 is involved in post-transcriptional regulation of rRNA biogenesis

7 RESULTS AND SUMMARY

7.1 PAPER I

Earlier in this thesis it was mentioned that actin interacts with the *Chironomus tentans* hrp65-2 and the human hnRNP U through a conserved actin binding motif (Percipalle et al. 2003; Kukalev et al. 2005). Since these interactions are essential for RNAP II transcription elongation it was hypothesized that they are required for recruitment of RNAP II coactivators (Percipalle and Visa 2006; Visa and Percipalle 2010). This idea was supported by the discovery that the actin-hrp65-2 interaction is required to recruit the HAT p2D10 in *Chironomus tentans* (Sjölinder et al. 2005).

In the first paper study we explored the possibility the mammalian actin-hnRNP U complex functions in a similar way to the *Chironomus tentans* actin-hrp65-2 complex, recruiting a specific HAT to RNAP II genes.

7.1.1 The HAT PCAF associates with actin in mammalian nuclei

To identify if nuclear actin associates with transcriptional co-activators, we applied DNAse I affinity chromatography assays to nuclear protein extracts from HeLa cells. As expected, actin co-precipitated the RNAP II and hnRNP U. Among the coprecipitated proteins we also identified the HAT PCAF. In contrast, other HATs such as CBP and TFIIIC220 (mammalian homologue of the *Chironomus tentans* P2D10) could be detected in the same DNase I pulldown experiments. Supporting these results, IPs with a PCAF specific antibody coprecipitated RNAP II, hnRNPU and actin.

7.1.2 Actin-hnRNPU interaction is required for the association with PCAF

To test whether PCAF association may be functionally linked to the actin-hnRNPU interaction we used bacterially expressed hnRNPU deletion constructs encoding different domains of the protein in pulldown assays. It turned out that only the

C-terminal hnRNP U construct which encompasses the actin-binding motif QRTQKK and RNA binding domain coprecipitated actin and PCAF from nuclear extracts. To further evaluate whether the association of PCAF is dependent on the actin-hnRNP U complex formation, we designed and raised an antibody, which we termed CED17, specific to the hnRNP U QRTQKK actin binding motif. We used this antibody in DNase I affinity chromatography and pulldown experiments with the hnRNP U constructs, as a competitor for the actin-hnRNP U interaction. Interestingly, pre-incubation of CED17 with nuclear extracts prior to DNAse I and hnRNPU-C pulldown experiments considerably decreased the amount of PCAF coprecipitated in a dose dependent manner. Gel filtration assays on nuclear extracts indicated that actin, hnRNPU, RNAP II and PCAF co-elute with an apparent molecular mass of 2-3MDa. The addition of CED17 to nuclear extract altered the elution profile, revealing decreased levels of hnRNP U and PCAF in the high molecular weight fractions and elevated levels in low molecular range. These results indicated that actin, hnRNP U, PCAF and RNAP II are part of the same complex. Furthermore they also suggested that the actin-hnRNP U complex is needed for stable association of PCAF with the RNAP II multi-protein complex.

7.1.3 The actin-hnRNPU interaction is needed for *in vivo* recruitment of HATs

To investigate whether the actin-associated PCAF is present in its active form, we incubated nuclear extracts with increasing amounts of CED17 and subjected them to DNAse I pull downs. We next performed in-liquid HAT assays on the precipitated proteins supplemented with purified histones. Using liquid scintillation we could prove that the levels of HAT activity directly correlate with the levels of precipitated PCAF confirming that actin-associated PCAF *in vitro* can function as a HAT. To see if the actin-hnRNP U complex functions *in vivo* as a recruitment platform for HATs, we microinjected CED17 in nuclei and cytoplasm of DRB synchronized HeLa cells and followed transcription by BrUTP incorporation. Upon injection of CED17 into cytoplasm no inhibitory effect at the transcriptional level could be observed. In contrast, microinjection of CED17 into cell-nuclei led to efficient transcriptional reduction. Interestingly, cells which were subsequently treated with TSA, an inhibitor histone deacetylases (HDACs), did not show any reduction in their

transcription rates at all, enforcing the assumption that the actin-hnRNP U complex facilitates recruitment of HATs to the transcription sites.

7.1.4 Actin, hnRNP U and PCAF associate with the RNAP II phospho-Ser2 CTD and are significantly enriched at coding regions of constitutively expressed genes

To address where and when actin-based PCAF recruitment takes place, we used a bacterially expressed construct encompassing the full S-tagged human RNAP II CTD. To mimic the different states of the RNAP II CTD we first phoshorylated the CTD with commercially available kinases CDK7 and CDK9 on Ser5, Ser2 or both serine residues. Next we used these phosphorylated constructs with HeLa nuclear extracts and performed pull down assays using protein S agarose beads. Ser2 and Ser2/5 phosphorylated CTD precipitated actin, hnRNP U together with PCAF. In contrast the same proteins were not coprecipitated with Ser5 phosphorylated CTD or unphosphorylated CTD. This observation prompted us to further investigate whether actin-hnRNP U may function as a platform for co-transcriptional recruitment of PCAF during RNAP II elongation. ChIP assays against RNAP II, hnRNP U, PCAF and actin resulted in enrichment of these proteins at promoter and gene coding sites of constitutively transcribed housekeeping genes. Using commercial antibodies against hnRNPU and PCAF we revealed high levels of occupancy at gene coding regions in contrast to low levels at gene promoter sites. Surprisingly, CED17 which binds the hnRNP U actin binding epitope, precipitated promoters but hardly any gene coding regions, indicating that the physical contact between actin and hnRNP U is established during transcriptional elongation whereas hnRNP U at promoters is not bound to actin. The actin-hnRNP U mediated recruitment of PCAF to gene coding regions, prompted us to investigate whether in coding region there is a causal link between hypo-acetylated chromatin and PCAF occupancy. Therefore we applied ChIP assays on HeLa cells pre-incubated with the HDAC blocking reagent TSA. Consistently, we found that TSA induced chromatin hyperacetylation, leading to a significant decrease of hnRNP U and PCAF occupancies.

