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Nucleolar reorganisation promotes repair of rDNA double strand breaks by homologous recombination throughout the cell cycle

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December 2013

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National University of Ireland, Galway

A thesis submitted to the National University of Ireland, Galway for the degree of Doctor of Philosophy
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<td>53BP1</td>
<td>Tumor suppressor p53-binding protein 1</td>
</tr>
<tr>
<td>ActD</td>
<td>Actinomycin D</td>
</tr>
<tr>
<td>ARF</td>
<td>alternate reading frame</td>
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<tr>
<td>ATM</td>
<td>Ataxia-telangiectasia mutated</td>
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<td>ATR</td>
<td>ATM and Rad3-related protein</td>
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<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<td>BLM</td>
<td>Blooms syndrome protein</td>
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<td>bp</td>
<td>base pair</td>
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<td>BRCA1</td>
<td>Breast cancer type 1 susceptibility protein</td>
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<td>BRCT</td>
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<td>BrUTP</td>
<td>5-Bromouridine 5’-triphosphate</td>
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<td>bovine serum albumin</td>
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<td>complementary DNA</td>
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<td>centimetre</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CRISPR</td>
<td>Clustered Regularly Interspaced Short Palindromic Repeats</td>
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<td>cockayne syndrome protein B</td>
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<tr>
<td>CtIP</td>
<td>CtBP-interacting protein</td>
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<tr>
<td>Da</td>
<td>dalton</td>
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<td>DAPI</td>
<td>4’-6-diamidino-2-phenylindole</td>
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<td>DFC</td>
<td>dense fibrillar component</td>
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<td>DMEM</td>
<td>dulbecco's modified eagle's medium</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DNA-PK</td>
<td>DNA dependent protein kinase</td>
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<td>Dnmt1</td>
<td>DNA methyltransferase</td>
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<td>dNTPs</td>
<td>deoxynucleotide triphosphate</td>
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<td>DRB</td>
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<td>ds</td>
<td>double stranded</td>
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<td>double strand breaks</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>eccDNA</td>
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<td>Extracellular signal-regulated kinases</td>
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<td>equal sister chromatid recombination</td>
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<td>EU</td>
<td>5-ethynyl uridine</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FC</td>
<td>fibrillarin component</td>
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<td>fluorescent ubiquitination-based cell cycle indicator</td>
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<td>human telomerase reverse transcriptase</td>
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<td>Immunoglobulin G</td>
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<tr>
<td>IGS</td>
<td>intergenic spacer</td>
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<td>PARP</td>
<td>poly (ADP-ribose) polymerase</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>phosphatase 2A</td>
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<td>promoter RNA</td>
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<tr>
<td>Rb</td>
<td>Retinoblastoma protein</td>
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<td>RFB</td>
<td>Replication fork barrier</td>
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<tr>
<td>SNF2h</td>
<td>sucrose nonfermenting 2 homologue</td>
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<tr>
<td>snoRNA</td>
<td>small nucleolar RNA</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>snRNA</td>
<td>small nuclear RNA</td>
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<td>X-ray repair cross-complementing</td>
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<td>zinc finger nuclease</td>
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I am very grateful to my supervisor, Professor Brian McStay, for this opportunity. You would also have time for questions and discussion. Especially I’d like to thank you for all your guidance and allowing me to give my own twist to this project.

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To the girls of TC1, you made boring TC1 times fun. Thank you!

Abstract

The nucleolus is the largest functional domain within the nucleus and is the site of ribosome biogenesis. It has a distinct structure and houses ribosomal RNA gene (rDNA) transcription, pre-ribosomal RNA (pre-rRNA) processing, and pre-ribosome assembly. The rDNA repeats encode the major rRNA species and are organized into large head-to-tail tandem arrays located at the nucleolar organiser regions (NORs). In humans, the ~300 rDNA repeats are distributed among five NORs on the short arms of the acrocentric chromosomes. In most human cells a majority of NORs, but usually not all, are active, and coalesce to form between 1 and 3 nucleoli. Nucleoli are spatially isolated from the rest of the nucleoplasm by a shell of heterochromatin. The enormous demand for ribosomes by actively growing cells means that rDNA repeats are the most actively transcribed genes in all eukaryotic cells. The resulting vulnerability of rDNA combined with emerging roles for the nucleolus in stress sensing prompted us to investigate the response of nucleoli to the presence of double strand breaks (DSBs) in the rDNA.

The 28S rRNA coding sequences contain the 15bp recognition sequence for the homing endonuclease I-PpoI. I have developed a broadly applicable mRNA transfection procedure that can efficiently introduce I-PpoI into a wide variety of human cell lines, thus inducing DSBs specifically within the rDNA repeats. Analysis of how nucleoli deal with DSBs in the rDNA has uncovered a complex response. The presence of DSBs results in activation of ATM and inhibition of transcription. As a direct consequence of transcriptional inhibition, rDNA repeats withdraw from the nucleolar interior to caps located at the nucleolar periphery. Importantly each cap represents the rDNA from a single NOR. Positioning of damaged NORs on the nucleolar surface renders their damaged rDNA accessible to repair factors, normally excluded from the nucleolar interior. Evidence suggests that repair is carried out by homologous recombination (HR), independently of the stage in the cell cycle. This complex nucleolar response highlights the fact that a spectrum of DSB repair mechanisms have evolved to maintain integrity of the genome.
Chapter 1

Introduction

The nucleolus is a visible subnuclear structure that can be seen under a visible light microscope. Fontana first described the nucleolus in 1781 as “un corps oviforme”. Throughout the 18th and 19th century many authors reported sightings of nucleoli in different species which are reviewed by Montgomery (Montgomery, 1898). But despite numerous observations of nucleoli the function and composition of the nucleolus was unknown. In 1931 Heitz compared mitotic cells from different plant species. The stained chromosomes contained gaps or constrictions. All chromosomes have a primary constriction where the centromere is located and a few chromosomes have a secondary constriction. He noticed that the number of secondary constrictions is always similar to the number of nucleoli in early telophase nuclei [Figure 1.1]. Also the location of these nucleoli is dictated by the position of secondary constriction bearing chromosomes in the preceding metaphase. Depending on whether the secondary constriction was located close to the centromere or closer to the telomere, the nucleolus would form in a different part of the nucleus [Figure 1.1]. The size of the nucleolus is also directly correlated to the size of the secondary constriction. A large secondary constriction gives rise to large nucleoli, while small secondary constrictions gave small nucleoli [Figure 1.1]. Finally haploid cells have half the number of nucleoli in early telophase, whereas triploid or tetraploid cells have respectively 3 and 4 times as many nucleoli as haploid cells. All this evidence lead him to conclude that nucleoli are encoded by the chromosomes with secondary constrictions (Heitz, 1931).

