Non-protein-coding RNA
Transcription and regulation of ribosomal RNA

Stefanie Böhm
To my loved ones.
Summary

Cell growth and proliferation are processes in the cell that must be tightly regulated. Transcription of ribosomal RNA and ribosomal biogenesis are directly linked to cell growth and proliferation, since the ribosomal RNA encodes for the majority of transcription in a cell and ribosomal biogenesis influences directly the number of proteins that are synthesized.

In the work presented in this thesis, we have investigated the ribosomal RNA genes, namely the ribosomal DNA gene and the 5S rRNA gene, and their transcriptional regulation. One protein complex that is involved in RNA polymerase I and III transcription is the chromatin remodelling complex B-WICH. B-WICH is composed of the proteins WSTF, SNF2h and NM1. RNA polymerase I transcribes the rDNA gene, while RNA polymerase III transcribes the 5S rRNA gene, among others. In Study I we determined the mechanism by which B-WICH is involved in regulating RNA polymerase I transcription. B-WICH is associated with the rDNA gene and was able to create a more open chromatin structure, thereby facilitating the binding of HATs and the subsequent histone acetylation. This resulted in a more active transcription of the ribosomal DNA gene. In Study II we wanted to specify the role of NM1 in RNA polymerase I transcription. We found that NM1 is not capable of remodelling chromatin in the same way as B-WICH, but we demonstrated also that NM1 is needed for active RNA polymerase I transcription and that NM1 is able to attract the HAT PCAF. In Study III we investigated the intergenic part of the ribosomal DNA gene. We detected non-coding RNAs transcribed from the intergenic region that are transcribed by different RNA polymerases and that are regulated differently in different stress situations. Furthermore, these ncRNAs are distributed at different locations in the cell, suggesting that they are involved in different processes. In Study IV we showed the involvement of B-WICH in RNA Pol III transcription and, as we previously had shown in Study I, that B-WICH is able to create a more open chromatin structure, in this case by acting as a licensing factor for c-Myc and the Myc/Max/Mxd network.

Taken together, we have revealed the mechanism by which the B-WICH complex is able to regulate RNA Pol I and Pol III transcription and we have determined the role of NM1 in the B-WICH complex. We conclude that B-WICH is an important factor in the regulation of cell growth and proliferation. Furthermore, we found that the intergenic spacer of the rDNA gene is actively transcribed, producing ncRNAs. Different cellular locations suggest that the ncRNAs have different functions.
List of articles included in this thesis


Fatemeh Sadeghifar, **Stefanie Böhm**, Anna Vintermist and Ann-Kristin Östlund Farrants. The B-WICH chromatin-remodelling complex regulates RNA polymerase III transcription by promoting Max dependent c-Myc binding. (To be submitted before the defence).

**Stefanie Böhm**, Judith Domingo Prim, Anna Vintermist, Neus Visa and Ann-Kristin Östlund Farrants. Non-coding RNAs from the rDNA intergenic repeat are transcribed by RNA polymerase I and II and have different functions. (To be submitted before the defense).

and parts of


**Work not included in this thesis**


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<tr>
<td>CSB</td>
<td>Cockayne syndrome protein B</td>
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<tr>
<td>HAT</td>
<td>Histone acetyl transferase</td>
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<td>HDAC</td>
<td>Histone deacetyl transferase</td>
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<td>HP1</td>
<td>Heterochromatin protein 1</td>
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<tr>
<td>IGS</td>
<td>Intergenic spacer sequence</td>
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<td>ISWI</td>
<td>Imitation switch</td>
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<td>ncRNA</td>
<td>Non-protein-coding RNA</td>
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Introduction

The transcription of ribosomal RNA is crucial for cell growth and proliferation, since ribosome biogenesis correlates directly with protein synthesis. The transcription of the ribosomal RNA genes, therefore, must be tightly regulated, considering that ribosomal transcription makes up the majority of transcription in a cell. In human cells the 5S rRNA, which is part of the ribosome, is transcribed by RNA polymerase III, while the ribosomal DNA gene is transcribed by RNA polymerase I. The ribosomal DNA gene comprises 43 kb, divided into a coding region of length 13 kb and an intergenic spacer sequence of length 30 kb. Many factors regulate Pol I and III transcription, including c-Myc, p53 and the B-WICH complex. B-WICH binds to the Pol I and III genes and regulates their transcription. The mechanism by which B-WICH is involved in regulating the ribosomal RNA is, however, not known. We have investigated the role of B-WICH in ribosomal transcription, and we have explored the intergenic spacer sequence of the ribosomal DNA gene.

RNA world

Transcription is essential for cell survival. RNA is transcribed from a DNA template in the nucleus and the RNA may be one of two kinds. Transcription can result in a messenger RNA (mRNA), when the mRNA codes for a protein. In this case, the mRNA is just an intermediate product, translation follows and the result will be a protein. The other kind is the non-protein-coding RNA (ncRNA). In this case, the RNA itself is the functional and regulating molecule. To the class of ncRNAs belong, for example, the rRNA and the tRNA that are involved in mRNA translation. In eukaryotes, many ncRNAs belong to the class of small ncRNAs, such as the snRNA, snoRNA, miRNA, piRNA and siRNA (Bernstein and Allis 2005, Huttenhofer,
Schattner et al. 2005, Mattick and Makunin 2006). The functions of these small ncRNAs have been characterised in detail. While snRNAs play a role in splicing and the snoRNAs are involved in rRNA processing, the miRNA, piRNA and siRNA have a function in translational silencing (Mattick and Makunin 2006). Beside the small ncRNAs another class exists: the long intergenic ncRNAs (lincRNAs). These can be several kb long and are involved in many functions, such as epigenetic regulation, transcriptional repression, and gene dosage compensation (Guttman, Amit et al. 2009).

Non-protein-coding RNA (rRNA and tRNA)

The term “non-protein-coding RNA” (ncRNA) is, as the name implies, RNA that is not translated into a protein. NcRNAs are neither intermediate products nor waste products. The functions of many such ncRNAs have been determined. However, there are still many ncRNAs that need to be characterised in their function. Transfer RNA (tRNA) and ribosomal RNA (rRNA) are two ncRNAs that have long been known, and have been studied in detail. tRNA carries the amino acid and interacts with the mRNA, while rRNA assembles the ribosome, together with ribosomal proteins. These RNAs are transcribed from particular genes, the tRNA genes and the ribosomal gene (Steitz and Moore 2003, Noller 2005).
Ribosomal RNA

In mammalian cells, the ribosomal RNA is transcribed from two different genes: The ribosomal DNA gene, from which the 45S pre-rRNA is transcribed, and the 5S rRNA gene. Both genes are arranged in tandem repeats and exist in many copies on the chromosomes. The ribosomal DNA gene is transcribed by RNA polymerase I, while the 5S rRNA gene is transcribed by RNA polymerase III. However, after transcription these ribosomal RNAs are combined to assemble the ribosome.

