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„Long non-coding RNAs in the intergenic spacer of 47S pre-ribosomal RNA genes"

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Master Thesis

Long non-coding RNAs in the intergenic spacer of 47S pre-ribosomal RNA genes

Department of Molecular Biosciences

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Abstractum

Die 43kb rDNA setzt sich aus der 13.3kb langen codierenden Sequenz der 47S pre-ribosomal RNA (rRNA) und dem nicht kodierenden intergenetischen Spacer (IGS) zusammen, welcher die aufeinanderfolgenden, repetitiven DNA Einheiten voneinander trennt.

Lange Zeit wurde davon ausgegangen, daß der intergenetische Spacer zwischen ribosomalen repetitiven Einheiten nicht transkribiert wird (Grummt and Pikard, 2003), bis Zentner et al., (2011) den Beweis erbrachte, daß die Transkription des IGS in geringem Maße stattfand. Im Mausgenome bindet, 2kb vom Transkriptionsstart der 47S pre-RNA entfernt, eine Promoter assoziierte RNA (pRNA) an die TIP5 Untereinheit des nucleolar remodeling Komplexes (NoRC). Dieser wird dadurch zum core Promoter der 47S pre-rRNA vermittelt. Die Bindung von NoRC and den core Promoter induziert die Formation von Heterochromatinstrukturen, welche die Transkription der 47S pre-rRNAs inhibieren. (Guetg et al., 2010). Im menschlichen Genom wurde keine Promoter assoziierte RNA entdeckt, doch Tschurikov et al., (2015) legten nahe, daß regulatorische ncRNAs im humanen IGS vorkommen und Teil der Transkriptionskontrolle von rDNA Einheiten sind. Darüber hinaus zeigte Audas et al., (2011), daß Umwelteinflüsse, wie beispielsweise Ansäuern oder Hitzeshock, die Transkription dreier langer, nicht kodierender RNAs (lncRNAs) beeinflussen.

Stephanie Böhm entdeckte drei weitere lncRNA, welche dem humanen IGS entstammen. Ihr Name orientiert sich zum einen an ihrer Transkriptionsrichtung, zum anderen am Locus ihres Transkriptionsstarts bezugnehmend auf den 47S pre- rRNA: 19 antisense RNA (19asRNA), 32 antisense RNA (32asRNA) und 38 sense RNA (38sRNA).

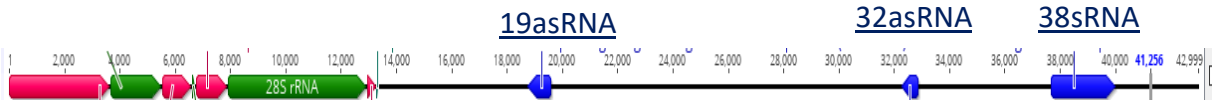

Figure1: IGSlncRNAs mapped to the rDNA repeat unit

Das erste Ziel meiner Masterthese ist die Definition der Sequenz aller drei lncRNAs. Des weiteren untersuchte ich ob die Loci dieser lncRNAs in die Replication, in Reparaturmechanismen oder in die Architektur des Chromatins involviert sind. Die dritte Aufgabe meiner Masterthesis beschäftigt sich mit der Frage, ob die Expression dieser lncRNAs abhängig vom Zellzyklus ist.

Abstract

The 43-kb rDNA unit contains the 13.3kb long 47S pre-ribosomal RNA (rRNA) coding sequence and a non-coding intergenic spacer (IGS) which separates the adjoining DNA repeats. It has long been assumed that the intergenic spacer of ribosomal repeat units is transcriptionally silent (Grummt and Pikard, 2003), but Zentner et al., (2011) has demonstrated that the IGS is transcribed at a very low level. In the mouse genome a promoter associated RNA (pRNA), transcribed 2kb from the transcription start site of the 47S pre-RNA binds the TIP5 subunit of the nucleolar remodeling complex (NoRC) and guides it to the core promoter of the 47S pre-rRNA. Binding of NoRC to the core promoter leads to formation of heterochromatin structures, which inhibits transcription of 47S pre-rRNAs (Guetg et al., 2010). In the human genome such a promoter associated RNA has not been found, but Tschurikov et al., (2015) proposed that regulatory ncRNAs arise from the IGS and take part in transcription control of rDNA units. Furthermore Audas et al., (2011) showed that three IGS long non coding RNAs (lncRNA) respond to environmental clues, such as acidosis and heat shock.

Stephanie Böhm discovered three lncRNA transcribed from the IGS. They are termed after their direction of transcription and the location with respect to the 47S pre- rRNA transcription start site: 19 antisense RNA (19asRNA), 32 antisense RNA (32asRNA) and 38 sense RNA (38sRNA).

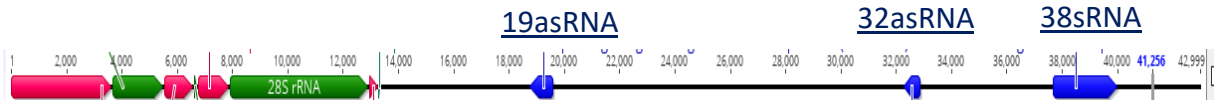

Figure1: IGSlncRNAs mapped to the rDNA repeat unit

The first aim of my master thesis is to define the sequence of all three lncRNAs. Secondly I want to investigate if the loci of these lncRNAs are involved in replication, repair or chromatin architecture. The third aim is to find out if the expression pattern of these lncRNAs is cell cycle dependent.

Acknowledgments

I want to thank my Professor Ann-Kristin Östlund Farrants for giving me the great opportunity to do my master thesis at Stockholm University. It has been an amazing experience to work in your lab on such an interesting project. I also would like to express my gratitude to my supervisor Jacklyn Quin for her continual support and her trust in me. Additionally, I want to thank Yuan Guo and Antoni Ganez Zapater, it has been a privilege to learn from you.

Finally, I would like to express my thankfulness to Dr. Silvia Bulgheresi for taking over the supervision of my master thesis at Vienna University.

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Introduction

Ribosomal biogenesis

The ribosome consist of ribosomal proteins (RP), responsible for maintaining the ribosomal structure and regulating the translational process and ribosomal RNA (rRNA), acting as a ribozyme which catalyzes, together with aminoacyl tRNA, the polypeptide formation (Kobayashi, 2014).

The ribosome comprises 79 ribosomal proteins (RP´s) and four distinct rRNAs. Three rRNAs are transcribed by RNA polymerase l (Pol l) in a long polycistronic pre-ribosomal RNA, the 47S ribosomal RNA (47S rRNA) (Henras et.al, 2015). Processing of the 47S rRNA into the 28S, 18S and 5.8S rRNA, the recruitment of the r-proteins and the assembly together with the 5S rRNA, (transcribed by pol lll) into the pre-40S and pre-60S ribosomal subunits occurs in the nucleolus (Henras et.al, 2015; Thomson et al., 2014). The nucleolus shows a tripartite structure tightly linked to a specific function. RNA pol l transcription occurse at the interface of fibrillar centers (FC) and dense fibrillar components (DFC), posttranscriptional maturation take place within DFC and the final assembly with ribosomal proteins occurs in the granular component (GC) (Jacob et al., 2013). After export through the nuclear pore complex into the cytoplasm, translational competence is achieved by final maturation steps (Zemp et al., 2007).

Its central importance in protein synthesis, cell growth and proliferation, makes ribosomal biosynthesis the most energetically demanding process in a cell (Thomson et al., 2014). Up to 80% of the overall cellular transcription output is generated from rDNA genes, encoding the 47S pre-rRNA transcript (Zentner et. al., 2011). And about 50% of the total protein mass in a cell is made of ribosomal proteins (Kobayashi, 2014).

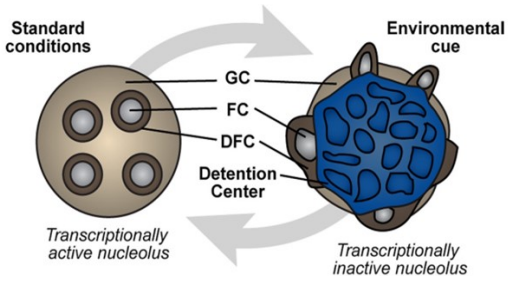


Figure 2: Structure of the nucleolus under standard conditions and after stress response (Jacob et al., 2013)

Ribosomal DNA unit

The diploid human genome has approximately 400 ribosomal DNA units arrayed in tandem repeats on the short arm of five acrocentric chromosomes 13, 14, 15, 21 and 22 (Birch and Zomerdijk et al., 2008; Shaw and Doonan, 2005). One single rDNA unit is 43-kb long and contains a 13.3kb rRNA coding sequence and a non-coding intergenic spacer (IGS) which separates the adjoining rDNA coding sequences.

The rDNA coding region is flanked by external spacers and contains two internal spacers separating the 28S, 5.8S and the 18S rDNA gene loci. The core promoter is located close to the transcription start site (TSS) of the 47S pre-RNA and comprises two elements, known as core promoter element (CPE) and the upstream control element (UCE).

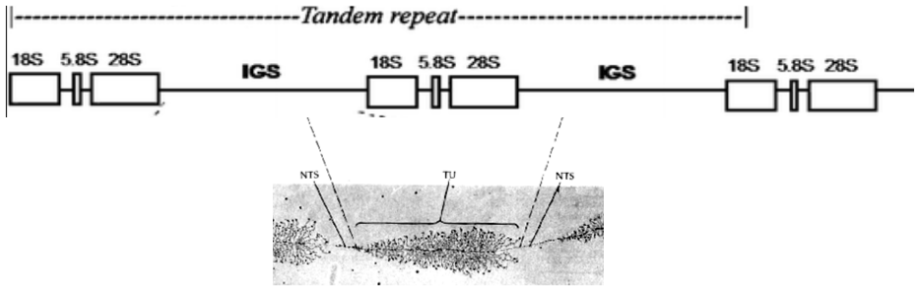


Figure 3: ribosomal DNA repeat unit (Scott F. Gilbert 2013)

Despite the fact that rDNA repeats are the most actively transcribed loci in the genome, only half of them are transcriptional competent and associated with euchromatic histone modifications (e.g. H3K4Me3) and the RNA Pol l transcription factor UBF which binds to the UCE (Quin et. al 2014). DNA methylation, histone hypoacetylation and specific histone methylations (e.g. H3K9me2/3) are connected to heterochromatin formation and therefore transcriptional repression (Stewart et. al 2005; Kouzarides, 2007). However rDNA repeats are proposed to perform additional functions to 47S transcription. It has been shown in yeast that silent rDNA units serve as a template for DNA damage repair and contribute to genome integrity (Kobayashi, 2010).