7.1.5 Actin-hnRNP U mediated recruitment of PCAF to gene coding regions is directed through Ser2 phosphorylation at the RNAP II CTD.

To confirm the involvement of the actin-hnRNP U interaction in PCAF recruitment to the RNAP II CTD and active gene, we performed ChIP assays on DRB treated cells where CDK9 activity is specifically blocked. As expected we observed a significant decrease of phospho-Ser2 CTD signals along gene coding regions indicating a general reduction in the occupancy of elongating RNAP II. Interestingly, this observation was accompanied by a general decrease of signals for hnRNPU, PCAF concomitantly with a reduction in the levels of acetylated lysine 9 at histone 3 (H3K9ac).

7.1.6 PCAF together with actin and hnRNP U associates with nascent mRNA during transcription.

Upon RNAse treatment on mildly permeabilized living HeLa cells, we found that the nuclear distributions of actin, hnRNP U and PCAF is considerably lower, indicating that the localization of these proteins is globally dependent on RNA. To gain molecular insights, we performed RNA immunoprecipitation (RIP) assays with antibodies against actin, hnRNP U and PCAF to test whether they are in physical contact with mRNPs. Analyses of the RIP assays by RT-PCR showed that actin, hnRNP U and PCAF coprecipitated with H2B and S19 mRNAs. Consistent with previous work in Chironomus tentas and mammalian cells, these observations support the idea that the functional pathway of actin in HAT recruitment is conserved from insect to humans.

7.2 PAPER II

In the cell nucleus actin seems to be present in a highly mobile and in a low state (McDonald et al. 2006). This observation has led to the possibility that the nucleus hosts a monomeric G-actin pool to feed dynamic polymeric actin structures. In support this idea, F-actin stabilizing and nucleating proteins such as N-WASP and ARP2/3 complex are present in the nucleus where they have been implicated in RNAP II transcription elongation (Wu et al. 2006; Yoo et al. 2007). Besides other proteins,

which induce actin polymerization such as formin-like mDia proteins or JMY (Miki et al. 2009; Zuchero et al. 2009), a set of G-actin binding and F-actin severing proteins were described to be present in the nucleus (Percipalle 2009). Interestingly two recent studies provided evidence that in the nucleus F-actin formation is important for transcriptional activation of RNAP I and RNAP II transcribed HoxB genes (Ye et al. 2008; Ferrai et al. 2009). However none of the studies provided mechanistic insights into how nuclear actin dynamics regulates transcription and it is controlled.

7.2.1 *In vivo* alteration of actin polymerization affects RNA synthesis

To test whether regulation of actin dynamics plays a general role in transcription, we made use of actin-specific drugs. We treated living HeLa cells with latrunculin A (LatA), cytochalasin D (CytD) and Jasplakinolide (Jasp) and subsequently monitored transcriptional activity through a short ^{33}P-alphaUTP pulse. Interestingly, latrunculin A mediated depletion of polymerization-competent G-actin resulted in a general decrease of transcription. CytD and Jasp treatment respectively led to no effect and to a slight increase in global transcription. To confirm these initial observations we performed a run on assay on HeLa cell grown on coverslips incubated with LatA, CytD and Jasp. Transcription was monitored through a pulse with the cell permeable UTP analogue flouro-Uridine triphosphate (Furd), which was subsequently detected by immunofluorescence and confocal microscopy. To ensure that elongating RNAP II transcription was monitored cells were synchronized with DRB and after release of the DRB block they were treated with LatA, CytD and Jasp. Recovery of nascent transcription was observed within 30 min after DRB block release. Independently of the DRB block cells revealed a 70-80% reduction in FUrd incorporation after LatA and an increment of 10-20% in the presence of CytD and Jasp.

To confirm that the above effects were on RNAP II transcription, we extracted total RNA from LatA, CytD and Jasp treated cells and subsequently analyzed reverse transcribed cDNA by PCR. In these screens we monitored two regions of EP300 mRNA which encodes the HAT p300 and is ubiquitiously expressed. In N6-random primed cDNA preparation we obtained similar results as in the previous experiments, indicating that LatA inhibits whereas CytD and Jasp do not affect or induce marginal increase in transcription rates, respectively. Results obtain from oligo-d(T) primed cDNA confirmed the results for LatA with hardly any detectable

transcripts and for CytD with nearly same levels as in control cells. However, oligo-d(T) primed cDNA derived from Jasp-treated cells revealed a 60-80% reduction for EP300 mRNA amplicons. To exclude that we were observing reverse transcription artifacts, we subjected living cells to ^{33}P-UTP chase experiments and applied total RNA preparations to oligo-dT cellulose beads. The resulting poly(A) containing mRNA was analyzed by liquid scintillation. In line with the results obtained from our PCR screens LatA-treated and Jasp-treated cells revealed hardly any or only a marginal enrichment of poly(A) containing RNA, whereas CytD treated cells did not seem to be affected at all.

Altogether the above observations indicate that mRNA synthesis needs a G-actin pool presumably to feed dynamic polymeric actin. These findings prompted us to find out how these mechanisms are regulated.