Around the same time, Barbara McClintock described a similar phenomenon in Zea mays. Maize only has 10 different chromosomes with a single secondary constriction on chromosome 6. In prophase, a nucleolar body is always associated with the condensing chromosome 6. By studying a mutant with a reciprocal translocation between chromosome 6 and 9, she noticed that the nucleolar body associates with both chromosomes. The breakage is in the region that associates with the nucleolar body leaving part of the nucleolar organising body on the original chromosome 6 and part on chromosome 9. Both chromosomes are involved in the formation of 2 nucleoli and therefore she concludes that these regions have elements that are involved in the formation of nucleoli. The nucleolar organiser bodies are referred to nowadays as nucleolar organiser regions (NORs).
Figure 1.1: A summary figure of Heitz’s important findings. The metaphase chromosomes with the secondary constrictions (the gaps) and the corresponding telophase nucleoli of 1 of the sister cells with the early nucleoli. Different plant species have secondary constrictions of different sizes and therefore different sized nucleoli in early telophase. The position of the secondary construction relative to the centromere determines the position of the nucleoli in the sister nucleus. Also the number of secondary constrictions is the same as the number of nucleoli in early telophase. Springer and Heitz, E. (1931), Die Ursache der gesetzmassigen Zahl, Lage, Form und Pflanzlicher Nukleolen, Planta. 12, 775-844, is given permission to the publication in which the material was originally published, by adding: with kind permission from Springer Sciences and Business Media.

1.1 The ribosomal genes are located in Nucleolar Organiser Regions

Despite secondary constrictions being linked to nucleoli, the function of nucleoli was still unknown. In the 1960’s cell fractionations made it possible to compare different compartments with each other. Cytoplasmic RNA has a similar sedimentation profile and base composition as nucleolar RNA while nuclear RNA is different (Edstrom et al., 1961). Additionally a Xenopus leavis mutant was identified which had no nucleoli due to a deletion of the NORs. The tadpoles had a deficiency in ribosomal RNA (rRNA) synthesis (Brown and Gurdon, 1964), which supports the hypothesis that rRNAs are synthesized in nucleoli.

But definitive evidence came from hybridisation with radioactively labelled rRNA on metaphase spreads and interphase cells. The rRNA hybridized at the secondary constrictions on the acrocentric chromosomes (Henderson et al., 1972; Ritossa and Spiegelman, 1965). In
interphase cells, radioactive rRNA preferentially hybridized in nucleoli (McConkey and Hopkins, 1964; Perry, 1962), proving that rRNA is synthesized in nucleoli and the rDNA genes are located on NORs.

Ribosomal RNA is transcribed as a 35S/45S precursor which is processed and modified into the mature 18S, 5.8S and 28S ribosomal RNAs which are found in ribosomes (Liau and Perry, 1969; Scherrer et al., 1963; Weinberg et al., 1967). The processing and modification of the pre-ribosomal RNA is discussed in more detail in section 1.2.2.

In yeast the ribosomal DNA (rDNA) is located in a single locus at chromosome XII (Petes, 1979) The 9.1 kilo-base (kb) rDNA repeat unit has the 35S and the 5S. The 35S is transcribed by RNA polymerase I, while the 5S is transcribed separately by RNA polymerase III (Bell et al., 1977). In higher eukaryotes the 5S rDNA gene is no longer part of the rDNA repeats at the NORs (Grozdanov et al., 2003; Maden et al., 1987). The intergenic spacer in between the 35S/45S genes contains multiple functional elements (Gonzalez and Sylvester, 1995; Grozdanov et al., 2003). Several species have multiple promoters at the 5’end of the 35S/45S gene. These spacer promoters are thought to enhance transcription of the 35S/45S genes (Kuhn and Grummt, 1987; Morgan et al., 1983; Moss, 1983; Paalman et al., 1995). Adjacent to the spacer promoter are enhancer elements. In *Xenopus leavis* and mice, these enhancers have been shown to bind UBF and promote transcription of nearby promoters in cis (Labhart and Reeder, 1984; Pikaard et al., 1990). At the 3’end of the 45S gene, a series of repeats (Sal boxes) mark the transcription termination region (Grummt et al., 1985). Additionally, near the 5’ end of the promoter there is another single termination sequence (T₀)(Gonzalez and Sylvester, 1995; Grozdanov et al., 2003; Grummt et al., 1986; McStay and Reeder, 1986). This termination sequence is important for regulating the activity status of the adjacent repeat, which is discussed in more detail in section 1.4.

### 1.1.1 The human ribosomal genes

The human 45S rDNA genes are localised on NORs on the human chromosomes 13, 14, 15, 21 and 22. They are transcribed in a telomere to centromere orientation (Henderson et al., 1972; Worton et al., 1988). An rDNA repeat is ~45kb and consists of the pre-RNA (13-14kb) separated by the intergenic spacer [Figure 1.2](Gonzalez and Sylvester, 1995; Maden et al., 1987).

The human intergenic spacer has Alu elements scattered throughout and 2 long repeats (LR). It also contains a cell division cycle protein 27 (cdc27) pseudogene which is not transcribed. In contrast to *Xenopus* and mice, the intergenic spacer of the human rDNA repeat does not have any identifiable enhancer elements and has no spacer promoter.
Figure 1.2: The features of the rDNA repeats. The intergenic spacer has 2 long repeats (LR) and many Alu repeats. There is also a processed cdc27 pseudogene. The terminator sequences are located at the end of the transcribed region and an additional single terminator sequence is located near the promoter. Figure after Gonzalez and Sylvester, 1995.
Terminator repeats are located at the end of the transcribed sequence and a single repeat near the promoter T₀ (Gonzalez and Sylvester, 1995). The sequence of the rDNA repeat does not have any EcoRV restriction sites allowing entire NORs to be released from the rest of the genome (Gonzalez and Sylvester, 1995; Maden et al., 1987). The released NORs were run with pulse-field gel electrophoresis (PFGE) and subsequent southern blots gave estimates of 300-350 copies per haploid genome. The size of the each individual NORs differs greatly ranging from 50kb to >6Mb (mega-base) (Sakai et al., 1995; Schmickel, 1973; Stults et al., 2008).