The 5S rRNA gene

5S rRNA genes are very small genes. Like the rDNA genes, they are arranged in tandem repeats and mostly localised on chromosome 1 (Sorensen, Lomholt et al. 1991). The 5S rRNA is 120 bp long and has a molecular weight of 40 kd. In contrast to the long ribosomal RNA, the 5S rRNA is transcribed by RNA polymerase III (Pol III). In humans, the 5S rRNA is transcribed in the nucleus, while in yeast the gene is located in the nucleolus, beside the 35S rRNA gene (Bell, DeGennaro et al. 1977). The 5S rRNA in humans is, however, shuffled to the nucleolus after its transcription. There it combines with other ribosomal RNAs to assemble the ribosome. The 5S rRNA, together with the 5.8S and the 28S rRNAs, and several ribosomal proteins, form the large ribosomal subunit; while the 18S rRNA, together with other ribosomal proteins, forms the small ribosomal subunit (reviewed in (Birch and Zomerdijk 2008)).

The ribosomal DNA gene

The ribosomal DNA genes (rDNA) are located on several chromosomes (13, 14, 15, 21 and 22) in sections that are known as the “nucleolar organized region” (NOR), around which the nucleoli are formed. In humans, 300-400
copies of the rDNA are arranged in tandem repeats, but approximately 50% of the genes are actively transcribed in differentiated cells. Hence, the ribosomal DNA can exist in an active state or an inactive state. The active state, however, can be pseudo-silenced, but is still transcriptionally competent (Sanij, Poortinga et al. 2008). The rDNA is transcribed as a large pre-transcript, the 47/45S rRNA. The 47/45S rRNA pre-transcript is transcribed by the RNA polymerase I (Pol I) with its own transcription machinery. The pre-transcript is then processed in 5.8S, 18S and 28S rRNA (reviewed in McStay and Grummt 2008).

The rDNA gene comprises 43 kb and is divided into a coding region, which covers about 13 kb, and an intergenic spacer sequence (IGS), which covers 30 kb. Many Alu elements are located in the intergenic spacer of the human gene repeat, to which they contribute 5474 bp. A pseudogene of the cell cycle protein Cdc27 is located from bp 33,673-36,005. A p53 binding site is located at around 39.5 kb (Gonzalez and Sylvester 1995). p53 is a tumor suppressor protein that controls the transcription of the rDNA gene (Ko and Prives 1996).

**Figure 1: Schematic illustration of the human rDNA repeat unit**

**Non-protein-coding RNA (lincRNA)**

Non-coding RNAs are often transcribed from intergenic regions and introns, and they may be transcribed in the antisense direction relative to the direction of transcription of genes. Pseudogenes are also transcribed into RNA.
The number of ncRNAs in an organism rises with its complexity. Just a small number of ncRNAs exist in prokaryotes, and the numbers of ncRNAs are higher in eukaryotes (Mattick and Makunin 2006). For many of the identified lincRNAs, no function has yet been discovered. The term “long ncRNA” applies when the transcript is more than 200 nt long. Several well-characterised long ncRNAs in mammals exist, such as Xist, MALAT, HOTAIR and Kcnq1ot1. Xist is an ncRNA of length 17 kb. It is transcribed from the X chromosome and it is involved in gene compensation. It silences one X chromosome in female mammals (Heard 2004). MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) is an ncRNA of length 8 kb that is expressed in human tissue and overexpressed in cancer (Ji, Diederichs et al. 2003). MALAT1 is involved in alternative splicing (Tripathi, Ellis et al. 2010). The long ncRNA HOTAIR is involved in gene repression (Rinn, Kertesz et al. 2007; Khalil, Guttman et al. 2009). It is expressed from the HOXC genes and is one of many ncRNAs that interact with the polycomb repressing complex 2 (PRC2) (Tsai, Manor et al. 2010). Kcnq1ot1 is an ncRNA of length 91 kb, transcribed in the antisense direction relative to the Kcnq1 gene. It is involved in genomic imprinting, and in the silencing of genes in the Kcnq1 domain (Pandey, Mondal et al. 2008). Mohammad et al. (2010) showed that Kcnq1ot1 is associated with chromatin and that it interacts with Dnmt1 (Mohammad, Mondal et al. 2010). Since ncRNAs are involved in a variety of functions, they can be distributed throughout the cell (Fig. 2). It has been proposed that more or less the whole genome of eukaryotes is transcribed. New techniques, in which the whole transcriptome is analysed, make it possible to detect more ncRNAs (Okazaki, Furuno et al. 2002). Of course, not all transcripts are functional. For RNA polymerases, it might just be energy-saving to transcribe the whole genome, including the intergenic regions. Many cryptic unstable transcripts (CUTs) are produced and these must be degraded. This is taken care of by surveillance mechanisms in the cell. Machinery known as the “exosome complex” degrades
most of the instable transcripts in eukaryotic cells (Wyers, Rougemaille et al. 2005, Houseley and Tollervey 2009).

Figure 2: Locations of different ncRNAs in the cell

RNA surveillance mechanism

The exosome complex, which in humans is also known as the PM/Scl-complex (polymyositis/scleroderma-complex), is a multisubunit complex that consists of different RNases. This RNase complex has a 3-5 exoribonuclease activity and is able to degrade mRNA that is not processed correctly (Vanacova and Stefl 2007). The exosome is needed also for the processing of the ribosomal and snoRNAs, and is present both in the nucleus and in the cytoplasm (Shen and Kiledjian 2006).

The exosome and its subunits were first identified in yeast in 1997. The human exosome was subsequently characterised, and it became clear that the subunits are conserved between yeast and human. The PM/Scl-complex exists of six core proteins that form a doughnut-shaped hexamer, with three further subunits on the top. The hexameric ring is built by hRrp41, hRrp42,
hMtr3, hRrp43, hRrp46 and the PM/Scl-75 (hRrp45) (Fig. 3). Characteristic for these subunits is that they contain an RNase PH (phosphorolytic exonuclease) domain. These RNases use inorganic phosphate to cleave RNA. However, the exonuclease activity is derived only from the hRrp41/hRrp45 dimer; the other subunits do not have an exoribonuclease activity (Shen and Kiledjian 2006, van Dijk, Schilders et al. 2007). The proteins that form the top cap are hCsl4, hRrp40 and hRrp4 (Fig. 3). These proteins contain an S1 RNA-binding domain and two of them contain also a K-homology (KH) domain. These cap proteins are needed for a stable exosome complex to be formed. One further protein has been identified, PM/Scl 100, which is directly homologous to Rrp6 in yeast. hRrp6 belongs to the RNase D family – a hydrolytic exoribonuclease. It is associated with the exosome, but is not part of the core complex. It is more closely associated with a nucleosomal exosome complex. In yeast, another associated protein, Rrp44, carries the activity in the yExo11 (exosome) complex (Allmang, Petfalski et al. 1999). An orthologue has been identified in humans, hDis3, but this is not physically associated with the exosome (Houseley, LaCava et al. 2006, Vanacova and Stefl 2007).