7.2.2 Post-transcriptional gene silencing of cofilin-1 produces significant decrease RNA transcription in living cells

In mammals siRNA mediated post-transcriptional gene silencing of cofilin-1 is known to result in the formation of abnormal F-actin structures (Hotulainen et al. 2005). Since cofilin-1 is also found in the nucleus we decided to investigate the effect of siRNA mediated cofilin-1 knock down on general transcription levels in living cells. We applied a FUrd pulse in cofilin-1 silenced cells and monitored transcriptional activities by immunofluorescence and confocal microscopy. Interestingly, cofilin-1 silenced cells revealed a general reduction in the number of FUrd rich foci, which was observed to be concomitant with decreased steady state expression of cofilin-1. This finding suggested that cofilin-1 may be involved in transcriptional regulation. However these observations did not address whether the cofilin-1 regulates transcription by controlling actin.

7.2.3 Cofilin-1 is part of the same complex with actin and RNAP II

To investigate whether in living cells nuclear cofilin-1 directly interacts with actin and RNAP II, we performed gel filtration chromatography assays on nuclear extracts obtained from *in vivo* DSP-crosslinked HeLa. In agreement with the transient nature of the cofilin-1/F-actin interaction, cofilin-1 was co-eluted with RNAP II and

actin only from crosslinked cells with apparent molecular mass of 2-3 MDa. Immunoprecipitations using an anti-cofilin 1 antibody performed on nuclear extracts from DSP-crosslinked cells we found that cofilin-1 coprecipitated both with actin and RNAP II. However, when nuclear extracts were prepared in presence of 8M urea, cofilin-1 only co-precipitated with actin.

These results, taken together, suggest in the nucleus that cofilin-1 associates with actin and RNAP II. They also indicate that the nature of this interaction is transient and occurs via actin.

7.2.4 Cofilin-1 preferentially occupies coding regions of RNAP II genes in an actin-dependent manner

To address whether cofilin-1 associates with actively transcribed genes, we performed ChIP assays with antibodies specific to TATA box binding protein (TBP), phospho-Ser2 CTD, actin, H3, acetylated H3K9, cofilin-1, hnRNP U and PCAF. The precipitated chromatin was subsequently analyzed by PCR with primers specific to the 90kb long gene EP300. We tested gene promoter, exon 3, 6, 31 and 3'UTR as well as two non coding regions, flanking the 3'end of the EP300 gene cassette, termed 3'FL1 and 3'FL2. As expected the EP300 promoter site was co-precipitated strongly with antibodies specific to TBP, actin, H3, acetylated H3K9 but only marginally with antibodies specific to phospho-Ser2 CTD, hnRNP U and PCAF as well as cofilin-1. However, when analyzing gene coding regions we uncovered high co-precipitation efficiencies not only for phospho-Ser2 CTD, hnRNP U and PCAF but also for cofilin-1. Interestingly, when screening for region located towards the 3'end of the EP300 gene we observed a loss of signal for hnRNP U and PCAF at exon 31 and finally also a loss of actin and cofilin-1 in the 3'UTR. This result indicated that cofilin-1 may be coupled to the elongating RNAP II and actin along coding regions of actively transcribed genes. We confirmed this hypothesis since our anti-cofilin-1 antibody selectively precipitated coding regions of the S19, beta-tubulin and GAPDH genes and only marginally the corresponding promoter sites.

The above ChIP results prompted us to prove whether the presence of cofilin-1 at RNAP II gene coding regions correlates with the presence of actin. Therefore we applied ChIP to test the distribution of phospho-Ser2 CTD, acetylated H3K9, actin and cofilin-1, along the gene cassette of EP300, on cells treated with LatA, CytD and Jasp. LatA treatment resulted in a complete depletion of actin, cofilin-1 and

acetylated H3K9, an epigentic mark for active transcription, from EP300 gene promoter and coding regions. A similar effect was observed for the elongating form of RNAP II. However, exon 31, which is at the 3'end of the EP300 coding region, revealed significant phospho-Ser2 CTD enrichment and weak signals for cofilin-1. In contrast to LatA, the distributions of actin, cofilin-1, phosho-Ser2 CTD and acetylated H3K9 were as in control untreated cells, even though the detected ChIP signals were overall weaker. Interestingly, in Jasp-treated cells, when looking at gene coding region only promoter-proximal exon 6 showed levels of occupancy for cofilin-1, actin, phospho-Ser2 CTD and acetylated H3K9 which were comparable to control cells. However, a significant signal for acetylated H3K9 could be observed at the distal exon 31 only. To our surprise none of the drug treatments led to decreased signals for H3K9 acetylation in the EP300 3' flanking region, suggesting that this acetylation mark is created *in vivo* independent from actin or elongating RNAP II and does not represent a mark for active transcription.

All ChIP results taken together from drug-treated and untreated cells suggest that cofilin-1 preferentially occupies coding regions of actively transcribed genes. However, the distribution of cofilin-1 is dependent on the presence of actin and elongating RNAP II. Alteration of the delicate equilibrium between G- and F-actin, results in the release of actin, cofilin-1 and RNAP II these gene coding regions.

7.2.5 During transcription cofilin-1 regulates nuclear actin dynamics

The observation that cofilin-1 is found together with actin and elongating RNAP II at coding regions of transcribed genes raised the question as to whether cofilin-1 directly regulates actin dynamics during transcription. To address this question we investigated the effect of CytD and Jasp on transcription in cofilin-1 silenced cells.

As expected, cofilin-1 silenced cells showed only few FUrd rich foci in the nucleus. Interestingly upon treatment with Jasp transcriptional activation was observed 30 and 60 min after drug treatment and decreased after 120 min. CytD treatment revealed slower kinetic in transcription re-activation, but in contrast to Jasp-treated cells, transcriptional activity was maintained. Therefore since CytD could rescue a cofilin-1 knock out phenotype, we conclude that cofilin-1 is essential for

transcription by ensuring that actin filament formation as a consequence of G-actin recruitment is constantly maintained.