Regulation of 47S pre-rRNA transcription

The rDNA transcription rate is regulated by two different mechanisms: chromatin remodeling serves as a long term stable adjustment whereas changes in the transcriptional machinery is a more flexible and rapid regulation of transcription. (Raska et al., 2004).

Short term regulation of rDNA transcription

In order to assemble the pre initiation complex (PIC) of Pol l, synergistic binding of two transcription factors to the promoter elements is necessary: the upstreaming binding factor (UBF) and the selectivity factor 1 (SL1). Then Pol 1 gets recruited by the transcription initiation factor (TIF-1A). Posttranslational modifications of TIF-1A, SL 1 and UBF like e.g. acetylation of UBF by CREB-Binding protein (CPB), regulate rDNA translation by impairment or facilitation of Pol 1 binding, depending on nutrient and grow factor availability and external stress factors (Grummt 2003; Meraner 2006).
The maximal transcription rate is reached in S-phase and G2. During mitosis transcription of rRNA is inhibited through phosphorylation of SL1 and TTF-1 by cyclin cdk1, but resumes as soon nucleoli reform in late telophase, (Raska et al., 2004; Grummt, 2010).

Heterochromatic regulation of rDNA transcription

The nucleolar remodeling complex (NoRC) is a member of ATP dependent chromatin remodeling complexes and comprise the ATPase SNF2h and TIP5 (TTF-l interacting protein). NoRC is known to silence rRNA genes by triggering DNA methylation of CpG residues in the UCE, hypoacethylation of histone H4 and dimethylation of histone H3K9 of the promoter region, leading to heterochromatin formation which prevents binding of UBF and silence transcription of 47S-preRNA epigenetically (Mayer 2006).

Organization of the nucleolus is 47S pre-RNA transcription dependent

The most prominent structure within the nucleus, the nucleolus, acts as a central coordinator of the synthesis and assembly of the ribosome. The nucleolus comprise 4500 distinct proteins (Grummt, 2013; Ahmad et al., 2009) and its dynamic structural domain is formed around actively transcribed 47S ribosomal RNA gene repeats, known as the nucleolus organizer regions (NORs) (Quin et al., 2013). Therefore its assembly is dependent on RNA Pol l transcription.
Intact nucleolar structure and function are strongly linked with regulation of p53 “the guardian of the genome”. Accumulation of p53 triggers growth arrest and apoptosis. Under normal physiological conditions p53 levels are regulated by ubiquitinase ligase HDM2, which marks p53 for proteolysis. Under stress conditions 47S pre-RNA transcription is repressed and

ribosomal proteins associated with nascent 47S pre-rRNA bind instead to HDM2 and inhibit its activity, leading to accumulation of p53. Dependent on the gravity of damage, the cell has to decide either to arrest cell cycle and initiate repair mechanism or succumb to p53 apoptosis pathways (Grummt, 2013, 2010). Thus, there is a strong connection between ribosomal biogenesis, stress signaling, replication and DNA repair (Golomb et al, 2014).

Long non coding RNAs

Only around 2% of the human genome is translated into protein. Apart from the high abundant non-coding transcripts like tRNA, rRNA and spliceosomal RNA, the non-protein coding regions had historically been considered as non-functional evolutionary relicts referred to as "Junk DNA". But 60-70% of the mammalian genome is transcribed, at least 25% from both strands (Mattik and Makunin, 2006).

Long non-coding RNAs (lncRNA) exert a wide repertoire of biological functions and are defined only by their length which range from 200nt to over 100kb (Clark and Mattick 2011).The numbers of transcribed long non-coding RNAs in mammal genomes are at least as numerous as protein coding genes (Clark and Mattick, 2011). The fact that mammals have just slightly more protein coding genes than lower organisms but the proportion of non-coding sequences increase with increasing complexity of organisms, lead to the suggestion that these non-coding transcripts contain elaborate regulatory functions and contribute to the emergence of developmentally complex organisms (Mattick, 2009; Hüttenhofer et al., 2005)

Over the last decade the importance of lncRNA in fundamental processes such as cellular stress response, cell cycle control, DNA repair, transcriptional regulation chromatin modifications and cellular differentiation has become evident (Liu and Lu, 2012).

LncRNA can be distinguished in three categories based on their way of interaction. Nonfunctional lncRNAs as a result of transcriptional noise, functional lncRNAs exerting their function by the act of transcription itself rather than by their transcripts and functional lncRNAs which act in cis and/or trans by their transcripts. Although primary sequence are poorly conserved in lncRNAs they show a higher preservation of secondary structures, genome positions and short sequence motifs. Many known lncRNAs are tightly regulated and show a cell type and cell state specific expression pattern. Some of them exert fundamental organismal functions like dosage compensation of X-chromosomes in female mammals by the lncRNA XIST.

It is proposed that one major function of lncRNA is epigenetic regulation of chromatin by guiding protein complexes to their targets (e.g., promoter-associated RNA) or regulation of gene expression by antisense lncRNA transcription (e.g. XIST) (Quinn and Chang 2016; Clark and Mattik 2011).

Features of the IGS

In contrast to the highly conserved rDNA coding region, the IGS is not conserved between species and can vary in length and sequence within species, even within a single individual (Smirnov et al., 2016). This can be explained by the highly repetitive nature of IGS sequences, which includes many tandem repeats, inverted repeats and transposable elements like LINE´s, SINEs and Alu-elements (Zheng et al., 2014; Zentner et al., 2014).
The IGS sequence possesses a polar fork blocking site close to the transcription termination site 13.3kb downstream of the 47S pre-RNA TSS and multiple replication initiation sites (Dimitrova 2011).
It has long been assumed that the IGS region is non-functional and transcriptional silent (Grummt and Pikard, 2003), but Zentner et al. (2010) have demonstrated that the IGS is transcribed at a very low level 100 fold lower than 45S pre-rRNA transcript. These IGS transcripts are enriched in nucleoli (Mayer et al., 2006). Tschurikov et al., (2014) propose that regulatory ncRNAs arise from the IGS and take part in transcriptional control of rDNA units.

LncRNAs arise from the IGS

Promoter associated RNA is involved in maintenance of silent rDNA repeats in the mouse genome

An IGS rRNA is transcribed from the antisense strand of the spacer promoter, located approximately 2kb upstream of the 47S pre-RNA TSS in the mouse genome. This rRNA is processed into a 150-250nt. long pRNA, which has complimentary sequence to the rDNA promoter and is required to mediate the binding of NoRC to the rDNA promoter (Guetg et al.,

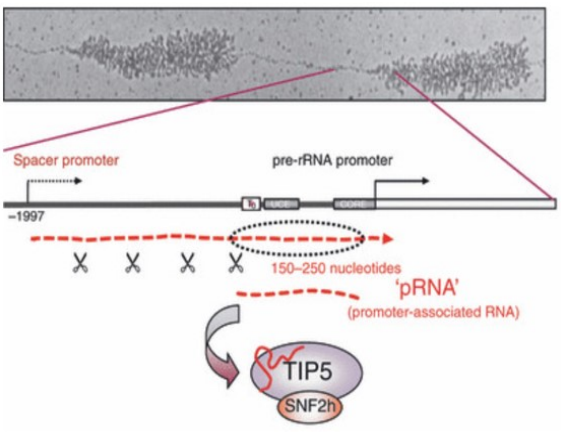


Figure 4: pRNA mediated heterochromatin formation of the rDNA promoter region in mice (Grummt 2010)

2010). Santoro et al., (2010) have shown that pRNA is only transcribed from heterochromatic rDNA clusters and mediate the inheritance of DNA methylation and heterochromatin formation of silent rDNA repeats. Transcription of pRNA is upregulated during mid and late S-phase which goes along with the fact that rDNA replication timing depends on chromatin structure. Silent rDNA cluster replicate in late S-phase whereas active rDNA repeats replicate in early S-phase (Dimitrova 2011).

IGS lncRNAs are involved in stress response in the human genome

Audas et al., (2012) have described three lncRNAs within the IGS region transcribed from stress-specific loci which respond to environmental cues such as acidosis and heat shock. These three IGS ncRNA are able to capture and immobilize proteins of wide variety, including E3 ubiquitin ligase (VHL) cell cycle regulators, chaperones (Hsp70) and DNA polymerase subunits (RPA16,RPA40) (Jacob et al, 2013). All these proteins have a specific nucleolar detention sequence which associate to the locations of IGS lncRNA transcription (Audas et al., 2012). This nucleolar sequestration of proteins, referred to as the nucleolar detention center (DC) alters the tripartite structure of the nucleolus and therefore its function. Audas et al. (2012) showed that the amount of 45S pre-rRNA transcripts is 85% decreased in both heat shocked and acidotic cells relative to untreated cells (Audas et al., 2011), corresponding with the observation that components of the ribosomal biogenesis machinery were also captured in the DC of heat-shocked and acidotic cells. The accumulation of heat shock proteins and the impairment of ribosomal synthesis allow the cell to attenuate cell cycle and focus the energy on repair mechanisms and stress recovery. Overall, these lncRNA regulate proteins function post-translationally by immobilizing and retaining them away from their downstream effectors (Audas et al., 2011). Audas et al. (2011) demonstrated using RT-PCR that acidosis treatment temporarily induces IGS28 transcription and heat shock induces the expression of IGS16 and IGS22. All three RNA are involved in formation of the nuclear detention center and sequester different proteins (Hsp70, VHL, POLD1) (Audas et al, 2011; Prasanth, 2012).