Figure 3: Composition of the exosome complex
ncRNA in the IGS of the rDNA gene

Non-coding RNAs in the rDNA intergenic repeat have been identified in several species. The IGS of the rDNA gene is transcribed in yeast. However, transcripts from the IGS are unstable and are thus substrates for the exosome complex. The ncRNA is rapidly degraded (Houseley, Kotovic et al. 2007). In mouse cells, the nucleolar remodelling complex (NoRC) contains ncRNA, also known as “pRNA”, which is transcribed from the intergenic region (IGS) of the rDNA gene. A spacer promoter is located 2000 nt upstream of the 45S pre-rRNA promoter. An ncRNA is transcribed from this spacer promoter, which is needed for the recruitment of NoRC to the chromatin. Furthermore, this ncRNA is essential for the silencing of the rDNA gene. The pRNA is 150-200 nt long and forms a secondary structure to which NoRC is able to bind. It has been proposed that there are more spacer promoters located in the IGS that show a related arrangement to the gene promoter (Mayer, Schmitz et al. 2006).

The CCCTC binding factor (CTCF) binds to the IGS of the rDNA in mouse cells upstream of the spacer promoter. The binding of CTCF to this region is essential, and it regulates the transcription of the pRNA. CTCF binds also to UBF, and it is required for UBF and Pol I to bind to the IGS near the spacer promoter. CTCF is also involved in the copy number control of the rDNA genes. Two binding sites of CTCF in the IGS have been identified in humans: one at H37.9 and one at H42.1. NcRNAs arising from these positions have not been identified (van de Nobelen, Rosa-Garrido et al. 2010).

Another approach to identify ncRNA in the IGS of human cells was used by Zentner et al. ChIP- and RNA-seq data from databases were aligned to the rDNA, trying to map histone patterns in the IGS and to identify possible ncRNAs. From the ChIP-seq data it could be concluded that the active histone modifications have a more distinct pattern over the IGS than the repressive histone modifications. The repressive histone modifications have a more
even distribution, while a peak of active histone modifications was found at the locations 28/29 kb and just upstream of the rDNA promoter. This suggests that promoters may be located at these positions. Small amounts of ncRNA are present at 28 kb and one small peak of RNA was visible at 43 kb, suggesting that this is the pRNA in human (Zentner, Saiakhova et al. 2011).

Audas et al. were able to detect ncRNAs that are transcribed from the IGS of the rDNA in humans. These ncRNAs are upregulated in different stress situations, binding to specific proteins, such as HSP70, in response to stress, and sequestering these proteins in the nucleolus (Audas, Jacob et al. 2012).

The RNA polymerases

There are three different RNA polymerases in eukaryotes: RNA polymerase I, II and III. They are responsible for transcribing different genes. While RNA polymerase II transcribes all protein-coding genes, RNA polymerase I transcribes the rDNA gene, and RNA polymerase III transcribes the 5S rRNA gene, the 7SL RNA gene, tRNA genes and snRNA genes.

RNA polymerase I transcription

RNA polymerase I (Pol I) transcribes only the rDNA gene. A basal machinery of proteins is needed for the initiation of Pol I transcription on active rDNA genes. The basal machinery is also known as the “pre-initiation complex” (PIC), and consists of Pol I-specific transcription factors and Pol I. The binding of UBF (upstream binding factor) to the enhancer, and the binding of SL1 (selectivity factor 1) to the promoter of the rDNA gene must precede the association of Pol I (reviewed in (Russell and Zomerdijk 2005)). UBF is a transcription factor that binds to the upstream core element that is localized upstream of the rDNA promoter. SL1, which contains a TATA-binding protein (TBP) and at least three TBP-associated
factors (TAFs), is a core-promoter-binding factor that binds to the core element and interacts with UBF, an interaction that is required for active transcription (Grummt 2003). SL1, in turn, attracts TIF-IA (in mice) or RRN3 (in humans), after which Pol I is recruited and transcription can be initiated (White 2005). TIF-IA must be phosphorylated to be able to bind to SL1, attract Pol I, and initiate transcription (Zhao, Yuan et al. 2003). Upon successful PIC formation and initiation of transcription, Pol I can escape from the promoter and continue to elongation, while UBF and SL1 remain at the promoter (Panov, Friedrich et al. 2001). The transcription termination factor (TTF-I) is located downstream of the rDNA transcription unit. It can bend the DNA and in this way pause Pol I. When TTF-1 works together with the transcript-release factor (PTRF), transcription can be terminated and the transcript released from the template DNA (Gerber, Gogel et al. 1997, Jansa and Grummt 1999).

UBF – a Pol I-specific transcription factor

The upstream binding factor (UBF) is a transcription factor that binds upstream of the rDNA gene promoter to the upstream core element (UCE) and contains HMG (high mobility group) boxes. It can use these HMG boxes to bend DNA. DNA can be wrapped around UBF just as it is wrapped around the histone octamer. A length of 140 bp can be wrapped around UBF and this conformation is called an “enhanceosome”. UBF is a chromatin remodelling factor itself, since it can modify the chromatin structure (Stefanovsky and Moss 2006). There are two splicing variants of UBF, UBF1 and UBF2, where UBF1 is active in rDNA gene transcription (O'Mahony and Rothblum 1991). UBF is needed for the PIC complex to form and to initiate Pol I transcription. However, UBF not only binds to the rDNA promoter, but throughout the whole rDNA repeat (O'Sullivan, Sullivan et al. 2002). UBF remains with the rDNA gene throughout the cell cycle (Roussel, Andre et al. 1993). Sanij et al. have shown that UBF is necessary to maintain rDNA genes in an
active state, and it is associated only with active copies of rDNA genes. If UBF is depleted in mice, linker histone H1 is able to bind to the rDNA, resulting in a more closed chromatin structure and the inactivation of rDNA transcription (Sanij, Poortinga et al. 2008).

RNA polymerase II transcription

RNA polymerase II (Pol II) transcribes all protein-coding genes, and ncRNAs. Some genes contain a TATA box, while others do not. The latter are known as “TATA-less” genes, and not much is known about them. The mechanisms by which transcription is initiated on TATA-rich promoters, in contrast, is well known. Different transcription factors that are specific for Pol II (TFIIs) are involved in the transcription of TATA box-containing genes (reviewed in (Orphanides, Lagrange et al. 1996)). TFIID binds first to core promoter elements. This complex contains the TBP-binding protein, which recognises the TATA box (Parker and Topol 1984, Nakajima, Horikoshi et al. 1988). TFIID recruits TFIIB to the promoter (Buratowski, Hahn et al. 1989, Maldonado, Ha et al. 1990), and the TFIIF/PolII complex is subsequently attracted to the DNA:protein complex (reviewed in (Young 1991)). However, transcription is not initiated until TFIIE and TFIIH bind, to form the preinitiation complex (Flores, Ha et al. 1990). TFIIA can join the PIC at any point, but the role of TFIIA in transcription initiation is uncertain (reviewed in (Orphanides, Lagrange et al. 1996)). Pol II harbours a C-terminal domain (CTD) that consists of heptad repeats of the consensus protein sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7. A CTD can contain up to 52 repeats (26 in yeast, 52 in human) of this consensus sequence (Allison, Moyle et al. 1985, Broyles and Moss 1986). The repeat may be chemically modified and play a role in Pol II progression through transcription. The most common modifications are phosphorylation on Ser2 or Ser5. When Pol II joins the PIC, it is
unphosphorylated on the CTD. However, phosphorylation on Ser 5 is then needed to initiate transcription (Sogaard and Svejstrup 2007). The degree of phosphorylation of Ser 5 decreases during elongation and Ser 2 starts to be phosphorylated. The Ser 2 phosphorylation increases towards the 3' end of the gene, and the Ser 5 phosphorylation is high at the 5' end. Many factors bind to the CTD tail: factors involved in initiation, elongation, RNA processing and termination (reviewed in (Heidemann, Hintermair et al. 2013)). Two termination pathways have been studied in detail: the poly (A)-dependent pathway and the Sen1-dependent pathway. The poly (A)-dependent pathway is often used for mRNA, and the Sen1-dependent pathway is used for ncRNAs (reviewed in (Kuehner, Pearson et al. 2011)).