Due to the repetitive nature of rDNA within NORs it is not possible with current sequencing technologies to obtain a precise description of the rDNA array. Moreover, the entire short arms of the human acrocentrics are emitted from even the most recent draft of the Human Genome (GRCh37- hg19). Nevertheless, molecular combing has revealed that long stretches of the normal canonical repeats are interrupted by rearranged and palindromic rDNA repeats. These noncanonical repeats are thought to be non-functional (Caburet et al., 2005).

The sequences distal and proximal of the NORs on the acrocentric short arms have recently been identified. These sequences are shared among all acrocentrics (Floutsakou et al., 2013; Worton et al., 1988). Proximal to the NOR, sequences are characterised by many segmental duplications from elsewhere in the genome. Towards the telomere the sequence is unique to the acrocentric chromosomes, which allows simultaneous detection of all NORs. These sequences are imbedded in the heterochromatin that surrounds the nucleolus (Floutsakou et al., 2013). The influence of this perinucleolar heterochromatin on the linked rDNA array is discussed below in section 1.8.3.

**Figure 1.3: The nucleolar proteome.** Only about a third of the proteins are linked to rRNA synthesis and processing. A large part of the proteome also consists of uncharacterised proteins. Reprinted by permission from Macmillan Publishers Ltd: Nature [Andersen, J.S., Lam, Y.W., Leung, A.K., Ong, S.E., Lyon, C.E., Lamond, A.I., and Mann, M. (2005). Nucleolar proteome dynamics. Nature 433, 77-83].
1.2 Proteomics of the nucleolus

The nucleolus contains many proteins besides proteins involved in transcription and processing. Mass spectrometry of isolated nucleoli has identified many proteins that are found in nucleoli. In the initial study, ~700 proteins were identified [Figure 1.3](Andersen et al., 2005). To date the nucleolar database contains more than 4500 proteins (Ahmad et al., 2009). It shows that the nucleolus has other functions beyond ribosome biogenesis. Cell cycle proteins, DNA repair and replications factors are found in nucleoli. An example is the phosphatase cdc14, which inactivates the mitotic kinases. During interphase it localises in the nucleolus and it stays associated with the NORs until anaphase when it is released to dephosphorylate the mitotic kinases (Visintin and Amon, 2000).

The proteome is not static, but highly dynamic. Stress conditions like inhibition of transcription can cause proteins to leave the nucleolus, while others are recruited to the nucleolus (Andersen et al., 2005). The subset of proteins that redistributes is specific to the stressor. After adenovirus infection (Lam et al., 2010), senescence (Kar et al., 2011), serum stimulation (Liang et al., 2012) or DNA damage (Boisvert et al., 2010; Boisvert and Lamond, 2010) a unique set of proteins is recruited to or depleted from the nucleolus.

![Figure 1.4: Yeast rDNA transcription can be visualised directly by Miller spreads.](image)

*Figure 1.4: Yeast rDNA transcription can be visualised directly by Miller spreads.* The DNA strand in the middle is coated by transcribing RNA polymerases. The ribosomal RNAs are released from the polymerases. Near the end of the transcribed region, the processing machinery is recruited to the pre-ribosomal RNA. Subsequently these terminal balls have been shown to be the small subunit (SSU) processome containing the U3 small nucleolar RNA (snoRNA)(Dragon et al., 2002; Miller and Beatty, 1969). Picture from French et al, 2003 and reprinted with permission from ASM. Bar ± 1μm.

1.2.1 The basal transcription machinery

The rDNA is heavy transcribed and this can be visualised in Miller spreads (Miller and Beatty, 1969) [Figure 1.4]. The rDNA is coated by many polymerases with the RNA molecules spreading out from them. Near the end of the transcribed region, pre-ribosomal RNA molecules have large particles attached to the end of the RNA molecule, the so-called terminal balls, which consist of the early processing machinery (Dragon et al., 2002).
The rDNA promoter consists of 2 elements; the upstream control element (UCE) and the core element [Figure 1.5]. Upstream binding factor (UBF) binds to both elements and recruits selectivity factor 1 (SL1) to the promoter (Bell et al., 1988). SL1 is a complex comprised of TATA–box binding protein (TBP) and 5 TBP associated factors (TAFs), TAF$_{110}$, TAF$_{63}$, TAF$_{48}$, TAF$_{41}$ and TAF$_{12}$ (Comai et al., 1994; Denissov et al., 2007; Gorski et al., 2007; Zomerdijk et al., 1994). The SL1 complex is highly species-specific (Bell et al., 1989). Hence a mouse rDNA promoter will not be transcribed in a human cell.

The rDNA is transcribed by RNA polymerase I (also called RNA polymerase A (RPA)). The mammalian RNA polymerase I complex consists of 13 subunits, some of which are shared which RNA polymerase II and III. The RNA polymerase I specific subunits are RPA$_{195}$, RPA$_{135}$, RPA$_{43}$, Polymerase associated factor 53 (Paf53) and Paf49 (Russell and Zomerdijk, 2005).

The majority of the polymerases are not able to initiate transcription. Only the fraction of the polymerases that is associated with RRN3, is able to be recruited to a promoter bound by UBF and SL1. The polymerase associated which RRN3, UBF and SL1, forms the pre-initiation complex (PIC). After initiation the RNA polymerase I leaves UBF, SL1 and RRN3 at the promoter (Aprikian et al., 2001; Bier et al., 2004; Russell and Zomerdijk, 2005). The polymerase is recruited to the promoter by interacting directly with UBF or indirectly via RRN3 (Hanada et al., 1996). RRN3 interacts with RPA$_{43}$ and the SL1 subunits, TAF$_{110}$ and TAF$_{68}$ (Stepanchick et al., 2013; Yuan et al., 2002). Recently RRN3 has also been shown to have DNA binding activity (Stepanchick et al., 2013). In the PIC, especially RRN3 and UBF are highly regulated by growth and stress signals (Bodem et al., 2000; Buttgereit et al., 1985; O'Mahony et al., 1992b). This is discussed in more detail in section 1.5.2. Transcription is terminated at the terminator elements which are bound by transcription terminator factor 1 (TTF1)(Evers and Grummt, 1995; Evers et al., 1995).