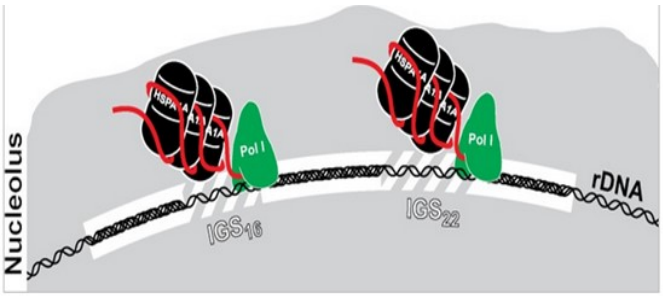


Figure5: Function of IGS16 and IGS22 RNA; Sequestration of proteins after heat shock (Place and Noonan 2014)

Newly discovered IGS lncRNAs

In my master thesis, I will define the sequence and investigate the function of three newly discovered lncRNAs: IGS19asRNA, IGS32asRNA and IGS38sRNA, named after the direction of transcription and the location with respect to the 45S rRNA. Due to previous research and experiments of the Group Östlund-Farrant, many characteristics of these three IGS lncRNA are already known (Stefanie Böhm, 2014).

	IGS_{19as}RNA	**IGS_{32as}RNA**	**IGS_{38}RNA**
Length (nc)	500	800	1300
Polymerase	Pol II (poly A-tail)	Pol I	Pol II (poly A-tail)
Direction of transcription	Antisense	Antisense	Sense
Regulation	Up regulation after heat shock	Up regulation after adding glucose	
Posttranscriptional regulation	Exosome	Exosome	Exosome
Transcription start site	19833kb from TSS		
Active Histone modification across the IGS	H3K4me3	H3K9me3	
Location of the mature ncRNA	Nucleus and cytoplasm (less abundant)	Only in the nucleus	Nucleus and cytoplasm (equal distributed)

Table 1: Characteristics of IGSlncRNAs

Taken together, these three lncRNA respond to different stress signals, have different locations and are transcribed by different polymerases, which provides evidence that all three lncRNAs exert different specific functions and indicates that they are functional transcripts.

Experimental outline

1. Determining the sequences of IGS19asRNA, IGS32asRNA, and IGS38asRNA and mapping them to the rDNA unit.
2. Investigation of their function, by ChIP (chromatin immunoprecipitation) with different antibodies (yH2Aα, CTCF, Polε, SMC1, Rad51) involved in replication, repair and regulation. I performed CHIP on cell cultures following different replication stress treatments, such as starving, thymidine and aphidicolin.
3. Determine if the expression pattern of these lncRNAs is cell cycle dependent. I performed RNA extraction of synchronized HeLa cell cultures followed by cDNA synthesis, qPCR and flow cytometric analysis.

MATERIALS AND METHODS

Cell line: Adherent human cervical cancer cells (HeLa). Cells were cultivated in Dulbecco´s Modified Eagle´s Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin. Cells were cultivated at 37^0C and at 5% CO_2.

Cell treatments: **Cell cycle synchronization**: cells were treated in 2mM thymidine for 16h, three times washed with PBS, released in DMEM+FBS for 12 hours, treated again with 2mM thymidine for 16h, released in DMEM+FBS. **Replication stress:** cells were treated for 17h with 2mM thymidine or 1µg/ml aphidicolin for 17h.

Antibodies: Primary antibodies: H2AX, Pol ε, CTCF, SMC1, Rad51 from abcam

Primers used for lncRNA within the humen rDNA repeat (accession number: u13369.1)

IGS19as forward		**Sequence (5'->3')**	**Start**	**Stop**
IIa F	GATGAGTTGTGCGCGGTG	Plus	18972	18989
IV	GAGGCACCATGCCTGCTTG	Plus	19348	19366
VI	CCACCTCAGCCTCCAGAG	Plus	19524	19541
VII	GACAGAGTTTCACTCTCGTG	Plus	19612	19631
IGS19as reverse				
lla R	GGCATATCTCCTTGACCGGTG	Minus	19120	19100
IV	CACTGCCGGGGTGACGG	Minus	19467	19451
VI	CACGAGAGTGAAACTCTGTC	Minus	19631	19612
VII	CAATCCCGGCTACTCAGGAG	Minus	19732	19713
VIII	GAGGTCGGGAGTTCGAAACC	Minus	19825	19806
IGS32as forward				
P1	GCTAATGATAGTGAAAGTGAAG	Plus	32045	32066
P2	CCTAGTAAAAGCTGGCCGATC	Plus	32153	32173
P5	GATCCATTAAACAACATATTCGC	Plus	32488	32510
P8	GAGTAGCTGGGACTACAGG	Plus	32812	32830
IGS32as reverse				
P7	CCTGTAGTCCCAGCTACTC	Minus	32830	32812
P8	GTCACGAGGTCAAGAGATCG	Minus	32925	32906
P11	GACAGTGTTCCAGTTTACGG	Minus	33231	33212
IGS38 forward				
A	CTAGGAAATCGCCACTTTGAC	Plus	38101	38121
F	CAAGAGCGAAACTCCGTCTC	Plus	38621	38640
38.5 (205)	CTTTGGGAGGCCAAGGCC	Plus	38776	38793
H	CACAATACACAACATTAGCCGG	Plus	38852	38873
Mf	CTGGGCCCGATTGTTCTTC	Plus	39307	39325
IGS38 reverse				
A	GTATTGTAATGGGTTGTATTGAC	Minus	38234	38212
H	CAACCTCCGCCTCCCAG	Minus	38965	38949
Mr	GTCCCCAACAACAACAAGGC	Minus	39437	39418

Other primers

Name	Forward 5`-3	Reverse 5`-3
ARP	GCACTGGAAGTCCAACTACTTC	TGAGGTCCTCCTTGGTGAACAC
Actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTAC
Gr. Prom	AGGGACAGGTCGCCAGAGGA	GCGATGGTGGCGTTTTTGG

Polymerase chain reaction (PCR): Tag polymerase (30cycles, 19as, 38sRNA)/phusion polymerase (40cycles, 32as RNA) was used to generate PCR products. Geneious software was used for mapping Sequences/PCR product to rDNA repeat unit (accession number: u13369.1).

Quantitative real-time polymerase chain reaction assay: RT-qPCR was performed in duplicates, using SYBR-green (PCR kappa mix) H_2O and Primers. Rotor gene 6000 series software was used for data interpretation. Reverse transcription control was performed in all samples using acidic ribosomal protein (ARP) primers. Non template controls have been used for each primer pair.

RNA extraction and cDNA synthesis: Cells were lysed by TRIzol lysis reagent. RNA extraction was performed by subsequent chloroform and isopropanol precipitation. RNA pellets were washed in 75% ethanol and dissolved in 10µl H_2O, concentration was measured by Nano Drop. Reverse transcription controls were made for all cDNA samples and treated with DNase. Superscript was used together with random primers and dNTPs to produce cDNA at 50^0C, reaction was stopped at 65^0C.

Chromatin immunoprecipitation (ChIP): Cells were crosslinked with 1% formaldehyde for 10 min, reaction was terminated by 0,125M glycine and washed with PBS. Cells were treated with 3ml ChIP buffer 1 and scraped, collected and centrifuged for 10min at 4^0C. Pellets were resuspended in 5ml ChIP buffer 2 to repeat the same procedure and finally stored in 2ml ChIP buffer 3. Cells were pulse sonicated (30s pulse 60Hz/ 30s rest) for 45min, removed of cell debris and incubated in unblocked A/G sepharose for 1 hour. The IP was performed in 1% Triton-100 and 0.1% DOC overnight, to which 15 µl blocked A/G sepharose was added. The IP was washed five times with RIPA buffer, cross-links were reversed by incubating samples at 65^0C overnight. DNA was extracted with phenol-chloroform and ethanol precipitation, pellet resuspended and stored in 100 µl H2O.

Flow cytometry: Cells were fixed in 80% ethanol for at least 4h, sinned down and resuspended in PBS (cell density 0.5-4 million/ml). Cells were stained with propidium iodide (1µg/ml) for 15min and analyzed with fluorescent activated cell sorter (FACS). FlowJo software was used for data interpretation.

Cloning: TOPO TA Cloning Kit was used for ligation of PCR product inside TOPO vector, transfection into *E. coli* (DH10B strain) by electroporation. *E. coli* were plated out on 1.5% LB-agar dishes with 50ng/ml kanamycin. 50µl of X-gal (20mg/ml) and 50µl ITPG (100mM) were spread over agar for blue white screening. After 16h positive colonies were picked and recultivated overnight in LB with 50ng/ml kanamycin, followed by plasmid DNA extraction using Viogene Mini Plus Plasmid DNA extraction system.