7.2.6 Cofilin-1 is required for the association of elongating RNAP II with active genes

To obtain insights into how cofilin-1 affects RNAP II during transcription cofilin-1 silenced, control and actinomycin D-treated cells were subjected to ChIP experiments. The lack of cofilin-1 in living cells resulted in a general depletion of actin from EP300 promoter, as well as promoter-proximal and distal exons. The same effect was observed for the phosphorylated RNAP II. Interestingly H3K9 acetylation levels were significantly reduced in cofilin-1 silenced cells.

Showing that lack of cofilin-1 results in loss of phopshorylated RNAP II and actin from EP300 coding regions suggests that cofilin-1 is needed during transcription to avoid uncoordinated actin filament formations which would otherwise inhibit and destabilize the elongating RNAP II complex at the gene.

7.3 PAPER III

Actin together with nuclear myosin 1 (NM1) is needed for RNAP I mediated transcription. Recent evidence indicated that actin and NM1 are not only engaged in RNAP I transcriptional initiation (Philimonenko et al. 2004) but also are found along gene coding regions. Actin cooperates with NM1 in the recruitment of the chromatin remodeling complex B-WICH to elongating RNAP I (Percipalle et al. 2006). NM1 is co-transcriptionally assembled into B-WICH and is found in direct association with the core components WSTF and the ATPase SNF2h. B-WICH contains several other nucleolar proteins including the RNA helicase II (DDX21) which is involved in pre-RNA processing. Interestingly, besides nucleolar proteins pre-rRNA has been found to associate with B-WICH (Cavellán et al. 2006). Therefore it has been suggested that NM1 presumably together with actin may be involved in post-transcriptional events of rRNA processing (Percipalle 2009). This hypothesis prompted us to test whether NM1 is present in pre-ribosomes, how this association is eventually mediated and if NM1 is engaged in any of the post-transcriptional processes taking

place during rRNA maturation and formation of export competent pre-ribosomal subunits.

7.3.1 NM1 associates with pre-ribosomal subunits, rRNA processing intermediates and mature rRNA transcripts.

To investigate whether NM1 is associated with rRNA we performed sucrose gradient sedimentation experiments on transcriptionally active, isolated nucleoli. To our surprise we found that NM1 was present in fractions corresponding to pre-60s and pre-40s ribosomal subunits and was absent if nucleolar extracts were treated with RNase. This observation motivated us to further investigate whether NM1 is found in direct contact with rRNA transcripts. To address this question we performed RIP experiments on native non-crosslinked HeLa nuclear extracts. Interestingly, NM1 could be co-precipitated with 18S and 28S rRNA as well 47S, 41S and 36S pre-rRNA transcripts. These results suggested that NM1 might be directly associated with RNAP I transcripts. However we could not distinguish whether NM1 genuinely associated with mature transcripts 18S, 28S and 5.8S or whether association was the result of NM1 binding to pre-rRNAs. We therefore made use of ActD which specifically blocks RNAP I transcription if utilized at low concentrations. Under these conditions it is also known that rRNA processing is not affected. A time course experiment revealed a significant drop in 47S levels already after 30 min of ActD treatment, whereas 36S and 32S were affected after 1h treatment. Although the levels of rRNA precursors in nuclear extracts and NM1 immunoprecipitates decreased throughout the ActD time-course, the levels of mature rRNAs were not affected at all. In contrast no 5S rRNA or 7SL RNA was detected suggesting a specific association of NM1 with RNAP I transcripts. Altogether these observations suggest that NM1 may be associated with transcripts throughout the entire rRNA processing pathway.

7.3.2 NM1 is involved in pre-rRNA maturation

The association of NM1 with mature as well as pre-rRNA, raised the possibility that NM1 is involved in the rRNA processing pathway *per se*. A classical approach would have been to test nuclear accumulation of certain pre-rRNAs in a NM1 knocked-down background. However, NM1 is essential for RNAP I transcription, thus

its absence would result in decreased synthesis of 47S rRNA itself which would impair a readout for downstream processing products. To bypass this problem we designed a novel assay on isolated nuclei. Briefly, we isolated nuclei from ActD treated HeLa cells, permeabilized the nuclei mildly with Triton-X and pre-incubated the nuclei on ice in the presence of antibodies against DDX21 (an RNA helicase involved in rRNA processing), NM1, actin and non-specific IgG as control. After 30 min preincubation, nuclei were placed in a 37C water-bath and were analyzed at different time points by PCR for their content of precursor and mature rRNAs. In the controls the presence of 47S, 41S and 36S precursors was significantly decreased after 30 min, and was not detectable already after 60 min. In contrast mature 28S rRNA was not affected throughout the whole time chase. Inhibition of NM1 and actin resulted in a significant and specific stabilization of 36S pre-rRNA throughout the entire chase experiment. Interestingly, a similar result was obtained upon inhibition of DDX21, consistent with its role in rRNA processing.

These results suggest that NM1 cooperates with actin during rRNA processing. To prove this hypothesis we next tested whether inhibition of the myosin ATPase activity by BDM or alteration of actin dynamics through LatA or CytD impaired rRNA processing. For this purpose living HeLa cell were treated with ActD and then with BDM, LatA or CytD. After 1h incubation we extracted the total RNA and analyzed rRNA by PCR. As expected no 47S, 41S and hardly any 36S pre-rRNAs could be detected, indicating that all precursor rRNA had been processed. Additionally we could not observe any reduction in the levels of mature 28S rRNA upon ActD showing that the lack of pre-rRNA is not due to unspecific RNA degradation. Surprisingly, we obtained significant enrichments for 36S pre-rRNA when cells were treated with LatA and BDM whereas CytD treatment did not have any effect. CytD functions as inhibitor of actin polymerization, stabilizing polymerization competent G-actin and therefore does not affect nuclear dynamics. LatA in contrast, depletes actin from the actin-cycle through formation of a stable LatA-actin-GDP complex.