**Figure 1.5:** A model of the pre-initiation complex of a RNA polymerase I. UBF binds to the upstream control element (UCE) and the core promoter. Selectivity factor 1 (SL1) binds to UBF. This recruits RRN3, which interacts with both the polymerase and SL1 and also interacts with the DNA directly (Russell and Zomerdijk, 2005; Stepanchick et al., 2013). Picture adapted from Stepanchick et al, 2013 and reprinted with permission from ASBMB.
Such high levels of transcription give a lot of torsional strain. This strain is released by DNA topoisomerase I activity, which is highly enriched in nucleoli (Christensen et al., 2004; Leppard and Champoux, 2005; Rose et al., 1988). Especially during elongation, inhibition of topoisomerase I stalls the RNA polymerase I (Zhang et al., 1988).

UBF binding is not restricted to the rDNA promoter, but is bound across the rDNA repeat (O’Sullivan et al., 2002). Therefore UBF is expected to have other roles beyond initiating transcription. In an effort to better understand the role of UBF in organising rDNA chromatin, arrays of a heterologous UBF binding sequence, Xenopus enhancer (Xen) elements, were integrated in non-acrocentric chromosomes. These integrated arrays which were of the order of 1 Mb in length formed novel secondary constrictions during metaphase. The basal transcription machinery was recruited to these arrays despite that the fact they are transcriptionally silent. These proteins remained associated with the novel secondary constriction throughout mitosis. These arrays did not recruit any processing machinery and did not form nucleoli and were hence referred to as pseudoNORs (Mais et al., 2005).

The formation of the secondary constriction is dependent on the binding of UBF. Upon knock-down of UBF, NORs in interphase cells are silenced and dissociate from nucleoli (Grob et al., 2014). This shows that UBF is important for maintaining open chromatin at the NORs.

The pseudoNORs do not form fully functional nucleoli. To form a proper nucleolus, transcription is needed. NeoNORs have the mouse rDNA gene flanked by Xen elements. When integrated in a non-acrocentric, these arrays form nucleoli either as a stand-alone nucleolus or part of a bigger nucleolus. All proteins that are normally found in the human nucleolus also localise to the NeoNOR nucleolus. They are transcribed and processed and the mouse rRNA is incorporated in ribosomes (Grob et al., 2014).

### 1.2.2 Processing of ribosomal RNA transcripts

Human rDNA is transcribed as a 47S precursor (Liau and Perry, 1969; Penman et al., 1966; Scherrer et al., 1963; Weinberg et al., 1967). Quickly after completion of transcription, the 5’ external transcribed spacer (ETS) is cleaved at the primary cleavage site and in the 3’ETS generating the 45S [Figure 1.6](Mullineux and Lafontaine, 2012).

The 5’ETS is further cleaved to generate the 41S particle. This is subsequently processed in the internal transcribed spacer (ITS1) downstream of the 18S separating it from the 5.8S and the 28S. The resulting 20S is processed into the mature 18S. The 32S is cleaved into the 28S and the 7S. The latter is trimmed to form the mature 5.8S. Most of these processing steps take place in the nucleolus, while a few take place in the cytoplasm (Maden et al., 1972; Mullineux and Lafontaine, 2012). The cleavages are a combination of endo- and exo-
nucleolytic cleavages. The ETSs and ITSs are quickly degraded after cleavage by proteins like the exosome (Lebreton et al., 2008).

The pre-rRNA is packaged in large ribonucleoprotein (RNP) complexes consisting of processing factors and ribosomal proteins. Ribosomal proteins are assembled on the rRNA in throughout all processing steps (Granneman and Baserga, 2004). After the processing the 18S is packaged into the 40S ribosomal subunit, while the 28S, 5.8S together with the imported 5S is packaged into the 60S ribosomal subunit (Granneman and Baserga, 2004).

In yeast, the U3 small nucleolar RNA (snoRNA) regulates the cleavages in the 5’ETS which is important for 18S processing. The U3 snoRNA is part of a big RNP complex called the small subunit (SSU) processome, which contains many processing factors including the UTP proteins (Dragon et al., 2002; Grandi et al., 2002). When the complex is disrupted, the terminal balls in the Miller spreads are lost (Dragon et al., 2002).

Some of the UTPs also have a role in transcription. The transcription UTPs (t-UTPs) form a separate subcomplex in yeast, which is recruited to the rDNA even in the absence of transcription (Gallagher et al., 2004).

In mammalian cells transcription factors, such as UBF are recruited to the rDNA promoter on plasmids independent transcription, while the recruitment of processing factors is dependent on transcription (Kopp et al., 2007).

Apart from the cleavages the pre-rRNA is also chemically modified by pseudouridylation and 2’-O-methylation. The modifications are guided by box H/ACA and box C/D snoRNAs respectively. The box H/ACA snoRNAs are in complex with Nhp2p, Nop10, Gar1p and Dyskerin, which catalyses the isomerisation of uridine residues (Granneman and Baserga, 2004). The box C/D snoRNAs are part of a complex containing Nop56, Nop58, 15.5K and fibrillarlin, a RNA methylase (Granneman and Baserga, 2004; Watkins et al., 2000). This complex associates with the Treacher Collins syndrome protein, Treacle (TCOF1). Treacle interacts with both UBF and Nop56, linking the transcription machinery to the modification of the pre-rRNA (Gonzales et al., 2005; Valdez et al., 2004). The correct 2’-O-methylation is important for cap independent internal ribosomal entry site (IRES) mediated translation (Basu et al., 2011).
Figure 1.6: A schematic overview of the processing of ribosomal RNA in human cells. Based on a figure from Mullineux and Lafontaine, 2012.

1.3 Internal structure within the nucleolus

The improved resolution offered by electron microscopy (EM) provides a more detailed picture of the internal structure of the mammalian nucleolus. Under EM, 3 distinct compartments were identified in the mammalian nucleolus; the fibrillar component (FC), the dense fibrillar component (DFC) and the granular component (GC) (Bernhard and Granboulan, 1963; Swift, 1963) [Figure 1.7]. The FC’s are the clear areas surrounded by darker DFCs. The FC’s contain proteins involved in transcription like UBF, DNA topoisomerase I and RNA polymerase I (Casafont et al., 2006; Christensen et al., 2002; Raska et al., 1989; Roussel et al., 1993). The DFC’s contain early processing factors like the U3 snoRNA, which is involved in the first processing steps (Puvion-Dutilleul et al., 1997; Raska et al., 1989). Late processing and ribosome assembly factors are found in the GC (Sirri et al., 2008).