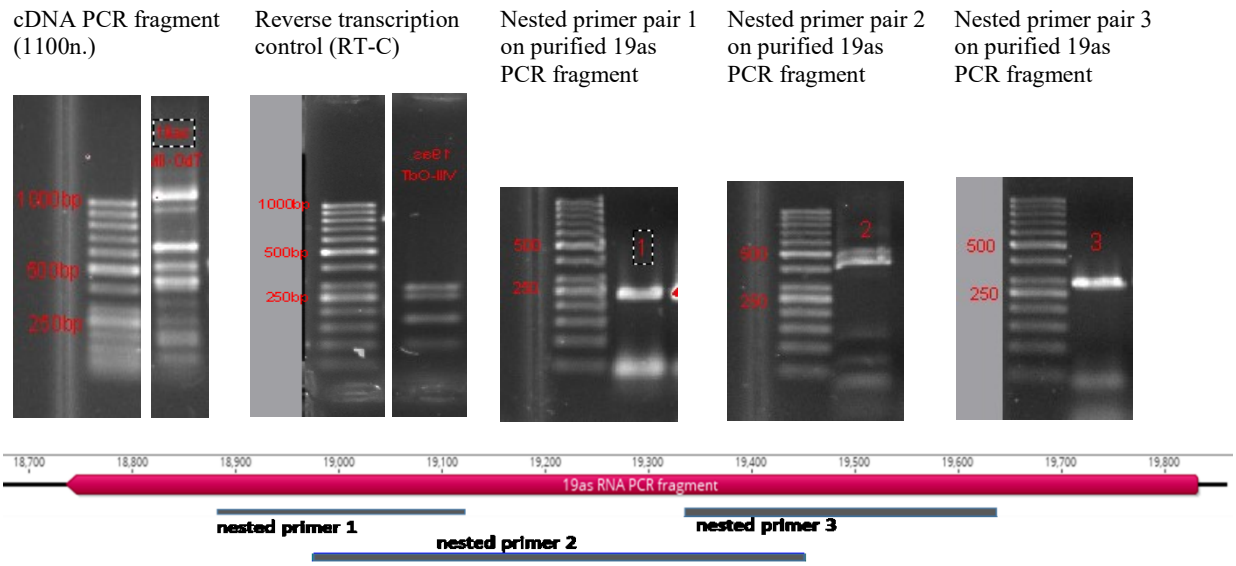
Results: Sequencing and PCR products

Due to the highly repetitive structure of the lncRNA loci, sequencing of PCR amplified cDNA fragments has shown no results, most likely because of secondary structures. Therefore the cDNA fragment has been ligated into a TOPO vector, and cloned in *E. coli*.

19as lncRNA

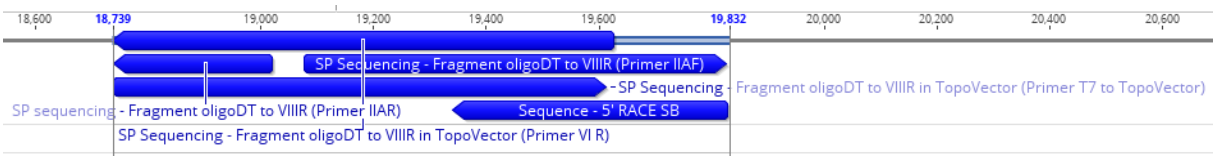
According to previous results from southern blots and 5´RACE sequencing, the expected length of the 19as RNA was 500bp. However, using a forward primer from the 19as TSS and oligo dT as a reverse primer, a specific, 1100 bp long PCR fragment has been obtained.

Figure 6: PCR products 19as RNA, mapped to the rDNA repeat using geneious software



This fragment was ligated into a TOPO vector and cloned in *E. coli*. By antibiotic selection and blue white screening, a colony carrying the vector with the 19as PCR fragment could be picked. After extraction of the plasmid it has been sequenced with primers laying within the 19as fragment and primers binding to the vector DNA flanking the 19as insert. Four reads from a cDNA fragment could be aligned to the IGS. The transcribed 19as RNA is around 1100bp long, the TSS is located at 19833bp+ and the TTS at 18736bp+ from the TSS of the rDNA repeat.

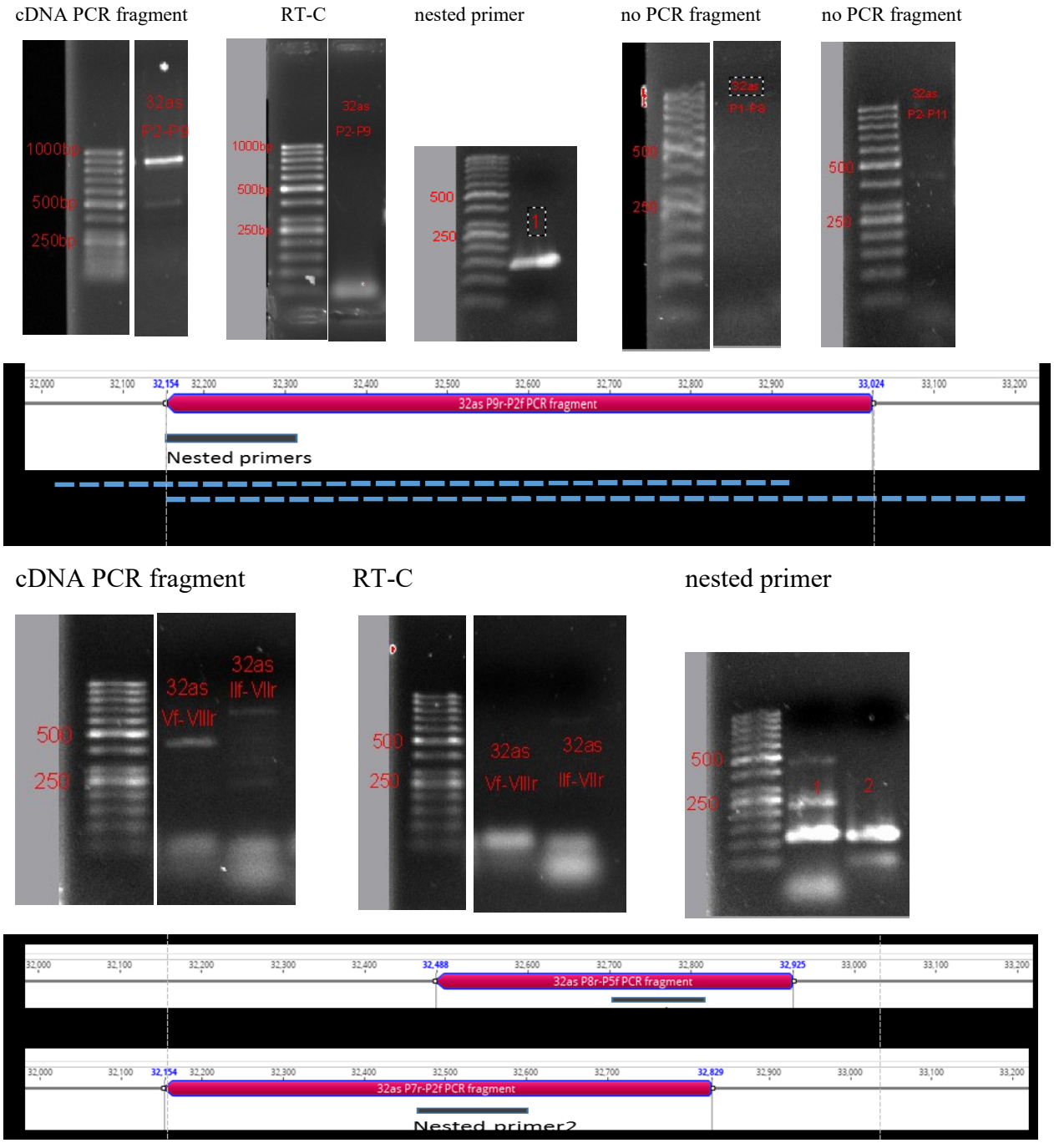
Figure 7: Sequence reads 19as RNA mapped to the rDNA repeat using geneious software



32as lncRNA

Three specific PCR products could be generated with different primer pairs binding 32kb downstream of the TSS of the rDNA unit. No PCR product has been obtained by using primers laying slightly outside the sequence of the longest PCR fragment.

Figure 8: PCR products 32as RNA, mapped to the rDNA repeat using geneious software