Taking the above results together, we conclude that NM1 plays an essential role during maturation of 36S pre-rRNA, presumably together with actin.

7.3.3 NM1 localizes at the nuclear periphery and is associated with the nuclear pore complex

The engagement of NM1 in rRNA processing steps and its association with mature transcripts suggested that NM1 might accompany rRNA transcripts throughout processing, assembly and nuclear export of ribosomal subunits. In support of this hypothesis NM1 was found at the nuclear periphery in this study NM1, and was also found to be a component an emerin-associated proteome (Holaska and Wilson 2007).

To start addressing whether NM1 accompanied rRNA to the nuclear periphery, we performed a double immunostaining on interphase HeLa cells with antibodies to NM1 and Nup107, Nup153 and Tpr. Interestingly, we found that NM1 partly colocalized with Nup153 and Tpr which are components of the nuclear pore basket. We next examined whether NM1 is present at the nuclear pore per se. For this investigation we used *Xenopus leavi* oocytes which have a very well studied morphology of the NPC. Briefly, we manually isolated the nuclei in native conditions and directly incubated them with the NM1 antibody conjugated to 8nm colloidal gold. We subsequently analyzed these nuclei by thin-section immuno-electron microscopy. In support of the result obtained in HeLa cells by confocal microscopy, the electron-micrographs indicated that NM1 is present at the nuclear pore basket. Interestingly, a major portion of NM1 signals was detected at the nuclear pore in a distance of 80-90nm to the central plane, which is also the expected location for the RNA binding nucleoporin NUP 153 (Nakielny and Dreyfuss 1998; Fahrenkrog and Aebi 2003). To further confirm this result we performed field emission scanning electron microscopy on isolated nuclear envelopes and nuclear content. The specimen stained with Nup153, NM1 and actin-specific antibodies was visualized by gold labeled secondary antibodies. The results obtained from feSEM confirmed that NM1 is indeed associated with the nuclear pore basket. Interestingly, our NM1 antibody also stained actin containing pore-linked filaments, previously described to form a network between NPC and nuclear bodies in Xenopus leavis oocytes (Kiseleva et al. 2004). However the function of theses filaments and its presence in somatic cells of higher eukaryotes is not clear and has to be further investigated.

7.3.4 NM1 physically interacts with CRM1 and Nup153 and accompanies rRNA transcripts to the nuclear pore.

Previous studies have revealed that nuclear export of ribosomal subunits is mediated via exportin 1(CRM1) and facilitated by Nup153 (Thomas and Kutay 2003; Soop et al. 2005).

Given our observations on the association of NM1 with rRNA transcripts and its presence at the basket of the NPC we evaluated whether NM1 associates with Nup153 and CRM1. Indeed we found that NM1, CRM1 and Nup153 can be coprecipitated from nuclear extracts obtained from DSP-crosslinked HeLa cells. We next tested whether rRNA-associated NM1 may simultaneously be in complex with both CRM1 and NUP153. For this purpose we performed successive RNA immunoprecipitation assays (reRIP) on formaldehyde-crosslinked HeLa cells. Briefly, we first subjected nuclear extracts from crosslinked cells to immunoprecipitations with anti-NM1, CRM1 and NUP153 antibodies and analyzed the precipitated rRNA by RT PCR. Consistent with our previous results and already published data endogenous NM1, CRM1 and NUP153 precipitated 18S and 28S rRNAs. For each immunoprecipitation we next tested the individually eluted material with the remaining two antibodies. Surprisingly, we found that from the NM1-precipitated fraction subjected to immunoprecipitation with antibodies to CRM1 and NUP153 we could precipitate 18S and 28S rRNA. The same result was obtained when subjecting CRM1 eluted material and Nup153 eluted material to immunoprecipitations with the remaining antibodies. These findings confirmed that NM1, CRM1 and Nup153 can form a complex on the same rRNA molecule.

Taking all results together, we suggest that NM1 has an essential role in rRNA biogenesis where it most likely cooperates with actin. NM1 accompanies the transcripts from nucleolar synthesis sites throughout all processing steps and pre-ribosomal assembly all the way to the NPC where it may be engaged in CRM1-mediated rRNA export.

8 GENERAL DISCUSSION

Our results show that actin and NM1 are key players in RNA biogenesis. By studying the engagement of actin at constitutively expressed RNAP II genes we provided evidence that actin is not only required for transcription initiation (Philimonenko et al. 2004) but it also has an essential role in the elongation phase. All genes, which were investigated, revealed that actin occupies both promoter sites and gene coding regions but it is absent in intergenic regions (Papers I, II). We could show that in mammals, actin in complex with hnRNP U is recruited to the elongating RNAP II *in vivo* and *in vitro* to function as a recruitment platform for the HAT PCAF. This mechanism seems to be required for basal transcription and is highly conserved from insects to humans as found in the *Chironomus tentans* model system (Sjölinder et al. 2005). This observation is of particular importance since eukaryotic chromatin in actively transcribed genes is hypoacetylated at coding regions. At these sites, decreased histone acetylation levels are considered to function as a barrier for cryptic initiations (Joshi and Struhl 2005). It has been suggested that recruitment of HAT co-activators to the elongating RNAP II complex is needed for efficient transcription through gene coding sites (Mellor 2006; Selth et al. 2010). Actin-hnRNP U mediated PCAF recruitment to the Ser2 phosphorylated CTD *in vivo* and *in vitro* (Paper I) is absolutely in line with this concept. In fact it suggests a central role for actin in facilitating migration of the elongating RNAP II through a hypoacetylated nucleosome barrier.