The rDNA localises to the FC, while rRNA is found in the DFC and GC (Koberna et al., 2002; Shaw et al., 1995; Thiry and Thiry-Blaise, 1991), leading to a debate where transcription takes place. But the consensus is that transcription takes place at the interface between the FC and DFC (Cmarko et al., 2000; Hozak et al., 1994; Raska et al., 1989; Shaw et al., 1995).
et al., 1995). Modification and cleavage of the pre-rRNA start in the DFC and continues in
the GC (Biggiogera et al., 1989; Puvion-Dutilleul et al., 1997).

**Figure 1.7: The internal structure of the nucleolus.** Cells stained for the UBF (FC- red) and Nop52
(GC- green) show a different nucleolar staining. FC and DFC proteins have a punctate nucleolar
pattern, while GC proteins stain the entire nucleolus. The internal structure can be seen in detail under
EM. Multiple FC’s are surrounded by the darker DFC’s which reside in the GC. Transcription takes
place at the boundary of the FC/DFC (Cmarko et al., 2000; Raska et al., 1989). Early processing takes
place in the DFC (Puvion-Dutilleul et al., 1997; Raska et al., 1989), while late processing and assembly
into mature ribosomes takes place in the GC. Left cell staining: M.van Sluis, right EM picture adapted
with permission from Hozak et al, 1994.

1.3.1 **Structure is dependent on ongoing transcription**

The internal structure of the nucleolus is dependent on ongoing transcription. Low dose
ActinomycinD (ActD) specifically inhibits RNA polymerase I transcription (Perry and
Kelley, 1970; Reich et al., 1961). Upon transcriptional inhibition, the nucleolus undergoes
several structural changes. The FC’s condense into larger darker foci [Figure 1.8]. By 30
min the segregation of the nucleoli is almost complete. One hour after Actinomycin D the
FC/DFC compartment is segregated to the nucleolar periphery. (Goldblatt and Sullivan,
1970).

Not all GC proteins are retained in the interior of the nucleolus. Many proteins like NPM are
released into the nucleoplasm, while others like p14ARF stay in the GC. The caps contain
many proteins form the FC and DFC, but also recruit proteins from Cajal bodies and
Promyelocytic leukemia (PML) bodies from the nucleoplasm. The function of these proteins
that are recruited is unknown. The reorganisation of the nucleolus is an energy-dependent
and therefore possibly a regulated process (Shav-Tal et al., 2005).
Figure 1.8: Morphological changes in nucleoli upon Actinomycin D (ActD) treatment. EM pictures are from rat livers injected with 1.25 µg ActD /g body weight. The rats were sacrificed at various times (Goldblatt and Sullivan, 1970). When transcription is inhibited, the FC’s condense into larger darker and larger foci. By 30 min the segregation of the nucleoli is almost complete. 60 Minutes ActD the FC/DFC compartment is segregated to the nucleolar periphery. The GC however does not segregate and remain in the nucleolar interior although many GC proteins are released into the nucleoplasm (Shav-Tal et al., 2005). Reprinted from Goldblatt, P.J., and Sullivan, R.J. (1970), Sequential Morphological Alterations in Hepatic Cell Nucleoli Induced by Varying Doses of Actinomycin D, Cancer Research 30, 1349-1356, with permission from AACR.

1.3.2 Nucleolar structure through the cell cycle

The nucleolar structure changes as the cell progresses through the cell cycle [Figure 1.9]. Upon entry into mitosis, the human nucleolus dissolves and transcription is inhibited (Gebrane-Younes et al., 1997). This reorganisation of the nucleolus is governed by the cyclin-dependent kinases (cdks) (Sirri et al., 2002; Sirri et al., 2000). The targets of the cdks are discussed in more detail in 1.5.1.

Although transcription is inhibited, the transcription machinery stays associated with the NORs. UBF, DNA topoisomerase I and SL1 remained at the NORs while proteins like fibrillarin and nucleophosmin dissociate from the nucleolus (Angelier et al., 2005; Christensen et al., 2002; Jordan et al., 1996; Roussel et al., 1996; Roussel et al., 1993; Scheer and Rose, 1984; Suja et al., 1997; Weisenberger and Scheer, 1995). Live cell imaging has revealed that some RNA polymerase I subunits leave the NORs from prometaphase until telophase. The disassociation of the DFC and GC proteins coincide with the breakdown of the nuclear membrane. The released processing machinery stays associated in the perichromosomal layer during metaphase and anaphase. In telophase prenucleolar bodies
(PNBs) are formed containing the processing factors, before these proteins are recruited to the new formed nucleoli (Angelier et al., 2005; Leung et al., 2004). The assembly of the nucleoli in the daughter cells starts with the reassociation of RNA polymerase I to NORs (Leung et al., 2004). Not all processing factors reassociate with the nucleoli at the same time. Early processing factors like fibrillarin leave the PNBs earlier, while late processing factors like Nop52 and NPM remain in the PNBs and translocate to the newly formed nucleoli later (Angelier et al., 2005; Leung et al., 2004).

Initially a nucleolus forms around each NOR, but during early G1 multiple nucleoli fuse to form bigger nucleoli containing multiple NORs (Gautier et al., 1994; Gautier et al., 1992; Jimenez-Garcia et al., 1994).

The rDNA remains undercondensed in metaphase, which is visible as the secondary constriction. EM pictures of mitotic NORs show the rDNA as a fibrillar compartment surrounding a central axis (Heliot et al., 1997; Suja et al., 1997).

![Figure 1.9: Nucleolar morphology through mitosis.](image)

Figure 1.9: Nucleolar morphology through mitosis. In prophase, chromosomes start to condense, while transcription ceases. Proteins like UBF (green) stay associated with the active NORs while processing factors like Nop52 (red) remain associated with remaining rRNA. As cells approach metaphase the processing factors are located in the perichromosomal compartment. In telophase the pre-nucleolar bodies (PNBs) form, which are stained with Nop52. Around this time transcription resumes and the processing factors are recruited to the sites of transcription. Pictures kindly provided by Chelly van Vuuren.

1.4 Not all repeats are actively transcribed.

The rDNA arrays contain many repeats. Not all of these repeats are actively transcribed and about half of the repeats are silenced (Dammann et al., 1993; Dammann et al., 1995). This is due to the repeats being in excess. In Robertsonian translocations, the long arms of 2 acrocentric chromosomes are fused at the centromere resulting in the loss of both short arms.
These translocations have a prevalence of 1:1000 births, but there seems to be no associated phenotype (Hamerton et al., 1972). The loss of 2 NORs (1/5th of the repeats) does not have major functional consequences. This is further supported by observations in yeast. Normal yeast cells have 150 copies, but cells can survive with as little as 20 copies (Ide et al., 2010). To maintain similar levels of rDNA transcription, yeast cells can load more polymerases on fewer repeats (French et al., 2003). Similar mechanisms compensate for the loss of active repeats upon UBF knockdown (Sanij et al., 2008).