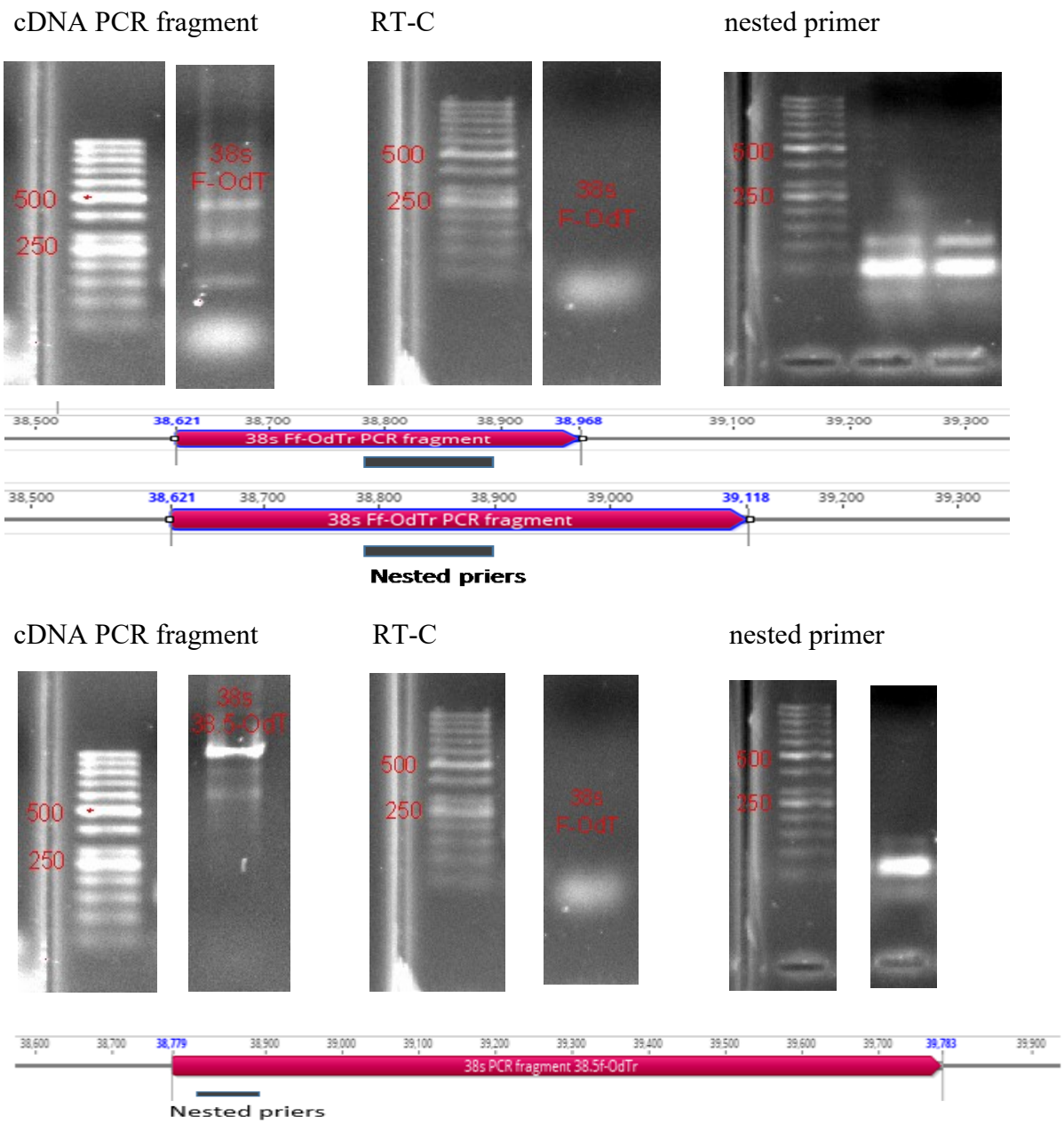


32as RNA is at least 860 nucleotide long, which corresponds well to the size received by northern blot (800nt). All fragments have been cloned in *E. coli*, but sequencing has not been successful.

38s lncRNA

For the 38s RNA two specific PCR products have been generated by the same primer pair. As the reverse primer was an oligo dT primer, it would be plausible that the 38s RNA is processed or has different TTS. Nested primers have confirmed the specificity of both PCR fragments.

Figure 9: PCR products 38ss RNA, mapped to the rDNA repeat using geneious software



Additionally, a 1000nt-long fragment has been generated, using an oligo dT reverse primer and a forward primer laying downstream of the one used above. The same nested primer pair has been used to test specificity. In total the PCR fragments received for the 38s RNA over span 1150bp.

Discussion

IGSlncRNAs co-localizes with Pleiades and correspond to active Histone modifications and DNase1 hypersensitivity

Strikingly Tschurikov (2015) has located nine hot spots of DSB (Pleiades) within the IGS, which co localize with all three IGSlncRNAs. The TSS of the 19as RNA is located within Pleiades R1. The 3`prime end of longest PCR fragment from 32as RNA colocalize with Pleiades R5 and the 5´prime end is close to Pleiades R4. The PCR products received for the 38s RNA span over Pleiades R8 and R9. Furthermore these regions show an active chromatin mark (HeK4me3) and DNase1 hypersensitivity, which indicates that these loci are accessible for DNA-protein interactions.

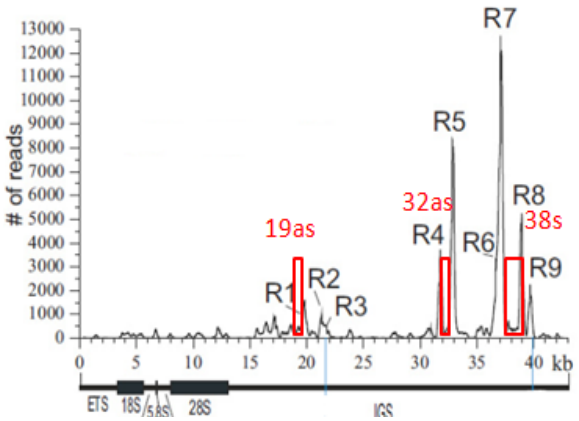


Figure 10 Co-localization of Pleiades with IGSlncRNAs (Tschrikov et al., 2015)

The origin of Pleiades is still an open question. It is well known that R-loops are a source of DBSs. They occur naturally during transcription and replication by forming a RNA:DNA hybrid and a displaced DNA strand, which is susceptible to DNA damage (Aguilera et al., 2012). The high transcription activity of rDNA repeats makes them prone to R-loop induced damage (Santos-Pereira 2015). The repetitive nature of IGS sequences, leading to secondary DNA structures is an additional source of replication stress (Mazouzi et al., 2014). There has been speculation weather R-loops trigger Pleiades, but the fact that all nine DBS hot spots are located within the IGS and not within the highly transcribed coding region contradict this hypothesis (Tschurikov 2015). Furthermore R-loops observed within the IGS do not co localize with Pleiades (Nadel et al., 2015). Incomplete shut down of transcription during mitosis is another possibility to explain DSB hot spots (Tschurikov et al., 2015).

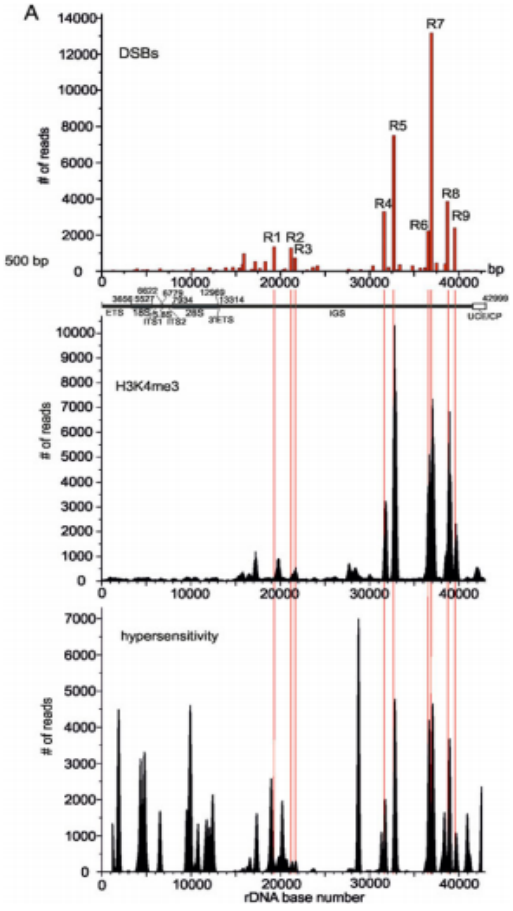


Figure 11: Pleiades coincide with H3K4me3 Histone marks and hypersensitive sites (Tschurikov et al 2015)

Another source of DSBs are collisions between replication and transcription forks. Within the rDNA coding region the transcription terminator Sal box T1 act as a polar replication fork barrier (RFB), preventing collisions of replication forks by inhibition of fork progression in the opposite direction of rDNA transcription. (Labib and Hodgson, 2007; Akamatsu et al. 2015). However this does not apply for the IGS region and the fact that in active rDNA units replications initiates randomly at different loci within the IGS makes frequent fork collision highly likely (Dimitrova 2011).

Tschurikov (2015) suggests that DBSs are only present in active rDNA repeats and connects them strongly to transcription, as they occur also in resting cells (CD4+), which are not replicating. However, the question if transcription leads to DSBs or DSBs activate transcription cannot be answered yet (Tschurikov (2015). Replication stress and changes in transcription activity in heat shocked cells increased DSBs at Pleiades (Tschurikov 2015). This raises the question if and how DSB´s at Pleiades flanking the IGSlncRNAs are involved their transcriptional regulation. Tschurikov (2014) propose that DSB signals for recruitment of regulatory proteins at specific sites, which correspond to the observation Audas (2012) has made with IGS 16as, IGS 22as and IGS 28as RNA (Audas et al., 2011).

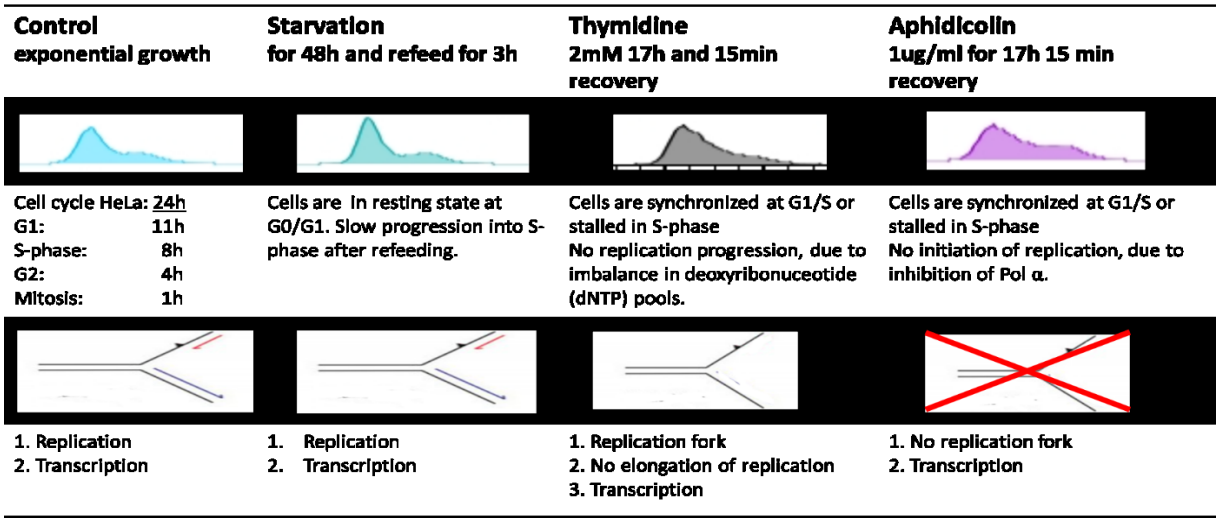
Interestingly each Pleiades is located within a transposable Alu element and all three lncRNA comprise Alu elements or part of them. Most recently Hu et al., (2016) have shown that a lncRNA, transcribed from the 5S rDNA loci in humans cells (HeLa, 193T) regulates alternative splicing of more than 200 exons by Alu/anti-Alu pairing with target genes and interacting with a splicing factor.