RNA polymerase III transcription

The third of the RNA polymerases is RNA polymerase III (Pol III). It is responsible for transcribing non-protein-coding RNAs that are shorter than 400 bp. The most well-known target genes are the 5S rRNA, tRNAs, 7SL RNA and snRNA (reviewed in (Schramm and Hernandez 2002)). It has recently been discovered that Pol III is responsible also for transcribing small miRNA. The promoters of Pol III transcripts vary more than the promoters of the other two RNA polymerases. Three types of promoter exist. Types I and II have internal promoters while type III has external ones (Fig. 4).

The type I promoter, the 5S rRNA gene, first characterised in Drosophila melanogaster, has different internal regulatory elements, the A box, the C box and an intermediate element (IE), which build to the internal control region (ICR) (Pieler, Hamm et al. 1987). Transcription factor IIIA (TFIIIA) binds first to this region, and recruits TFIIIC, which, in turn, recruits TFIIIB. TFIIIB, which contains TBP, then allows Pol III to bind and transcription can be initiated (Engelke, Ng et al. 1980, Sakonju, Brown et al. 1981, Lassar, Martin et al. 1983).
The type II promoter has internal A and B boxes. Examples of genes with such promoters are the Ad2 (adenovirus 2) VAI gene and the tRNA genes from *X. laevis* and *Drosophila melanogaster* (Galli, Hofstetter et al. 1981, Hofstetter, Kressman et al. 1981). TFIIIC binds to the promoter to form a DNA:protein interaction, and then recruits TFIIIB, which in turn recruits Pol III to initiate transcription (Bieker, Martin et al. 1985, Setzer and Brown 1985).

The third type of promoter contains external regulatory elements. It contains a TATA box, a proximal sequence element (PSE), and a distal sequence element (DSE), which is located upstream of the PSE. RNAs that are transcribed from a type III promoter include the U6 snRNA genes in humans. This small nuclear RNA (snRNA) is part of the spliceosome and is in this way involved in splicing (Kunkel and Pederson 1988). The PSE-binding protein (PBP) or the snRNA-activating complex (SNAPc) of a type III promoter makes the initial binding to the PSE-element, and the TBP component of an TFIIIB-like protein is recruited to the TATA box. These then recruit RNA polymerase III (Waldschmidt, Wanandi et al. 1991, Schramm, Pendergrast et al. 2000).

Another type of promoter exists that is most similar to type III. This promoter has internal A and B boxes and an internal TATA box. This type of promoter is present in the U6 RNA of *Saccharomyces cerevisae*. The TFIIIB-like protein binds to the TATA box independently of TFIIIA, TFIIIC and SNAPc, and attracts Pol III to initiate transcription (Kassavetis, Braun et al. 1990).

All Pol III genes have in common that they own a cluster of T residues downstream of their transcription unit, where transcription is terminated. Pol III recognises these T residues and terminates transcription. Another protein that is involved in transcription termination is the La protein. This protein was first identified as an autoantigen (Wolin and Cedervall 2002). It is now known to be involved in Pol III transcription by binding to the poly-U
tail at rRNA transcripts, which suggests that La plays a role in transcript release (Stefano 1984). La is still bound to the 5S rRNA after its release from the DNA template and helps to shuffle it to the nucleolus, where it is needed for ribosome assembly (Intine, Dundr et al. 2004).

Figure 4: Schematic overview over the different types of Pol III promoter

The RNA polymerases in the transcription of ncRNA

All three RNA polymerases are involved in the transcription of ncRNA. The RNAs that are transcribed by Pol I and III all belong to the class of ncRNAs, but Pol II also transcribes ncRNAs. Long non-coding RNAs such as Xist and Kcnq1ot1, and short RNAs such as miRNA and siRNA, are transcribed by Pol II (Huttenhofer, Schattner et al. 2005, Chaumeil, Le Baccon et al. 2006, Pandey, Mondal et al. 2008). The pRNA that is present in the IGS of the rDNA gene in mice is transcribed by Pol I (Mayer, Schmitz et al. 2006), whereas the ncRNAs in the IGS of the rDNA gene in yeast is tran-
scribed by Pol II (Houseley, Kotovic et al. 2007). Pol III transcribes the non-coding ALU RNA in humans that arises from SINES (Yakovchuk, Goodrich et al. 2009). Transcription of ncRNAs is not limited to one class of RNA polymerase.

**Regulation of Pol I transcription**

Ribosomal DNA genes are heavily transcribed, since ribosomal RNA is needed in large amounts to build ribosomes that can carry out protein synthesis. It is, therefore, important that Pol I transcription is carefully regulated and highly controlled. Numerous proteins regulate rDNA transcription: Tumor suppressors, oncogenes, phosphatases, and several pathways and factors that are required for rDNA transcription (reviewed in (White 2005)).

C-Myc regulates transcription in an activating manner. C-Myc is an oncogene and works as transcription factor. C-Myc has often a deregulated expression and is involved in numerous cancers (Vita and Henriksson 2006).

Together with Myc-associated factor X (Max), c-Myc can bind to E-box sequences (CGCGTG) and stimulate transcription (Blackwell, Kretzner et al. 1990, Papoulas, Williams et al. 1992). The ribosomal DNA gene contains several E-boxes to which c-Myc binds, thereby increasing the transcription of ribosomal RNA (Arabi, Wu et al. 2005). Grandori et al. showed that the increase in ribosomal transcription depends on c-Myc binding, and SL1 and Pol I being recruited to the rDNA gene (Grandori, Gomez-Roman et al. 2005).

Overexpression of UBF can stimulate Pol I transcription in cancer, and c-Myc regulates Pol I transcription by inducing overexpression of UBF (Poortinga, Hannan et al. 2004). Furthermore, the post-translational modifications of UBF play an essential role in regulating the transcription of the rDNA gene. For example, phosphorylation of UBF at the C-terminus allows the recruitment of SL1 to the promoter, thereby attracting Pol I to start tran-
scription (Tuan, Zhai et al. 1999). On the other hand, UBF can also be acetyl-
lated by the histone acetyltransferase CBP. This acetylation increases the
activity of UBF, and thus the transcription rate of the rDNA gene increases
(Hirschler-Laszkiewicz, Cavanaugh et al. 2001).

The tumor suppressor protein p53 is involved in the transcription of many
genes. It is involved also in Pol I transcription (Crighton, Woiwode et al.
2003; Sugimoto, Kuo et al. 2003). p53 can repress the transcription of the
45S pre-rRNA, and p53-deficient cell lines have elevated pre-rRNA levels
(Budde and Grummt 1999, Zhai and Comai 2000). The retinoblastoma pro-
tein (RB) is another tumor suppressor that represses Pol I transcription by
interacting with UBF, thereby preventing the recruitment of SL1 to the pro-
moter (Hannan, Kennedy et al. 2000). RB is active only in its
unphosphorylated state (Cavanaugh, Hempel et al. 1995). p53 and RB are
often disregulated in cancer, showing that they play important roles in the
control of cell proliferation by controlling rDNA synthesis.