Epigenetics play an important role in controlling the activity status of individual repeats (Lawrence and Pikaard, 2004). The rDNA is associated with various histone modifications, which are mainly located at the promoter and in the intergenic spacer (Zentner et al., 2011). In mouse, the promoter proximal T0 termination site has been shown to play a central role in controlling the activity status of repeats. TTF-1, when bound to T0, can recruit chromatin remodelling complexes (Langst et al., 1998; Langst et al., 1997). TTF-1 can activate transcription by recruitment of the histone methyltransferase G9a and Cockayne syndrome protein B (CSB) to the rDNA promoter (Yuan et al., 2007). However TTF-1 is also involved in silencing of repeats by recruiting the Nucleolar repressor complex (NoRC)(Nemeth et al., 2004; Strohner et al., 2004). The complex consist of TTF-1 interacting protein 5 (TIP5) and sucrose nonfermenting 2 homolog (SNF2h), the latter being a chromatin remodeler (Strohner et al., 2001).

The silent repeats are characterised by hypoacetylated histone H4 and hypermethylation of histone H3 lysine 9 (H3K9). To maintain this chromatin state, NoRC facilitates recruitment of the DNA methyltransferases Dnmt1 and Dnmt3b and Histone Deacetylase 1 (HDAC1) to the promoter (Santoro and Grummt, 2005; Santoro et al., 2002; Zhou and Grummt, 2005; Zhou et al., 2002). Especially methylation of CpG -133 is reported to correlate with transcriptional activity. Methylation prevents binding of UBF to the promoter (Santoro and Grummt, 2001). After replication NoRC association with the silent repeats ensures that the epigenetic marks are propogated through mitosis (Li et al., 2005; Santoro et al., 2010).

The mouse rDNA repeat has a spacer promoter 2kb upstream of the transcription start site. Transcripts from this spacer promoter are processed into 150-300 nucleotide (nt) promoter RNAs (pRNAs) with a hairpin structure (Mayer et al., 2008; Mayer et al., 2006). This pRNA is bound by Tip5 with induces a conformational change, likely allowing the binding of additional proteins such as Dnmt3b and Poly(ADP-ribose) polymerase (PARP1) (Guetg et al., 2012; Schmitz et al., 2010). The human promoter however does not have a spacer promoter and therefore it is unclear whether similar mechanism silence human rDNA.
How the silent repeats are distributed in the NOR and amongst the NORs is unknown. However not all NORs are active at any given time in the cell cycle. Active NORs can be identified on metaphase chromosomes by silver staining and a secondary constriction. The transcription machinery remains associated with active NORs through mitosis, while silent NORs are devoid of the transcription machinery (Roussel et al., 1996). Transcriptionally silent NORs do not have a secondary constriction and are not stained with silver. Depending on the cell type the number of silent NORs can vary (Hubbell, 1985).

Active NORs associate with the nucleolus, while silent NORs can dissociate from the nucleolus (Sullivan et al., 2001). In plant hybrids it has been shown that some NORs are dominant and therefore always active (McStay, 2006). Recessive silent NORs in plants are characterised by DNA methylation, hypermethylation on H3 and hypoacetylation on H4 (Lawrence et al., 2004). However the mechanisms involved in silencing entire NORs are less clear. It is speculated that a sequence outside of the NORs might function as a locus control sequence, however to date no such sequence has been identified (Floutsakou et al., 2013).

1.5 Regulation of transcription

Ribosomal RNA transcription is influenced by growth signals, the cell cycle and cellular stresses. There are two ways of regulating transcription quickly, changing the number of active repeats or the rate of transcription per gene. UBF seems to determine the number of active genes that can be transcribed. Upon UBF knockdown the number of inactive repeats increases (Sanij et al., 2008). However when UBF levels are reduced 4.5-fold, the cells can still keep growing despite the fact that entire NORs are silenced (Grob et al., 2014). Presumably the rate of transcription of the remaining active genes is increased to maintain the same rate of rRNA synthesis of fewer repeats, similar to what has been shown in yeast (French et al., 2003).

Growth factors and stress signals both seem to influence the formation of the pre-initiation complex. Many pathways converge on RRN3 and UBF. The casein kinase II (CK II) is a constitutive kinase, which controls a large variety of processes among which transcription of the ribosomal genes. The consensus sequence is [S/T]-X(2)-[D/E] which is present in many nucleolar proteins (Prosite: PD000006)(Montenarh, 2010).

When CK II is inhibited by 5,6-Dichloro-β-D-Ribofuranosylbenzimidazole (DRB), the nucleolar structure becomes enlarged and fragmented. Individual transcription units can be seen as “beads-on-a-string” (Haaf and Ward, 1996; Scheer et al., 1984). The effects of DRB are not restricted to the nucleolus. DRB also inhibits RNA polymerase II transcription making it difficult to determine whether the collapse of the nucleolar structure is a direct consequence of CK II inhibition (Montenarh, 2010).
A feature of many nucleolar proteins is acidic stretches which are present in for example Nucleolin, UBF, Treacle and Nop140. These stretches contain many potential CK II consensus sequences. CK II has been shown to phosphorlylate RRN3 on Ser170 and Ser172, which is needed for efficient transcription. RRN3 bind the polymerase dephosphorylated and the phosphorylation releases RRN3 from the polymerase to facilitate promote escape and elongation (Bierhoff et al., 2008; Fath et al., 2001). Free RRN3 is subsequently dephosphorylated by FCP1 before it can interact with RNA polymerase I again for re-initiation (Bierhoff et al., 2008). In addition, re-initiation also requires phosphorylation of CK II on the C-terminal tail of UBF (Voit et al., 1992). The phosphorylation enhances the stability of interaction between UBF and SL1 (Kihm et al., 1998; Lin et al., 2006; O'Mahony et al., 1992a).