In summary, IGS lncRNAs coincide with hots spots of DSBs and active Histone marks, which argues for a connection between IGSRNA´s, DBS´s and regulation of transcription inside rDNA repeats (Tschurikov et al., 2015). It is proposed that these lncRNA can immobilize and sequester regulatory proteins at specific sites as it has been shown for other IGS lncRNA (Place and Noonan, 2014). Finally, all IGS lncRNAs contain Alu sequences, which can be involved in modulating gene expression post-transcriptionally (Häsler and Strub, 2006).

Results: Immunoprecipitation

To investigate if DSB repair and chromatin architecture is connected with replication, three different replication stress treatments have been imposed on HeLa cells. Then all cell cultures have been stained with propidium iodide and analyzed with a fluorescent activated cell sorter (FACS).

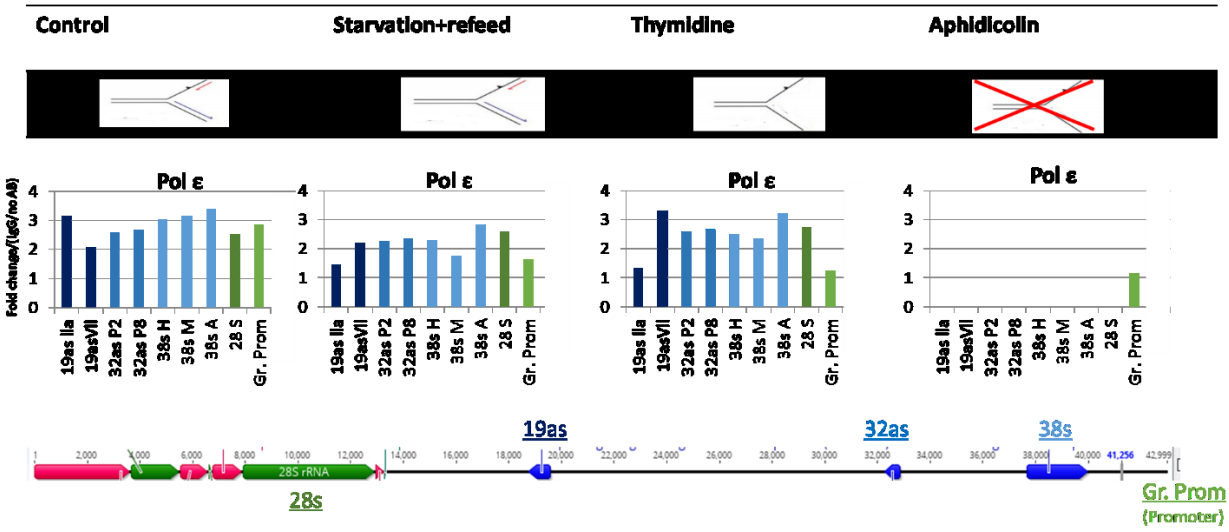
Figure 12: Cultivation of HeLa cells with different replication stress conditions



For qPCR analysis two primers were chosen for each lncRNA. In addition a loci within the 28S coding region and within the promoter region have been analyzed.

Proof of principle for stress treatments

Figure 13: ChIP results for Pol ε IP



As expected Pol ε is present in exponential growing cells and in serum starved cells after 3 hours of refeeding. Pol ε binds also to thymidine treated cells, as thymidine only prevent elongation of replication, but not the establishment of a replication fork. Aphidicolin inhibits

initiation of replication by binding to Pol α, therefore no replication fork could be established to which Pol ε would bind. By using a specific Pol ε antibody for immunoprecipitation the principle of these three different stress treatments could be established.

γH2AX, Rad 51 and SMS1 bind to lncRNAs loci

After DSB induction, serine 139 in histone H2AX gets phosphorylated (γH2AX), this post-translational histone modification spreads over 2kb of the DSB loci. γH2AX phosphorylation is required for recruitment and accumulation of DNA double strand break repair (DDR) proteins (Mendez-Acuna, 2010). BRCA2 is one of the first proteins binding to DSB, it recruits Rad51 to ssDNA (Renodon-Cornière et al., 2013). Rad51 promotes pairing of homologous sister chromatids and is involved in strand displacement (Alexander and Orr-Weaver, 2016). Sister chromatids are used as a template for homologous recombination repair (HR), thus close spatial proximity of sister chromatids is decisive for HR. The cohesion complex (**Smc1**, Smc3, Scc1/Mcd1/Rad21, and Scc3/Irr1) associates with chromosomes in late G1-phase, establish a physical linkage between sister chromatids during S-phase and dissociated before chromosome segregation in anaphase (Peters et al., 2008). Therefore, HR takes only place in S- and G2-phases.

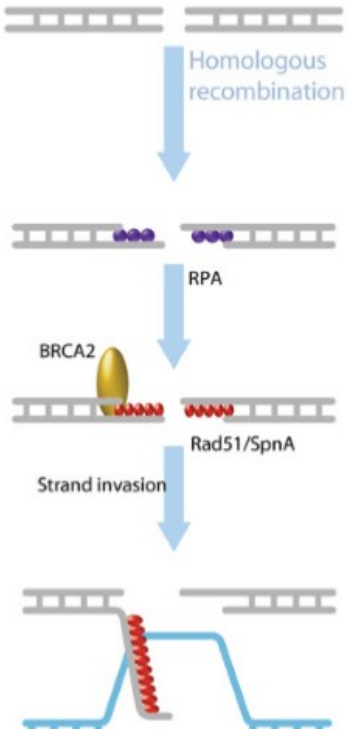
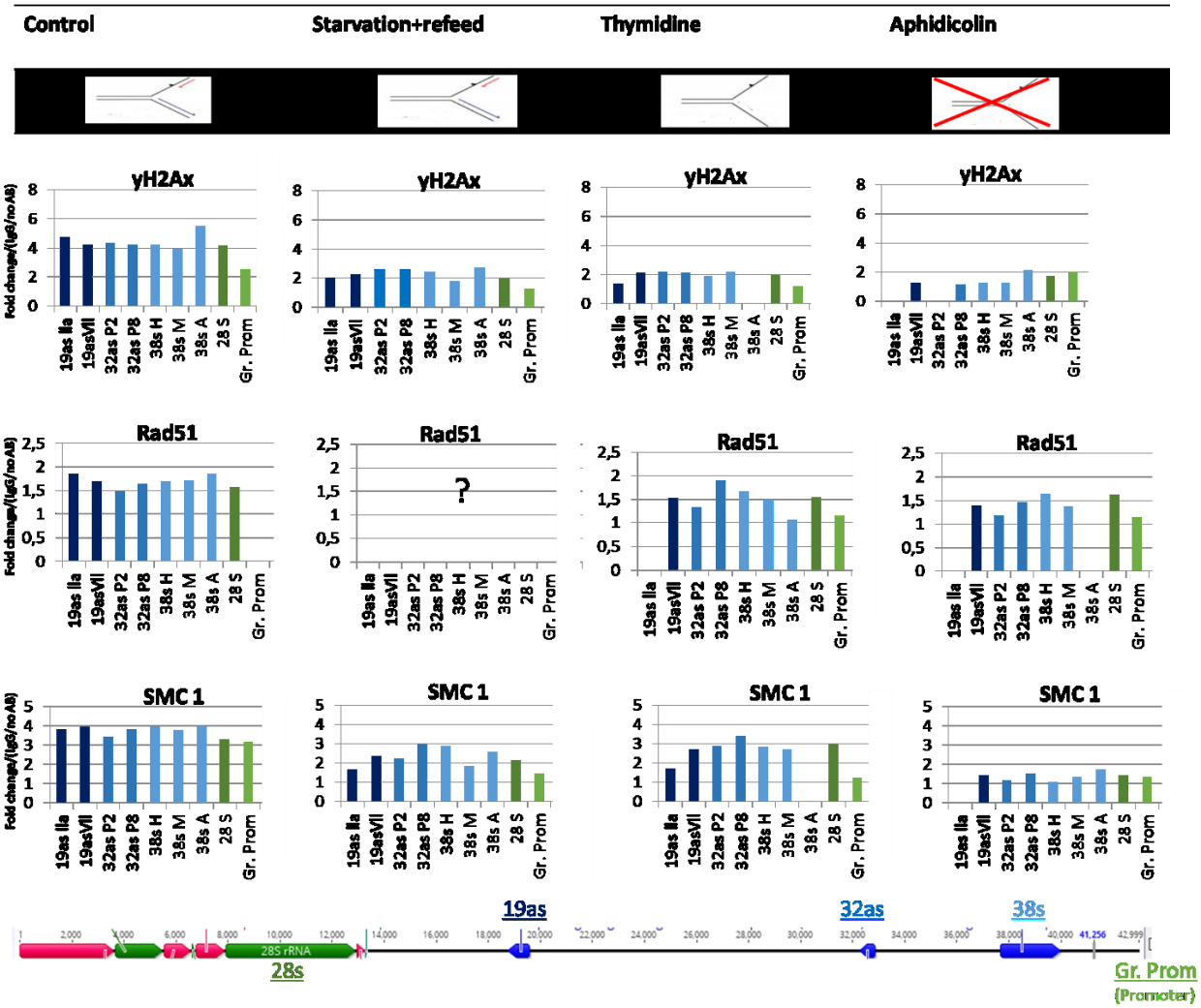


Figure 14: Homologous recombination repair (Alexander and Orr-Weaver, 2016)

Figure 15: ChiP results for γH2AX, Rad51 and SMC1 IP



γH2AX is present in all treatment groups, but the strongest signal has been received for exponential growing cells. DSBs are more frequent within the IGS during normal replication and cell cycle progression than in replication stress conditions. Moreover γH2AX corresponds well to the loci of all three lncRNAs. The signal is reduced at the promoter region, where no Pleiades is located. However the locus within the 28S rRNA coding region shows a similar signal for γH2AX as the lncRNAs loci do. One explanation would be that due to the high transcriptional activity within the rDNA coding region, DSBs caused by fork collision are more likely. Rad 51 is present in exponentially growing cells, thymidine and aphidicolin-treated cells, which proofs that DSBs are repaired by HR. In starved and re-fed cells no Rad 51 binding is present. This is most probably due to a technical problem, as pol ε has been present in these cells, demonstrating that cells have progressed into S-phase. SMC1 is present in all samples, but on a very low level in aphidicolin treated cells.