Different signaling pathways regulate rRNA synthesis. For example, the
TOR (target of rapamycin) pathway and the MAP pathway are involved in
cell growth and cell proliferation, and have a direct influence on Pol I trans-
scription. The TOR pathway can be inhibited by rapamycin and is stimulated
by nutrients. The TOR pathway is involved in the phosphorylation of UBF,
and in this way improves the binding to SL1 and stimulates Pol I trans-
scription (Hannan, Brandenburger et al. 2003). The MAPK pathway is stimulated
by growth factors and the extracellular signal-regulated kinase (ERK) is an
important kinase that acts in this pathway. ERK phosphorylates several fa-
cors that are involved and that regulate Pol I transcription, such as UBF, RB,
TIF-IA and TBP. ERK thus plays a major role in the regulation of ribosomal
transcription (Stefanovsky, Pelletier et al. 2001, Zhao, Yuan et al. 2003).
Regulation of Pol III transcription

Control and regulation of ribosomal transcription is essential for the cell, making it obvious that various factors that regulate Pol I transcription also regulate Pol III transcription.

C-Myc plays a role also in regulating Pol III transcription. It activates tRNA and 5S rRNA transcription. No E-box has been identified in the promoter region of the 5S genes, where c-myc binds in a complex with Max. However c-Myc binding to tRNA genes and 5S rRNA genes has been confirmed. C-Myc probably binds and acts at Pol III genes via its binding to TFIIIB (Gomez-Roman, Grandori et al. 2003).

Also the tumor suppressors p53 and RB regulate Pol III genes. p53 binds to TFIIIB, and prevents it from being recruited to promoters (Crighton, Woiwode et al. 2003). RB also binds to TFIIIB, making it impossible for TFIIIB to bind to TFIIIC and Pol III, and thus start transcription (Sutcliffe, Brown et al. 2000, Hirsch, Jawdekar et al. 2004).

As pointed out in the previous section, ERK has a great influence on factors that are involved in ribosomal transcription. This is the case also in Pol III transcription. ERK phosphorylates TFIIIB and enhances the binding of the transcription factor to Pol III (Felton-Edkins, Fairley et al. 2003). Also the TOR pathway is involved in Pol III transcription, but the mechanism behind is not clear. Strict regulation mechanisms are necessary for Pol I and III transcription, to make sure that cell growth, proliferation and cell cycle progression occur at the right times.
**Chromatin**

The RNA polymerases must overcome the structural barrier of chromatin to be able to access DNA and initiate transcription.

Chromatin is formed when DNA is wrapped around proteins to create a compact but dynamic structure. Two meters of linear DNA must be packed to fit into the nucleus. The most important proteins in the chromatin structure are the histones. Stable ionic bonds are formed between the positively charged histones and the negatively charged DNA. There are five types of histones: H1, H2A, H2B, H3 and H4. A nucleosome constitutes two H2A-H2B dimers and one H3-H4 tetramer, forming the histone octamer (Luger, Mader et al. 1997). 146 bp of DNA are wrapped around this octamer to form the nucleosome core, which is the smallest unit in the chromatin.

The DNA is wrapped around the histone octamer 1.7 times and builds the “10 nm-fiber” which has a “beads-on-a-string” appearance (Luger 2003). The nucleosomes in turn are packaged further to form the 30 nm fiber of the chromosome, this is achieved by the linker histone H1 that binds between nucleosomes (Wolffe and Hayes 1999). These fibers are then folded into looped domains, associated with other non-histone proteins. These looped domains form either the tightly packed heterochromatin or the more loosely packed euchromatin of the chromosome (Campos and Reinberg 2009).

**Chromatin remodelling complexes**

Histone modifications and covalent histone-modifying enzymes

To overcome structural barriers in the chromatin structure and expose DNA for transcription, replication or DNA repair, histone tails must be modified
by histone-modifying enzymes, or chromatin remodelling complexes must alter chromatin structures. It is the lysine-rich histone tails that can be chemically modified. Modifying enzymes are responsible for altering the histone tails. By modifying the histone tails, the chromatin structure can go through a conformational change. Histones can be acetylated, methylated, SUMOylated, ubiquitinated or phosphorylated. Here the focus is on acetylated and methylated histones. Acetylated histones play a role in chromatin accessibility and transcriptional activation, while methylated histones often play a role in heterochromatin formation and transcriptional silencing. Histone acetylation creates a more open chromatin structure known as an “active histone mark”. Methylation of histones can do both, depending on the location and the identity of the proteins that are recruited to the locations. It can open up the chromatin structure, or the chromatin can become more closely packed and then it is known as an “inactive histone mark” (reviewed in (Jenuwein and Allis 2001)). Examples of active histone marks are H3K9ac, H3K14ac and H3K4me3. The latter is often found on 5' ends of genes (McStay and Grummt 2008). H3K9me3 and H3K27me3 are often found at promoters in heterochromatin (reviewed in (Wang, Allis et al. 2007)).

Histone methyl transferases (HMTs) and histone demethylases methylate and demethylate lysine residues, respectively. HATs (histone acetyl transferases) and HDACs (histone deacetylases) acetylate and deacetylate histone tails, respectively (Davie 1998, MacDonald and Howe 2009). Three main families of HATs exist: the Gcn5-related N-acetyl transferase family, which includes HAT1, Gcn5 and PCAF; the CBP/P300 family; and the MYST family, which includes TIP60 and Mof. Gcn5, for example, acetylates the histone H3 at lysine 14 (Johnsson and Wright 2010), and Mof acetylates H4 at lysine 16 (Akhtar and Becker 2000). There are four classes of HDACs: class I, which contains HDAC1-3 and HDAC8; class II, which consists of
HDAC4-7 and 9-10; class III, which consists of Sirtuin 1-7; and class IV, which includes HDAC11 (Bolden, Peart et al. 2006, Kouzarides 2007).

Histone modifications can attract other proteins to maintain and stabilise a specific state on the chromatin. Methylation of lysine 27 on histone H3 (H3K27me3), for example, recruits the polycomb repressor complex (PRC), either PRC1 or PRC2. PRC2 is involved in gene repression. PRC2 contains the enhancer of zeste 2 (EZH2), which itself is a methyltransferase for H3K27me3 and is involved in stem cell renewal and embryonic development (Bracken, Dietrich et al. 2006, Sparmann and van Lohuizen 2006). Methylation of lysine 9 (H3K9me3), on the other hand, recruits HP1, which is important for heterochromatin formation (Grewal and Elgin 2007).

ATP-dependent chromatin remodelling complexes

In addition to histone-modifying enzymes, ATP-dependent chromatin remodelling complexes can change the chromatin structure. They use the energy of ATP hydrolysis to slide nucleosomes, and at least one ATPase subunit is needed in each such complex. Chromatin remodelling complexes play a major role in gene expression and they facilitate transcription, DNA replication and repair. The ATPase subunits all belong to the SWI2/SNF2 superfamily of proteins (Becker and Horz 2002). It got its name according to the first characterised ATP-dependent chromatin remodelling complex: the SWI/SNF complex in yeast (Cote, Quinn et al. 1994).