1.5.1 Regulation of transcription through the cell cycle

Transcription is not constant through the cell cycle. In G1 transcription levels are low. During late G1 to S-phase transcription levels increase reaching maximum during G2. At the beginning of mitosis, transcription is inhibited and it remains silent until late telophase when transcription is re-activated (Gebrane-Younes et al., 1997; Klein and Grummt, 1999) [Figure 1.10].

The changes in levels of rRNA synthesis are mainly determined by sequential phosphorylations on UBF. In G1, cyclin D-cdk4 and cyclin E-cdk2 phosphorylate UBF at Ser484, which increase transcription (Voit et al., 1999). Similarly in late G1 to early S-phase, Ser388 on UBF gets phosphorylated. This phosphorylation enhances the interaction between UBF and RNA polymerase I (Voit and Grummt, 2001). Apart from UBF, RRN3 is also targeted by cyclinE-cdk2 at Ser44, which also increases transcription (Mayer et al., 2004).

Not all phosphorylations have a positive influence on transcription. During mitosis TAF110 gets phosphorylated on Thr852 by cyclin B-cdk1, which silences transcription (Heix et al., 1998; Kuhn et al., 1998).

These phosphorylations are likely not the entire mechanism. In a large proteomics study, many cell cycle dependent phosphorylations sites on nucleolar proteins were detected (Dephoure et al., 2008; Olsen et al., 2010)[Table 1.1]. Phosphorylation of RRN3 Ser44 and UBF Ser484 were found to be enriched in G2-M cells verifying previous studies (Mayer et al., 2004; Voit et al., 1999). However additional phosphorylation sites on UBF (Ser638) and on RRN3 (Ser640) with a consensus cdk-site were detected. Apart from UBF and RRN3, many other proteins involved in rDNA transcription and processing were detected. The sites that the 2 studies have in common are listed in Table 1.1. TTF-1 has previously been found...
to be phosphorylated by cyclinB-cdc2 in G2/M phase (Sirri et al., 1999). The sites were unknown, but the proteomics studies provide a list of putative cdk-phosphorylation sites.

**Figure 1.10:** Phosphorylations of the basal transcription machinery by the cell dependent kinases. rRNA synthesis is low during G1, but increases in late G1 to S-phase reaching a maximum in G2. During mitosis transcription is silenced (Klein and Grummt, 1999). UBF is phosphorylated on Ser484 and Ser388 by Cdk4-cyclin D and Cdk2-cyclin E/A through G1 and S phase to activate transcription (Voit and Grummt, 2001; Voit et al., 1999). Transcription is further stimulated by the phosphorylation of Cdk2-cyclinE on RRN3 (Mayer et al., 2004). At the beginning of mitosis, UBF and RRN3 are dephosphorylated, while TAF,110 is inactivated by phosphorylation (Dephoure et al., 2008; Heix et al., 1998; Kuhn et al., 1998).

Apart from protein involved in rDNA transcription, processing factors are also phosphorylated [Table 1.1]. Nucleolin, Nucleophosmin (NPM) and UTP14A are all involved in processing of the pre-rRNA (Ginisty et al., 1998; Savkur and Olson, 1998). Nucleolin aids the SSU for efficient cleavage of the primary cleavage site (Ginisty et al., 1998). Nucleophosmin on the other hand is the endoribonuclease with catalysis the cleavage in the ITS2 in the 32S, generating the 7S and 28S (Savkur and Olson, 1998).

However during mitosis, NPM is phosphorylated by cyclinB-cdc2, which inhibits its RNA binding activity preventing cleavage of ITS2 (Okuwaki et al., 2002; Savkur and Olson, 1998). Phospho-proteomics studies have identified other potential sites that might be regulated by cdks [Table 1.1].
### Name | Cell cycle regulated phosphorylations found in both studies
---|---
Fibrillarin | 124
RRN3 | 44*, 640
Treacle | 107, 153, 156, 249, 310, 381, 503, 581, 582, 583, 1190
TTF-1 | 64, 65, 231, 481, 487, 872
UBF | 201*, 412, 484², 495, 638
UTP14A | 29, 52, 77, 405, 445, 448, 451, 453, 569
NPM | 67, 70, 125, 137, 139, 205, 208, 214, 225, 231, 250
Nucleolin | 67, 69, 76, 121, 145, 153

* just in Dephoure et al, 2008, known cdk site; Mayer et al, 2004

¹ known ERK site; Stefanosky et al, 2001

² known cdk site; Voit et al, 1999

*Table 1.1: Cell cycle regulated phosphorylations on nucleolar proteins. (Dephoure et al., 2008; Olsen et al., 2010)*

Apart from phosphorylation, there also is regulation by acetylation. Cells overexpressing histone acetylases or treated with Trichostatin A (TSA) have increased levels of rDNA transcription (Hirschler-Laszkiewicz et al., 2001). This is not just due to histone acetylation, but the histone acetylase P300/CBP-associated factor (P/CAF) can also acetylate TAF68. This increases the affinity of SL1 to DNA, thereby stimulating transcription (Muth et al., 2001). Similarly, UBF is also acetylated in S-phase to stimulate the interaction with RNA polymerase I (Meraner et al., 2006). HDAC1 has been shown to deacetelyated TAF68 and UBF (Meraner et al., 2006; Muth et al., 2001). Another deacetylase that is important for cell cycle dependent regulation is sirtuin SIRT7, which localises to nucleoli and remain associated with mitotic NORs. The activity is needed for resumption of transcription in late telophase (Grob et al., 2009).

#### 1.5.2 Growth factors and stress signals

Serum starved or confluent cells have low levels of rRNA synthesis while sub-confluent cells grown in the presence of growth factors have high levels of transcription. Growth signalling increases rDNA transcription while many stress signals decrease transcription. Upon stimulation with growth factors, protein levels do not change, but rather the phosphorylation of UBF and RRN3 increases [Figure 1.11] (Buttgereit et al., 1985; O'Mahony et al., 1992b).

Growth factors signal via the ERK (Extracellular signal-regulated kinases) pathway to stimulate entry into the cell cycle and increase rDNA transcription (Grummt, 2003). UBF gets phosphorylated on Thr117 and Thr201 upon stimulation of the ERK pathway with the growth hormone epidermal growth factor (EGF). Phosphorylated UBF bind DNA less
tightly, which is thought to aid in promoter escape (Stefanovsky et al., 2001). ERK2 and RSK have also been shown to phosphorylate RRN3 on Ser633 and Ser649, which disrupt the complex with RNA polymerase I (Zhao et al., 2003). Presumably this also aids in promoter escape.