Whether actin engaged in transcription is needed in its filamentous configuration or in a more globular monomeric form is the main question in the emerging nuclear actin field. Using a combination of actin-specific drugs and RNAi approaches we obtained experimental data indicating that during transcription actin undergoes dynamic polymerization which is highly regulated in order to have a continuous input of G-actin monomers (Paper II). Through a comparative analysis on the effects of actin-specific drug treatments on living cells we found that depletion of polymerization-competent G-actin by LatA inhibits global transcription. This observation is in line with data from the laboratory of Ingrid Grummt (Ye et al. 2008). However, we found that stabilization of actin filaments mediated by Jasp does not simply support transcription *per se* but it seems to deregulate elongation and termination as well as 3'end processing events. In fact we know that Jasp did not show any impairment in splicing. When analyzing the effects of CytD, we did not observe

any decrease in transcription rates and on the mRNA quality. This observation is of special importance since CytD has the dual functions of inhibiting actin-filament growth while acting as an actin nucleator (Goddette and Frieden 1986). This complex scenario led us to investigate whether there are proteins that regulate nuclear actin dynamics. Indeed we found that the F-actin severing protein cofilin-1, which is abundantly present in the cell nucleus (Pendleton et al. 2003) associates with actin and RNAP II, preferentially at coding regions of actively transcribed genes. We could also show that cofilin-1 is essential for efficient transcription elongation and for the association of elongating RNAP II complex on the gene (Paper II). Thus these results indicate that during transcription F-actin formation has to be controlled to maintain progression of the elongating RNAP II complex. In this context an, interesting observation was made in the laboratory of Gerald Crabtree and indicated that BRG1, which is the subunits of the human BAF chromatin remodeling complex can interact *in vitro* with F-actin at its pointed ends (Rando et al. 2002). This may indicate that co-transcriptional F-actin formation may play a role in chromatin remodeling during RNAP II transcription and that spatial regulation of BAF at the chromatin may be controlled by the activity of cofilin-1.

At a first glance our results on nuclear actin polymerization may not go in hand with recently published data (Ye et al. 2008; Ferrai et al. 2009). However taking into account that those findings were obtained in very special transcriptional models, namely transcription in chromosomal NORs regions in the case of RNAP I and RA-inducible HoxB genes, differences to our results derived from constitutively expressed RNAP II genes are not surprising. An explanation for eventual discrepancies may be found in recently published work in the nuclear structure and dynamics field. In this context a number of studies provided evidence that actin filament formation together actomyosin motor functions is involved in long range gene repositioning upon transcriptional activation (Chuang et al. 2006; Dundr et al. 2007; Hu et al. 2008). Therefore formation of actin filaments at gene promoter sites or regulatory elements as previously reported (Ferrai et al. 2009) may make perfect sense. In this context also transcriptionally active NORs, the foci of RNAP I transcription which are considered as prototypes for transcriptional factories (Grummt 2003), may be regulated by similar mechanisms during induction and enhancement of RNAP I transcription. In support of this view it is again not surprising that treatment of cells with CytD lowered transcription levels in the context of rRNA and HoxB genes expression (Ye et al. 2008; Ferrai et al. 2009). It also important to question whether reported effects derived from

inhibition of actomyosin motor functions at rRNA genes are based on impaired mobility of the elongating RNAP I transcription complex (Ye et al. 2008) or if they simply result from impaired nucleolar structural arrangements which could rather have an impact on transcriptional initiations at promoter sites. In agreement with the last point Cisterna and co-workers have recently reported that inhibition of actin and myosin does not simply block RNAP I transcription but induces severe structural changes in the nucleolar organization (Cisterna et al. 2009). Thus the state of the art in the context of actomyosin motors facilitating migration of an elongating RNAP complex is far from being understood.

When further analyzing the composition of the B-WICH complex it was found that it contains rRNA processing factors and pre-rRNA (Percialle et al. 2006; Cavellán et al. 2006). These observations provided the rationale for the third study (Paper III) reported in this thesis. We hypothesized that NM1 could be implicated in later phases throughout rRNA biogenesis and not only at the transcriptional level. Consistently, in this study we reported evidence that NM1 associates with all intermediate pre-rRNAs and mature 28S, 5.8S and 18S rRNAs but does not associate with 5S rRNA, which is transcribed by RNAP III. This suggests that NM1 associates with rRNA transcripts from their site of synthesis through assembly into export-competent pre-ribosomal subunits and perhaps accompanies them to the NPC.

Early cytological studies suggested that certain myosin species could be coupled to the NPC (Berrios and Fisher 1986; Berrios et al. 1991). Indeed NM1 together with actin is present in the nuclear periphery, in close proximity to the NPC. In addition NM1 localizes at the NPC basket where it forms a complex with Nup153 and CRM1 on the same mature rRNA transcripts. We also found that inhibition of NM1, actin or the actomyosin interaction results in stabilization of certain precursor rRNA species. Interestingly we found that NM1 is capable to bind rRNA in absence of chemical as well UV light mediated cross-linking suggesting that NM1 may be in tight physical contact with its associated rRNA. However we did not address whether this interaction is direct or mediated by an RNA-binding adaptor protein. A possible explanation for our result from Paper III is that NM1 and actin cooperate for early rRNP remodeling events that take place during 90s/SSU processosome and at later stages of pre-ribosomal assembly. Along these lines the association of NM1 with nascent 47S pre-rRNA as well its association with processing intermediates and mature transcripts in direct contact with the NPC export machinery supports the view of a

cotranscriptional pathway leading to maturation of ribosomal subunits (Granneman and Baserga 2004).