The SWI2/SNF2 superfamily contains four main subfamilies: the SWI2/SNF2-, the ISWI-, the CHD- and the Ino80-subfamily. Each of these subfamilies is able to promote nucleosomal sliding. Different chromatin remodelling complexes have been identified in different species such as yeast, Drosophila and humans/mice (Becker and Horz 2002). The SWI2/SNF2 group contains a bromodomain that allows them to bind to acetylated histones (Horn and Peterson 2001, Martens and Winston 2003).
Complexes containing SWI2/SNF2 are involved in the activation and repression of transcription (Cairns, Schlichter et al. 1999).

The subfamily ISWI is characterised by a SANT domain. ISWI-containing complexes are involved in different processes. Depending on the complex and the species, they can be involved in regulation of Pol II and I transcription (Santoro, Li et al. 2002, Morillon, Karabetsou et al. 2003), and in chromatin assembly and replication (Bozhenok, Wade et al. 2002). The CHD contains a chromodomain and can deacetylate histones (Feng and Zhang 2003). INO80 has a split ATPase domain and is involved in DNA repair (Shen, Mizuguchi et al. 2000).

**ISWI-containing complexes**

In this study we focus on the ISWI subfamily. ISWI stands for “imitation switch” because of its sequence similarity to the SWI2/SNF2. There are two isoforms of ISWI in mammals, SNF2h (sucrose non-fermenter homologue) and SNF2l (sucrose non-fermenter like). ISWI is part of numerous chromatin remodelling complexes, such as CHRAC and NURF (Vignali, Hassan et al. 2000). Here we will concentrate on the isoform SNF2h in mammalian cells and the chromatin remodelling complexes NoRC, WICH and B-WICH. NoRC consists of the ATPase SNF2h and TIP5 (Strohner, Nemeth et al. 2001). WICH consists of the ATPase SNF2h and WSTF. The WICH complex is recruited to replication foci by the DNA clamp protein PCNA and it is involved in replication. First, WSTF is attracted to PCNA and then WSTF recruits SNF2h (Poot, Bozhenok et al. 2004). The B-WICH complex consists also of the ATPase SNF2h and WSTF, and contains also NM1 and several RNA-processing proteins (Percipalle, Fomproix et al. 2006).
The WAL/BAZ family - WSTF

WSTF belongs to the WAL/BAZ family of proteins and is also known as “BAZ1B”. The WSTF gene is located on chromosome 7 and was given its name because there is a heterozygous deletion of the WSTF gene in patients who have Williams Syndrome (Lu, Meng et al. 1998, Peoples, Cisco et al. 1998). WSTF contains a number of different motifs that are conserved from *Xenopus laevis* to mammals. A PHD (plant homeodomain) domain and a bromodomain are located in the gene, and genes with those domains can bind to and/or modify chromatin. The WSTF bromodomain binds to H3K14ac (Kato, Fujiki et al. 2007). Proteins that harbour a bromodomain are involved in transcription (Barnett and Krebs 2011). Furthermore, WSTF contains a WAC domain. A WAC domain is often found in proteins that have kinase activity. Xiao et al. have shown that WSTF is a tyrosine kinase in mouse cells. WSTF is also involved in DNA damage response by phosphorylating H2A.X, a histone modification that is accumulated in DNA damage (Xiao, Li et al. 2009). WSTF itself can be post-transcriptionally modified. It is phosphorylated in the WAC domain at serine 158 by MAP kinases. This is, however, not necessary for WICH complex assembly, since WSTF binds to SNF2h in its unphosphorylated form (Barnett and Krebs 2011).

![Figure 5: Drawing of the WSTF gene including its conserved domains LH, WAC, DDT, BAZ1, BAZ2, WAKZ, PHD and the bromodomain](image)

Figure 5: Drawing of the WSTF gene including its conserved domains LH, WAC, DDT, BAZ1, BAZ2, WAKZ, PHD and the bromodomain
The role of B-WICH, NoRC and CSB in Pol I transcription

We have previously identified B-WICH as a chromatin remodelling factor in rRNA transcription. It is composed of the WICH complex and other nuclear proteins. These include Sf3b155/SAP155, RNA helicase II/Guα, proto-oncogen Dek, CSB, Myb-binding protein 1a and NM1. B-WICH binds to the rDNA promoter and throughout the coding region, and this has led to it being proposed that B-WICH plays a role in Pol I transcription. Knock down of WSTF, which recruits SNF2h and NM1, results in a decreased 45S pre-mRNA level, which confirms that the B-WICH complex is involved in Pol I transcription (Cavellán, Asp et al. 2006).

Other scientists have, however, shown that the CSB (Cockayne syndrome B) remodels chromatin over rDNA genes. CSB recruits the methyl transferase G9a to the rDNA gene, which creates a more open chromatin structure (Yuan, Feng et al. 2007). CSB was originally identified through the role it plays in DNA damage. CSB has an ATPase activity, and is involved in chromatin remodelling (Citterio, Van Den Boom et al. 2000), and in Pol II transcription (Balajee, May et al. 1997). Its role in Pol I transcription became clear later (Bradsher, Auriol et al. 2002). Despite its ATPase activity, CSB is not part of the SWI2/SNF2 superfamily of ATP-dependent chromatin remodelling complexes.

Transcription is repressed at inactive copies of the rDNA gene by the chromatin remodelling factor NoRC, which, in turn, recruits histone deacetylases that deacetylate histone H4. Furthermore, DNA methyltransferases and histone methyltransferases that methylate the DNA (CpG-islands), H3K9 and the H4K20 respectively, are involved in the repression (Mayer, Schmitz et al. 2006).
Figure 6: A mechanistic model for B-WICH and its role in Pol I transcription

Role of B-WICH in Pol III transcription

B-WICH binds also at Pol III genes such as the 5S rRNA and 7SL RNA gene. The B-WICH complex also facilitates chromatin remodelling and transcription at those genes, since the knock down of WSTF (recruiter of SNF2h and NM1) reduces the transcript levels of 5S rRNA and 7SL RNA (Cavellan, Asp et al. 2006).
The present investigation

Objectives/Aim of the studies
In these studies we focused on the ribosomal RNA genes: the ribosomal Pol I 45S rRNA gene and the 5S rRNA gene. We focused also on Pol III transcription, which transcribes the 7SL RNA and tRNAs, among others. One aim of these studies was to obtain more insight how ribosomal transcription is regulated: to determine the mechanism by which the chromatin remodelling complex B-WICH is involved in ribosomal RNA transcription. Another aim was to investigate the IGS of the rDNA gene. We studied the extent to which the IGS is transcribed and how non-coding transcripts from this region are regulated.