Taken together the ChIP results show that within the IGS DSBs occur more frequently when replication in not inhibited and SMC 1 is binding more efficiently when DNA replication is not compromised. Rad 51 is present in DSB loci and coincide with γH2AX.

Binding of CTCF depends on a functional DNA replication machinery

The CCCTC-binding factor (CTCF) has diverse regulatory functions and can act as an insulator, as a transcriptional activator or repressor by forming chromatin loops, and is often associated with cohesin. Furthermore CTCF mediates distant chromatin interactions and is involved in genome organization by separating active chromatin inside the loops from inactive chromatin domains outside loops and vice versa. (Oti, 2016), (Holwerda and de Laat, 2013). Moreover it has been shown than CTCF bind to the region of UCE and CPE, facilitates the recruitment of UBF and is involved in the regulation of the epigenetic state of rDNA repeats, by competing with cohesion for shared binding sites inside rDNA clusters (Huang et al., 2013; Tschurikov 2015).

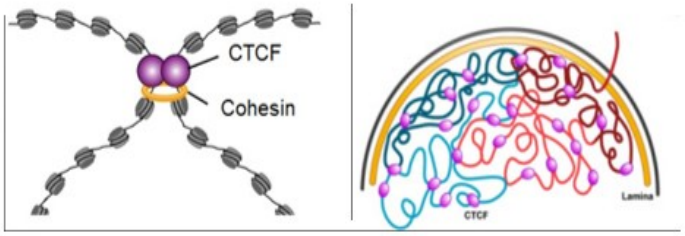
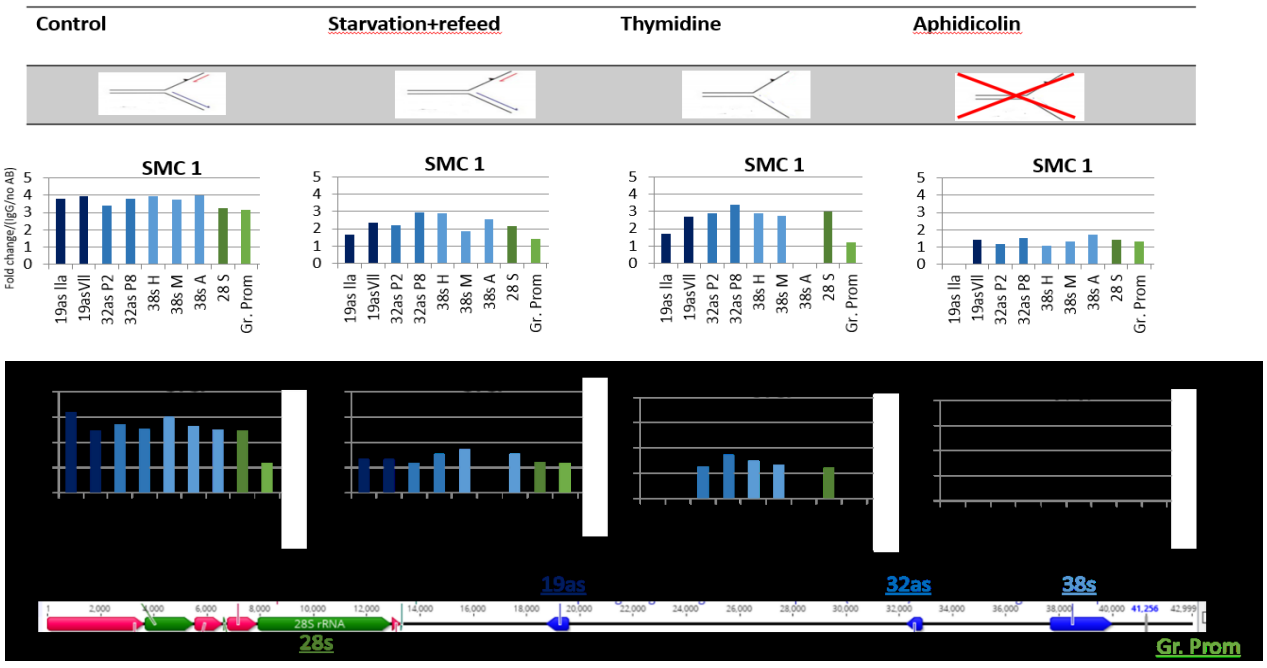


Figure 15: CTCF and SMS1 mediate chromosomal contacts and are involved in chromatin architecture (Li et.al 2013)

Figure 16: Results ChIP for SMC1 and CTCF IP



SMC1 and CTCF binding is connected to normal replication and cell cycle progression. Cells suffering from replication stress bind less SMC1 and CTCF at these specific loci inside the IGS. At least under replication stress conditions these two proteins do not compete with each other for shared binding sites at these specific loci within the IGS. Therefore it is more likely that they are working together, forming chromatin domains connected to replication.

Discussion

Pleiades coincide with H2AX CTCF and KAP1 binding sites

Tschurikov (2015) has demonstrated using -IFISH with a fluorescently labeled rDNA probe that the highest γH2AX signal is located inside central areas of the nucleoli, moreover γH2AX signals co localize with UBF binding sites, only accessible in active euchromatic rDNA clusters. Hence, only transcriptional competent rDNA repeats possess hot spots of DBSs.

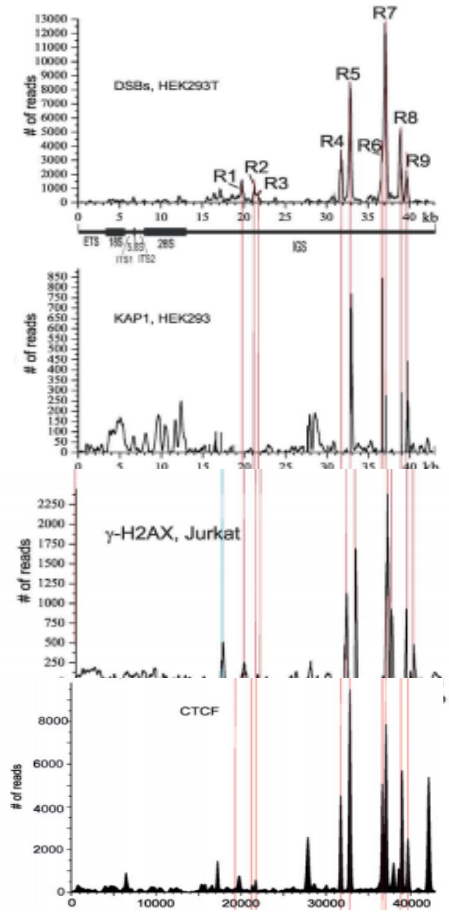


Figure 17: KAP1, yH2AX and CTCF coincide with Pleiades (Tschurikov et al., 2016, 2015)

KRAB-associated protein 1 (KAP 1) is known to be a master regulator of the genome and is involved in multiple cellular processes including regulation of epigenetic chromatin pattern, DNA repair, cell growth and differentiation. KAP 1 does not possess a chromatin binding domain but it induces gene silencing by binding to the chromo shadow domain of HP1. HP1α and KAP1 are enriched in heterochromatic regions (White et al., 2012). Furthermore Ayrapetov (2014) has shown that a protein complex consisting of H3K9 methyltransferase Kap 1 and HP1 is rapidly recruited to DSB sites, leading to a repressive chromatin structure at DSB, as a first "stabilizing" step in DNA double strand break repair (DDR).

It is unclear whether KAP1 associated with Pleiades is involved DDR, epigenetic regulation or both. Tschurikov suggests that KAP1 is associated with the inactive rDNA clusters, located in the nucleolar periphery and plays an important role in its suppression. The mechanisms which connect replication timing and chromatin structures are not fully understood yet (Li et al., 2005). According to the ChIP results, inhibition of replication impairs binding of CTCF and decrease SMC1 binding to these loci. Together with the fact that CTCF and SMC1 are involved in chromosomal architecture by mediating intra and interchromosomal contacts, both proteins and the lncRNA transcribed form these loci could be involved in regulation and maintenance of active or inactive rDNA clusters and their replication timing.

Genomic contacts of Pleiades sites within the rDNA and the whole Genome

By performing 4-C rDNA experiments Tschurikov et al., (2015) demonstrated many intra and interchromosomal contacts between Pleiades R4 and R5 (coincide with IGS32as RNA loci) and pericentromeric heterochromatin (PC-HC) regions, with chromosomal sites with DSB hot spots, but also between and within rDNA gene clusters. Contacts between PC-HC regions could be important for regulation or maintenance of active or passive rDNA repeats.

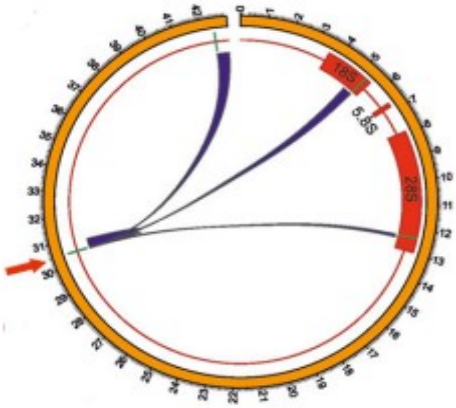


Figure 18: Intrachromosomal contacts between Pleiades R4 and rDNA repeat unit (Tschurikov et al., 2015)

As normal human cells possess only on nucleolus, it would be plausible that genomic contact between rDNA repeats and other genome loci helps to form a chromosomal compartment, which brings together all NORs from 5 acrocentric chromosomes. Internal interaction between Pleiades R4 and R5 and the 18S, 28S coding region and the rDNA promoter region are observed (Tschurikov et al., 2015)

This raises the question if IGS32asRNA, lying in between Pleiades R4 and R5, having H3K9me3 methylation bound to HP1α which recruits KAP1 and mediates the formation of heterochromatin structures, is involved in epigenetic regulation of the rDNA cluster or other genome loci.