Immediately after assembly both ribosomal subunits and mRNPs are released into the nucleoplasm where they head towards the NPC. There is evidence that these particles in transit to the NPC behave as freely diffusing particles (Politz et al. 2003; Shav-Tal et al. 2004). Our data are in agreement with this view since actin and NM1 are assembled as RNP components and they are not likely to facilitate directional movement during intranuclear transport. We rather speculate that the dynamic actin-NM1 interaction facilitates assembly and disassembly of RNA binding factors such as RNA helicases which are required for RNP maturation (Percipalle 2009). On the other hand, since both actin and NM1 accompany RNPs to the NPC they may also cooperate at the nuclear envelope. NM1 physically interacts with Nup153 and CRM1. These three factors together seem to form a complex on the same mature 18S and 28S rRNAs while they are excluded from the 5S rRNA synthesized by RNAP III. These observations altogether support an involvement of NM1 presumably together with actin in preparatory events that lead to CRM1 mediated nuclear export of ribosomal subunits.

In summary our work underscores the ever growing evidence that actin and myosin play essential functions during nuclear RNA biogenesis. The actin-NM1 synergy has been dissected in the case of rRNA biogenesis (Fomproix and Percipalle 2004; Philimonenko et al. 2004; Percipalle et al. 2006). However even though NM1 has been implicated in RNAP II transcription *in vitro* (Hofmann et al. 2004; Hofmann et al. 2006), it is still unclear how actin cooperates with NM1 in RNAP II transcription. During the period of this PhD training a number of reports have been published, showing that other myosin species are also involved in nuclear processes (Louvet and Percipalle 2009). For instance MyoVI was shown to associate with RNAP II and actively transcribed RNAP II gene coding and promoter regions (Vreugde et al. 2006). Considering that ongoing studies in our lab (not included in this thesis) suggest that NM1 occupies both promoter and gene coding region of RNAP II genes, it is possible that in RNAP II transcription actin synergizes with multiple myosin species for specialized tasks.

Based on our results and published evidence (see also Visa and Percipalle 2010), two possible models are hypothesized to depict the engagement of actin and myosin in transcription elongation and ribosomal biogenesis:

I. Actin together with specific hnRNPs such as hnRNP U assembles at Ser2-phopshorylated CTD to provide a platform for subsequent co-activator recruitment such as the HAT PCAF (Figure 4). This leads to acetylation of chromatin downstream the elongating RNAP II complex, thereby facilitating its migration through the nucleosomal barrier. Actin polymerization, which may be further enhanced through co-transcriptional recruitment of F-actin nucleation factors such as N-Wasp and ARP2/3, is controlled by transient interaction with actin-severing proteins such as cofilin-1, to avoid eventual sterical hindrance of the migrating RNAP II complex. Oligomeric actin may further lead to recruitment of chromatin remodeling complexes such as SWI/SNF to support migration of the polymerase complex or to re-establish nucleosomal structure after passage of the elongating RNAP II. Actin and associated co-factors are finally recruited to nascent pre-mRNPs where the HAT may perform factor acetyltransferase (FAT) activity to facilitate eviction of certain RNPs, actin(-oligomers) and the HAT itself during pre-mRNP assembly.

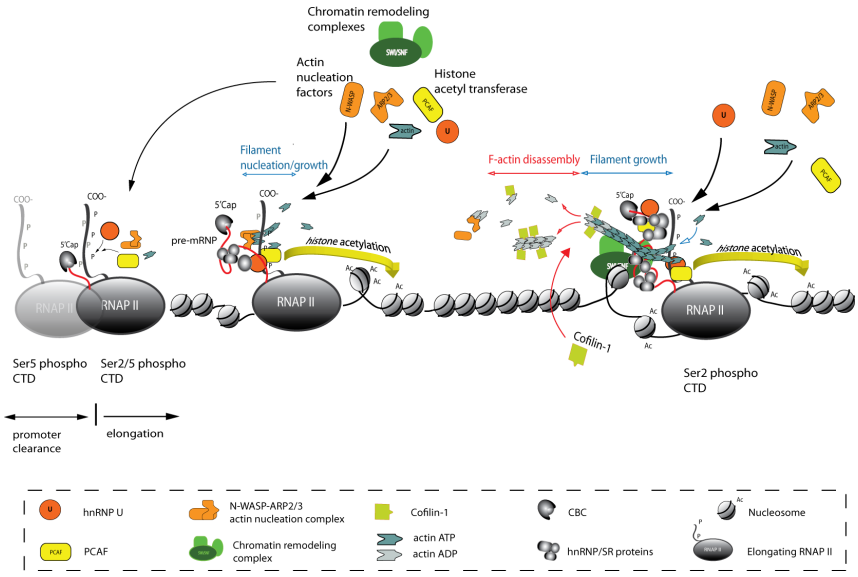

Figure 4. A model describing actin function and dynamics during RNAP II elongation. The assembly of actin and hnRNPU at phospho-Ser2 CTD of the elongating RNAP II functions as a platform for cotranscriptional recruitment of PCAF and it is essential for efficient transcription through the nucleosomal barrier (Percipalle and Visa 2006). Co-transcriptional actin polymerization is enhanced through the actin nucleation complex N-WASP-ARP2/3 and controlled by transient interaction with

cofilin-1. Cotranscriptional formation of short actin-filaments or oligomers may serve as attachment site for human SWI/SNF chromatin remodeling complex.

II. Actin in the form of short oligomers may cooperate with myosin as a dynamic molecular switch for the migration of RNAP I along the rDNA gene coding cassettes. One could imagine that actin and myosin interact as a transient bridge between polymerase and chromatin remodeling factors such as B-WICH facilitating chromatin remodeling activity downstream the elongating RNAP I complex for active transcription. Co-transcriptional actin filament growth is likely to be controlled as indicated in model I, in which actin severing proteins like cofilin-1 might play a central role (see model I).