Model system
These studies were conducted with two human cell lines: HeLa and HEK293T cells. HeLa is a cervical carcinoma cell line that is commonly used in research. It is an immortal transformed cancer cell line, the cells of which are derived from a patient called Henrietta Lacks, who died of cervical cancer in 1951. The cells have been kept in culture ever since. HEK293T cells are immortalized human embryonic kidney cells. They were obtained from a healthy aborted fetus in the 1970s and were transformed with sheared DNA of human adenovirus 5.
Study I

The chromatin remodelling complex B-WICH changes the chromatin structure and recruits histone acetyltransferases to active rRNA genes

Aim
In our first study, we investigated the coding region of the ribosomal DNA gene. We had shown earlier that the B-WICH (WSTF, SNF2h and NM1) complex is involved in RNA Pol I transcription (Percipalle, Fomproix et al. 2006), and the aim of Study 1 was to analyse the mechanism by which this participation of B-WICH occurs.

Results
In Paper I we showed that the B-WICH complex is associated with active copies of the rDNA gene, together with Pol I and UBF. DNase accessibility assays revealed that DNA is more inaccessible to DNase I when WSTF has been knocked down than in a knock down with a scrambled control. We conclude that WSTF can create a more open chromatin structure over the rDNA gene promoter and in the coding region. Since we could see an effect on chromatin structure by knocking down WSTF, we investigated the recruitment of proteins by WSTF. We showed with IPs that WSTF attracts SNF2h, NM1 and Pol I to the rDNA gene, while the UBF binding is independent of WSTF. We checked whether the B-WICH complex is involved also in the processing of the ribosomal subunits. Northern blots revealed that the rRNA processing intermediates did not change when WSTF was knocked down. We concluded that B-WICH does not play a role in rRNA processing, and that the role of B-WICH is limited to the chromatin structure. Histone acetylation is often correlated with active transcription (MacDonald and Howe 2009). Active histone marks were present on the
rDNA gene, and the H3K9ac had the highest decrease in WSTF kd cells. Inactive histone marks were associated also with the rDNA gene, since at least half of the copies are silent. WSTF kd resulted in a reduced silencing mark H4K20me3, while the H3K9me3 was unaffected.

For histones to be modified, different histone-modifying enzymes are needed. Histones are acetylated, for example, by HATs (histone acetyltransferases) (Davie 1998). As histone acetylation decreased in WSTF kd cells, we wanted to investigate whether this occurred at the level of HAT recruitment to the rDNA. Decreased H3-HAT binding to the rDNA promoter in WSTF kd cells was detected, which confirms that WSTF attracts H3-HATs to the rDNA gene. Mof was not affected upon WSTF kd, indicating that WSTF is not responsible for recruiting H4-HATs. Since HATs are dependent on WSTF at the rDNA gene, we next checked if the HATs would be bound by WSTF. Interaction between PCAF and WSTF was only detected at low salt concentrations, suggesting that the interaction is weak. WSTF does not interact with Gcn5 nor with p300. Finally, we examined whether a chromatin remodelling event precedes the acetylation of H3. With a MNase assay in scrambled control and WSTF kd cells, the B-WICH complex was needed to open up the chromatin structure 200 bp upstream of the rDNA promoter. A more closed chromatin structure at this position was detected when WSTF was knocked down.

In our studies we define a clearer picture of the mechanism by which the B-WICH complex regulates rDNA synthesis. We have shown by ChIP experiments that WSTF, a protein in the B-WICH complex, attracts HATs to the rDNA gene, and that the B-WICH complex is thus indirectly responsible for the acetylation of histones at rDNA. This action, in turn, creates a more open chromatin structure and more transcription can take place. We can, however, only speculate on the way in which B-WICH attracts HATs, since no direct interaction has been found with p300 or Gcn5, and a very weak interaction with PCAF. It is possible that B-WICH creates a more open
chromatin structure by remodelling chromatin, thereby establishing spots on the DNA for the HATs to bind. We can speculate further that this is unique for H3-HATs, since WSTF knock down did not influence the H4-specific HAT Mof.

Model:

![Model of B-WICH in rRNA transcription](image)

*Figure 7: Model of the role of B-WICH in rRNA transcription. The B-WICH complex is required for the access of B-WICH factors and H3-specific histone acetyl-transferase to the DNA, resulting in H3K9-acetylation.*
Study II

Nuclear myosin 1c facilitates the chromatin modifications required to activate rRNA gene transcription and cell cycle progression

Aim

The aim for our research in this paper concerned only the question of the mechanism by which NM1 is involved in Pol I transcription.

In Study I we show the mechanism by which the B-WICH complex is involved in Pol I transcription. In this paper we focus on NM1, a protein that is part of the B-WICH complex, recruited by WSTF to the Pol I gene.

This paper also included studies of the regulation of B-WICH in mitosis and interphase. Additionally the phosphorylation pattern of WSTF was characterised during the cell cycle.

Results

In Paper II we show that NM1 is needed for active Pol I transcription. Decreased 45S pre-rRNA levels are detected in NM1 knock down cells.

Just as in Paper I, we carried out MNase assays in NM1 kd and scrC cells. The degree of chromatin protection did not change following NM1 kd, suggesting that NM1 itself is not responsible for chromatin remodelling. The binding of WSTF-SNF2h or of Pol I to the rDNA gene was not affected when NM1 was knocked down. We concluded that WSTF acts at a step prior to NM1. Similar to WSTF kd, however, NM1 kd causes a decrease in the histone acetylation pattern. The binding of the HAT PCAF was down-regulated in NM1 kd cells, which suggests that binding of PCAF to the Pol I gene is an NM1-specific feature. Furthermore, WSTF binding to the Pol I gene was tested throughout the cell cycle with ChIP experiments. WSTF associates to the rDNA gene during the complete cell cycle, and does
not leave the gene during mitosis. During mitosis, however, WSTF associates with the Pol I gene in its phosphorylated form. WSTF is unable to bind SNF2h and NM1 when it is phosphorylated (Oya, Yokoyama et al. 2009). NM1 is needed for active Pol I transcription, but it is not a chromatin remodelling complex itself and cannot open up the chromatin on the rDNA gene. Since rDNA levels decrease upon NM1 knock down, NM1 is an important component of the B-WICH complex that is necessary to carry out Pol I transcription.

**Study III**

**Non-coding RNAs from the rDNA intergenic repeat are transcribed by RNA polymerase I and II and have different functions**

**Aim**
In this study we investigated the IGS of the rDNA gene. Interest into intergenic regions is increasing, as is interest into coding strands of DNA, since it has become clear that ncRNAs, including asRNAs, are transcribed from these locations. Furthermore, ncRNA is transcribed from the IGS of the rDNA gene in other species than humans (Mayer, Schmitz et al. 2006). NcRNAs have recently been identified in the IGS in human cells (Audas, Jacob et al. 2012). Here we wanted to investigate the extent to which the IGS of the rDNA gene in humans is transcribed, and whether these ncRNAs regulate Pol I transcription.

**Results**
In Paper III we show with RT-qPCR that more or less the complete IGS of the rDNA gene in human cells is transcribed. However, in a Northern blot
approach we could identify three specific ncRNAs by their size and direction of transcription. We found the IGS19asRNA, the IGS32asRNA and the IGS38RNA, which differ in length and transcription direction. Only IGS32asRNA was transcribed by Pol I, the other two were Pol II products. NcRNAs are sometimes unstable transcripts and degraded by the exosome complex (Houseley, Kotovic et al. 2007). NcRNAs identified in this studies showed greater expression when Rrp6, a protein of the exosome complex, was knocked down, which suggests that these ncRNAs are a substrate for the exosome.