Results and discussion: Expression pattern of lncRNAs during cell cycle progression

A double thymidine block has been used to synchronize cells at the G1/S border. RNA has been extracted from synchronized HeLa cells at different time points. Additionally cells have been stained with propidium iodide for flow cytometry analysis. This experiment has been repeated three times. For normalization the mean value of actin and ARP (acidic ribosomal protein) has been used.

G1/S	Mid/late S	G2	Late G2/early G1	Control
0 hours after release	6 hours after release	9 hours after release	13hours after release	Exponential growing cells

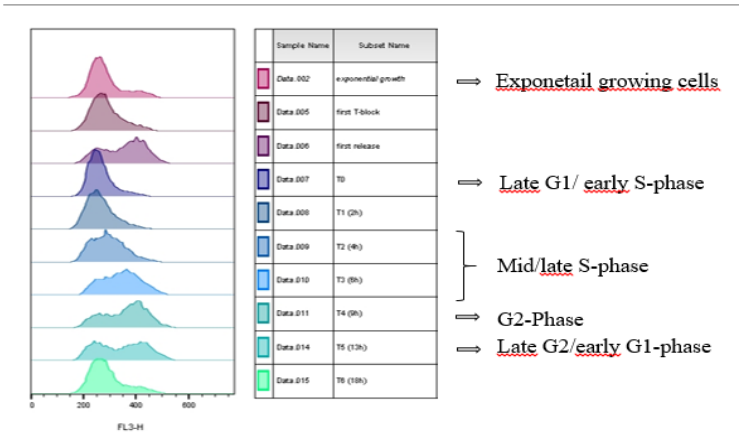


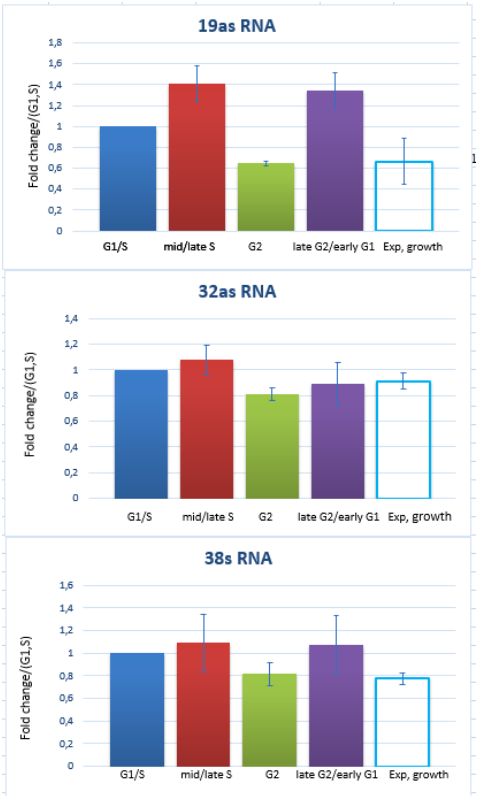
Figure 19: Flow cytometric diagram with synchronised HeLa cells

RNA 19as RNA shows a cell cycle dependent expression pattern, it is downregulated in G2-phase and shows a higher expression level during S-phase and in early G1 Phase. The fact that only expression of the 19as RNA seem to be cell cycle dependent, indicates that its function is different from the 32as and 38s RNA.

32as RNA does not show a cell cycle dependent pattern. This indicates that the 32as RNA is not directly involved in transcriptional regulation of the rDNA genes during cell cycle, as rDNA genes reach their highest transcriptional activity during S-phase and G2-phase.

The expression of the 38s RNA is slightly lower in G2 phase, but overall 38s RNA doesn´t show a strong connection between its expression levels and cell cycle progression.

Figure 20: Expression pattern of IGS lncRNA at different stages of the cell cycle



Summary

All three lncRNA could be mapped to the rDNA repeat unit and it has been shown that they coincide with hot spots of double strands breaks. It could be confirmed that γH2AX and CTCF are binding to these loci and that DSBs occur more frequently during normal replication and cell cycle progression rather than under replication stress conditions. Furthermore it has been shown the frequency of DSBs is higher at the lncRNA loci than at the promoter region, which corresponds to Tschurikovs (2015) reported hot spots of DSBs. The ChIP results additionally showed that CTCF binding is impaired, when formation of a replication fork is inhibited by aphidicolin treatment, indicating a connection between CTCF binding and regulation of replication.

It is not possible yet to assign a specific function to these newly discovered lncRNAs but taken into account that the 19asRNA transcription is upregulated after heat shock allows the speculation of its involvement in stress response, particularly as Audas (2011) has already shown that this is the case for IGS16lncRNA and IGS22lncRNA. The cell cycle dependent expression pattern of 19asRNA and its detection in the nucleolus and in the cytoplasm is an indication for additional functions.

Tschurikov et al., (2015) has shown that the loci of 32asRNA can form many inter and intrachromosomal contacts, particularly with pericentromeric heterochromatin (PC-HC) regions. Moreover the 32asRNA loci shows heterochromatic histone marks (H3K9me3) and bind to HP1α, which recruit KAP1, a mediator of heterochromatin formation (Böhm 2014) (Tschurikov et al., 2015) (White et al., 2012). Taken together this indicates the involvement of 32as RNA transcripts in maintaining and/or establishing heterochromatic structures.

Little is known about the 38sRNA, but its locus spans over two hot spots of DSBs and according to the PCR results, the transcript appears to be processed. The detection of 38sRNAs in the cytoplasm supports this speculation, as splicing and splicing related proteins are important export factors (Clark and Mattick, 2011).

The connection between DBSs and lncRNA is not known yet. On the one hand transcription of these lncRNA could cause DSBs due to collision of replication and transcription forks. But it would also be plausible that the occurrence of DSB at these loci serve as a signal for lncRNA transcription, which would argue for their involvement in stress response and/or DNA repair. The question which comes first: “The transcript or the break?” remains open (Tschurikov 2015).

Zusammenfassung

Den drei untersuchten lncRNAs konnten Loci in der rDNA-Repeat-Einheit zugeordnet werden. Es hat sich herausgestellt, daß diese Loci mit Hot-Spots von Doppelstrangbrüchen zusammenfallen. Darüber hinaus konnte bestätigt werden, daß γH2AX und CTCF an diese Loci binden und DSBs während normaler Replikation und Zellzyklusprogression häufiger auftreten als unter indizierten Zellstress- Bedingungen. Die Häufigkeit von DSBs innerhalb der lncRNA-Loci war höher, als an der Promotorregion der 47S-pre-rRNA. Dies bestätigt die von Tschurikov et al., (2015) entdeckten Hot Spots von DSBs, welche die drei lncRNAs flakieren. Die ChIP-Ergebnisse zeigten, daß CTCF nicht an die lncRNA-Loci bindet, wenn die Replikationsgabel durch Aphidicolin-Behandlung gehemmt wird. Dies verweist auf einen Einfluß der CTCF-Bindungen auf die Replikationsregulation hin.

Noch ist es nicht möglich den drei neu entdeckten lncRNA´s eine spezifische Funktion zuzuordnen. Die deutliche Hochregulierung der Transkription von 19asRNAs nach einem initiierten Hitzeschock verweist auf eine Mitwirkung in der Hitzeschock-Antwort. Audas et al., (2011) hat bereits nachgewiesen, das dies für IGS16lncRNA und IGS22lncRNA der Fall ist. Das Zellzyklus abhängige Expressionsmuster der 19as RNA und ihr Vorkommen im Nucleolus als auch im Zytoplasma ist ein Indiz für zusätzliche, unbekannte Funktionen.

Tschurikov et al., (2015) hat aufgezeigt, daß der Locus der 32asRNA verschiedenste Inter- und Intrachromosomale Kontakte vermitteln kann, besonders zu perizentrometrischem Heterochromatin (PC-HC). Zudem befinden sich auf dem Locus der 32asRNA heterochromatische Histon-Markierungen (H3K9me3), welche an HP1α binden, das wiederum KAP1, einen Vermittler von Heterochromatin-Strukturen, rekrutiert (Tschurikov et al., 2015) (White et al., 2012). Dies deutet stark auf eine Mitwirkung von 32asRNA Transkripten im Aufbau und Erhalt von Heterochromatin-Strukturen hin.

Über die Funktion der 38sRNA können nur wenige Aussagen gemacht werden. Der Locus enthält zwei Hot-Spots von DSBs. Die PCR Resultate deuten darauf hin, daß das Transkript prozessiert wird. Die Detektion von 38sRNAs im Zytoplasma unterstützt dies, da der Export ins Zytoplasma oft durch Splicing-Proteine und Splicing assoziierte Proteine vermittelt wird (Clark and Mattick, 2011).

Der kausale Zusammenhang zwischen DBSs und lncRNA ist nach wie vor ungeklärt. Die Transkription der lncRNA könnte die DSBs durch Kollision mit der Replikation- und Transkriptionsgabel verursachen. Es wäre aber ebenso denkbar, daß die DSBs an diesen Loci als Signal für die Initiierung der lncRNA Transkription dienen. Dies spräche für die Beteiligung

der lncRNA Transkripten an Streß-Antworten oder an DANN-Reparaturmechanismen. Die Frage was zuerst kommt: “Das Transkript oder der Bruch?” bleibt offen (Tschurikov 2015).

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