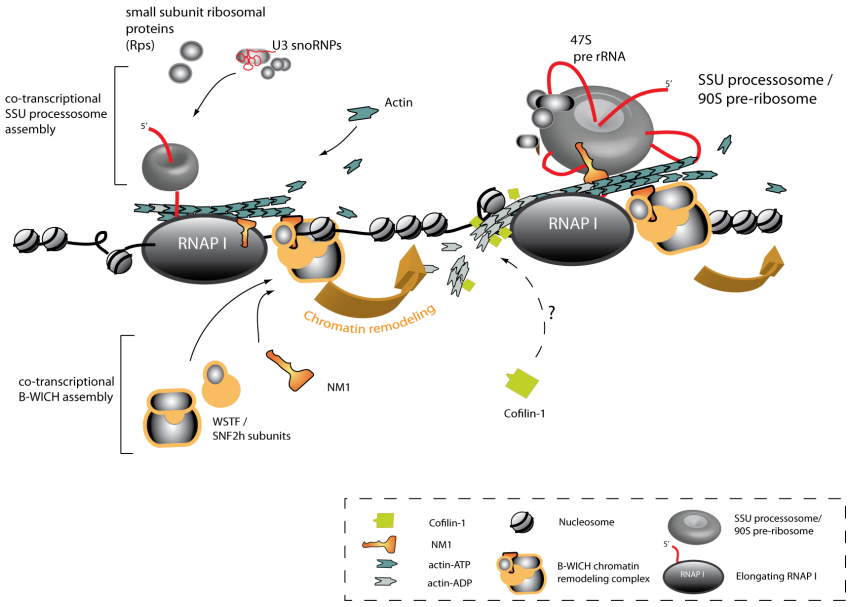

Figure 5. Actin and NM1 cooperate for RNAP I elongation. NM1, WSTF and SNf2h assemble co-transcriptionally into the B-WICH chromatin remodeling complex. B-WICH recruitment to active rRNA gene coding region is mediated via the dynamic actin-NM1 interaction that has been proposed to function as a molecular switch to facilitate migration of the RNAP I through chromatin (Percipalle and Östlund Farrants 2006). We speculate that actin polymerization may be controlled in a similar manner as for RNAP II. NM1 is cotranscriptionally associated with nascent pre-rRNA and becomes finally incorporated into SSU processosome (see also figure 6).

NM1 is subsequently recruited to the nascent precursor rRNA and assembles together with non-ribosomal and ribosomal proteins in the nascent SSU processosome. NM1 presumably cooperates with actin for rRNP remodeling events which take place during pre-ribosomal assembly and are essential for the formation of export competent 40S and 60S pre-ribosomes.

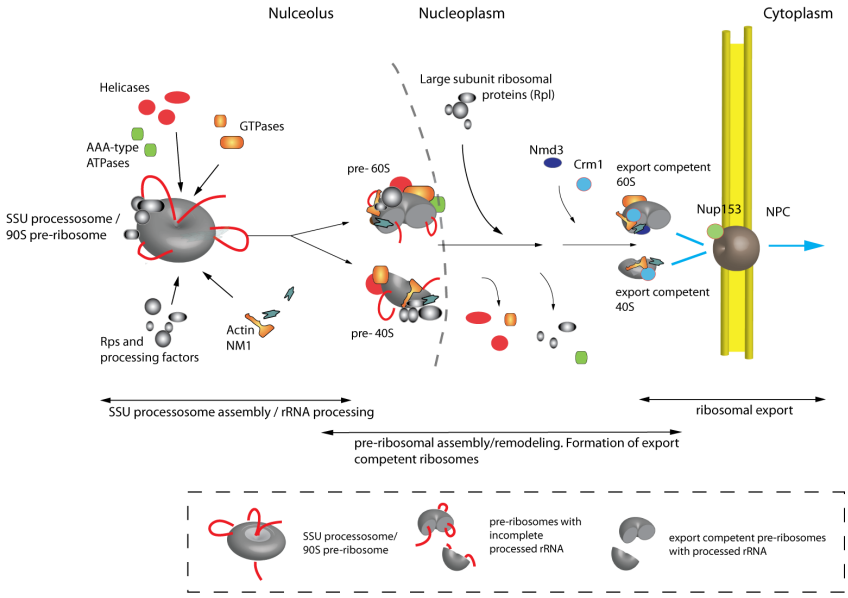

Figure 6. **Actin and NM1 cooperate in ribosomal biogenesis.** During transcription NM1 is recruited to 47S pre-rRNA and stays associated with the RNA throughout rRNA processing and pre-ribosomal assembly. The synergy between NM1 and actin may reflect the need for energy consuming mechanisms that are required during rRNP remodeling events that probably take place during rRNA processing and nuclear export. CRM/exportin-1, export receptor; Nmd3, non-sense-mediated decay protein 3 (adaptor for Crm1); Nup153, nucleoporin 153; NPC, nuclear pore complex; Rps, small subunit ribosomal proteins.

9 PERSPECTIVES

Multiple studies from recent years implicate that actin-based myosin motors are involved in many aspects of nuclear function. Since ATP consuming processes are found at multiple steps of RNA biogenesis it is not difficult to imagine the engagement of these factors in chromatin remodeling, transcription, RNA processing and RNP assembly. Nevertheless, we are just at the beginning of this exciting fast developing field. The discovery of additional myosin species in the nucleus beside NM1 emphasizes that the establishment of specific nuclear actin-myosin is likely to be regulated through complex mechanisms. Thus it is now time to define exactly which myosin species are needed for transcription, processing and packaging. It is important to further reveal if different myosins or actomyosin complexes show a degree redundancy or exclusively target products of certain polymerases. In these lines recent advances in the field of mass spectrometry, genome wide sequencing and bioinformatics will help to clarify these undefined aspects. Thus a combination of affinity chromatography approaches with mass spectrometry, Chip-seq and Rip-seq will help to define the general or specific engagements of particular actomyosin complexes in the cell nuclei.

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