FISH experiments revealed that IGS19asRNA is enriched at specific sites in the nucleus and its 5' TSS could be identified with a 5' RACE. The two antisense RNAs, at 19 kb and 32 kb from TSS, are differently regulated. The IGS19asRNA is up-regulated upon heat shock, as is also an ncRNA from the human IGS of the rDNA gene, which was identified by Audas et al. (Audas, Jacob et al. 2012). The IGS32asRNA was up-regulated upon glucose refeeding in the same way as the 45S pre-rRNA, while the level of IGS38RNA did not change in either treatment. Furthermore, we obtained a speckled nuclei staining with IGS19asRNA with FISH, suggesting that IGS19asRNA is associated with speckles. ChRIP experiments showed that IGS32asRNA remains at the chromatin and binds to HP1. The IGS32asRNA is one of the first ncRNAs that binds to HP1, and may play a role in transcriptional repression. However, the functions of these ncRNAs are still unknown and need to be elucidated. We cannot rule out that the ncRNAs regulate transcription of the 45S pre-rRNA or influence nucleolar stability. IGS32asRNA is transcribed in close proximity to the pseudogene Cdc27, and it is possible that it is involved in regulating this region. We found IGS19asRNA mostly outside the nucleoli in a speckled pattern, where it may be involved in regulating nuclear processes.
Study IV

The B-WICH chromatin-remodelling complex regulates RNA polymerase III transcription by promoting Max dependent c-Myc binding

Aim

We have previously identified the chromatin remodelling complex B-WICH. It binds to Pol III genes, such as the 5S rRNA gene and the 7SL RNA gene. Upon knock down of the WSTF, in which the subunit that recruit SNF2h and NM1 is removed, transcription of these genes is down-regulated (Cavellán 2006). It is not clear, however, how B-WICH down-regulates Pol III transcription. In this study we investigated the way in which B-WICH is involved in Pol III transcription.

Results

The B-WICH complex is able to influence the binding of the Pol III machinery to the 5S rRNA and 7SL RNA genes. It does not, however, recruit the TFIIIs and Pol III directly, which we can conclude since our results showed that the proteins do not interact.

We investigated the influence of the B-WICH complex on chromatin. B-WICH was able to influence the chromatin structure around the 5S rRNA and 7SL RNA genes at specific locations, as shown by MNase assays, confirming that B-WICH functions as a chromatin remodeller. The chromatin structure after WSTF knock down was a closed structure. WSTF was the protein that attracted the rest of the B-WICH complex to Pol I and III genes. The closed chromatin structure could be caused by changes in the histone modifications that occur. Similar to the regulation of Pol I genes, WSTF kd reduced the levels of H3K9ac and H3K14ac and abolished the binding of the H3-HATs p300 and Gcn5 to the 5S rRNA and 7SL RNA gene. The HAT
Gcn5 is part of the TRRAP complex (Kenneth, Ramsbottom et al. 2007). C-Myc is involved in the regulation of Pol III transcription, where it attracts the TRRAP complex and alters the binding of Pol III factors (Gomez-Roman, Grandori et al. 2003; van Riggelen, Yetil et al. 2010). These findings led us to investigate whether the B-WICH complex is involved in c-Myc binding to Pol III genes. We demonstrated that binding of c-Myc is reduced at Pol III genes upon WSTF knock down, thereby confirming that B-WICH is involved in the attraction of c-Myc. We also showed that c-Myc binds to the IGS of the 5S rRNA gene, in addition to the promoter binding. When WSTF is knocked down, c-Myc levels are even lower at the position in the IGS than they are at the promoter binding site. c-Myc binding was also affected at 7SL RNA genes when WSTF was knocked down. We identified an E-box in the IGS of the 5S rRNA gene. c-Myc binds to E-boxes with its binding partner Max (Wahlstrom and Henriksson 2007, Gallant and Steiger 2009). We also detected Myc:Max binding to this E-box in the IGS of the 5S rRNA gene, suggesting that c-Myc not only regulates transcription by binding at the promoter but also by binding to the IGS. We showed that c-Myc regulates histone acetylation and recruitment of Pol III to the 5S rRNA gene. However, knock down of c-Myc did not influence the binding of WSTF or SNF2h to the 5S rRNA gene, which suggests that B-WICH acts at a step prior to the step at which c-Myc acts.

We suggest that c-Myc can alter the 5S rRNA transcription in a B-WICH-dependent manner in two ways: by binding to the promoter, interacting with TFIIIB and thereby recruiting more Pol III, and in a Max-dependent way in the IGS. These findings show for the first time that also the 5S rRNA is under the control of the Myc/Max/Mxd network. As in Pol I transcription, the B-WICH here works as a factor that makes it possible for other proteins to bind to Pol III genes.
Conclusions

Study I
The B-WICH complex acts as a licensing factor by creating a more open chromatin structure over the promoter region of the rDNA gene. B-WICH is subsequently able to attract HATs to the rDNA gene, Gcn5, p300 and PCAF, by which process histone tails become more highly acetylated and activate transcription.

Study II
NM1 is part of the B-WICH complex and cannot remodel chromatin. Instead, NM1 can activate Pol I transcription by attracting the HAT PCAF. This results in higher acetylation of H3 histone tails, which stimulates transcription.

Study III
Three ncRNAs transcribed from the IGS of the rDNA gene were identified: the IGS₁₉₈ₙRNA, the IGS₃₂ₐₙRNA and the IGS₃₈ₙRNA. IGS₁₉₈ₙ₉ₙRNA and IGS₃₂ₐₙₙRNA are transcribed by Pol II, while IGS₃₂ₐₙₙRNA is a Pol I transcript. The IGS₁₉₈ₙₙRNA and the IGS₃₂ₐₙₙRNA are regulated upon heat shock stress and glucose refeeding, respectively. The IGS₁₉₈ₙₙRNA enters the nucleoplasm and gives a speckled pattern, suggesting that it binds to speckles. The IGS₃₂ₐₙₙRNA binds to the HP1 on chromatin and may be involved in transcriptional repression.

Study IV
As in Study I, the B-WICH complex is able to create a more open structure at Pol III genes. B-WICH is able to remodel a site at Pol III genes in which c-Myc binds to an E-box. C-Myc recruits the TRRAP complex in a
Max-dependent way, which in turn acetylates histones to activate transcription.

**Future perspectives**

We have determined the mechanism by which the B-WICH complex is involved in Pol I and III transcription, and we have determined the role of NM1 in Pol I transcription. We confirm that B-WICH plays an important role in the transcription of ribosomal genes, and thus a role in cell proliferation and cell growth. However, we do not know how WSTF is initially recruited to the Pol I and III genes. The question remains what recruits it to the rDNA gene, the 5S rRNA and the 7SL RNA gene?

We have also identified three ncRNA transcripts in the IGS of the human rDNA gene. We have characterised them and determined the function of IGS_{32a}RNA. The functions of IGS_{19a}RNA and IGS_{38}RNA, however, remain to be investigated. More experiments to investigate IGS_{32a}RNA:HP1 interaction and its outcome are needed.
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