Apart from ERK signalling, mammalian Target of rapamycin (mTOR) signalling is also a determinant of RNA polymerase I activity. The mTOR pathway responds to nutrient availability (Schmelzle and Hall, 2000). Most of the signalling is via RRN3, which affects the initiation efficiency of RNA polymerase I, but does not alter the number of active genes (Claypool et al., 2004). mTOR also causes hyper-phosphorylation on Ser199 on RRN3, which activates RRN3 (Mayer et al., 2004). The downstream target S6K1 also phosphorylates the C-terminal tail of UBF, which stimulates the interaction with RNA polymerase I (Hannan et al., 2003). Akt also promotes cell growth signalling via the mTOR pathway. But it also affects rDNA transcription directly by an unknown mechanism (Chan et al., 2011).

**Figure 1.11**: Growth factors upregulate rDNA transcription, while stress inhibits transcription. All pathways seem to on UBF and RRN3 (Drygin et al., 2010; Mayer et al., 2005; Schmelzle and Hall, 2000).
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Loss of signalling from growth signals inhibits RNA polymerase I transcription. Upon hypoxia or nutrient depletion signalling via mTOR ceases which leads to the loss of the stimulatory phosphorylations on UBF and RRN3. In addition stresses signalling through JNK can also directly inhibit transcription. JNK2 has been shown to phosphorylate RRN3 on Thr200, which impairs the interaction with RNA polymerase I (Mayer et al., 2005).

In addition, the large subunit of RNA polymerase I RPA195 can be ubiquitinated. Upon cold-shock transient mono-ubiquitination is thought to stabilise the polymerase when it encounters secondary structures that are harder to resolve at lower temperatures (Richardson et al., 2012). Similarly upon zinc deficiency, RNA polymerase I is ubiquitinated and degraded (Lee et al., 2013).

1.5.3 Tumour suppressors and oncogenes

Tumour suppressors like retinoblastoma protein (Rb) are important for cell cycle progression. In G1, Rb is hypophosphorylated and inhibits DNA replication. In late G1, Rb gets phosphorylated by cdk4-cyclinD which inactivates the protein. The phosphorylation of Rb lifts the inhibition on the transcription factors E2F, which enables the cell to enter S-phase [Figure 1.12] (Sherr and Weber, 2000). In many cancers, Rb is dysfunctional leading to uncontrolled proliferation (Weinberg, 1995).

Cell cycle progression is partially restricted by rDNA transcription levels. Rb has been shown to interact with UBF (Cavanaugh et al., 1995). When cells reach confluency, Rb accumulates in the nucleolus, where dephosphorylated Rb disrupts the interaction between UBF and SL1 (Hannan et al., 2000a; Hannan et al., 2000b; Voit et al., 1997). The related pocket protein p130 can also bind UBF and inhibit rRNA synthesis (Ciarmatori et al., 2001; Hannan et al., 2000a). By interacting with UBF, Rb is thought to recruit an HDAC, while in the absence of Rb, UBF is thought to be bound by the acetyltransferase CBP. The balance between these two proteins regulates the acetylation-deacetylation of the RNA polymerase I transcription machinery and possibly the surrounding chromatin (Pelletier et al., 2000).

The tumour suppressors p14ARF (alternative reading frame) and p53 also downregulate rRNA synthesis (Ayrault et al., 2006; Budde and Grummt, 1999; Zhai and Comai, 2000). p14ARF binds UBF and decreases the cell cycle dependent phosphorylations on Ser388 and Ser484 (Ayrault et al., 2006), while p53 disrupts interaction with SL-1 and UBF (Zhai and Comai, 2000).

Oncogenes also regulate ribosome biogenesis. c-Myc coordinates cell proliferation, differentiation, apoptosis and self-renewal. It regulates transcription of all three RNA polymerases, upregulating synthesis of ribosomal RNAs and ribosomal proteins (van Riggelen et al., 2010). Furthermore c-Myc also upregulates expression of essential RNA
polymerase I factors, like UBF, RRN3 and fibrillarin (Poortinga et al., 2004; Poortinga et al., 2011; Schlosser et al., 2003).

c-Myc binds to the E-boxes in the rDNA repeat and stimulates rRNA synthesis by increasing histone acetylation (Arabi et al., 2005; Grandori et al., 2005). It is known that c-Myc can recruit TRRAP, which associates with the histone acetyltransferases P/CAF and Tat interactive protein 60 kDa (Tip60) (Arabi et al., 2005; Frank et al., 2003; McMahon et al., 2000). Tip60 has been reported to be involved in rDNA transcription by interacting with and acetylating UBF (Halkidou et al., 2004).

**Figure 1.12:** The Rb-ARF-p53 pathways are all connected. Mitogenic signals stimulate Cdk4-cyclin D activity to phosphorylate Rb inactivating it at mid to late G1. The phosphorylation of Rb lifts the inhibition on the transcription factors E2F, which enables the cell to enter S-phase. The entry into S-phase is accelerated by Myc. Aberrant expression of either the E2Fs or Myc induces the tumour suppressor p14\textsuperscript{ARF}, which inhibits HMD2, the E3 ubiquitin ligase that degrades p53. Reprinted from Sherr, C.J., and Weber, J.D. (2000). The ARF/p53 pathway. Current opinion in genetics & development 10, 94-99, with permission from Elsevier.

All these proteins are connected to each other [Figure 1.12]. Upon phosphorylation of Rb, the E2F transcription factors together with Myc promote entry into S-phase. However the inappropriate expression of either protein can induce the expression of p14\textsuperscript{ARF}, which inhibits the E3 ubiquitin ligase Human double minute 2 homolog (HMD2). HMD2 targets p53 for degradation under normal circumstances, however upon expression of p14\textsuperscript{ARF} or after DNA damage, HDM2 is inhibited which stabilises p53 and induces a growth arrest or apoptosis (Sherr and Weber, 2000).
1.6 The nucleolus of cancer cells

It has been known for a long time that cancer cells have a larger and more amorphous nucleoli compared to the surrounding non-cancerous tissue (Pianese, 1896). The size of silver stained nucleoli correlates with the proliferation rate of cancer cells (Derenzini et al., 2000).

Cancer cells often have inactivated tumour suppressors and overexpressed oncogene to sustain proliferation (Hanahan and Weinberg, 2011). This high proliferation demands a high level of rDNA transcription. Oncogenes like c-Myc and tumour repressors like Rb and p53 both regulate rDNA transcription (section 1.5.3). Cancer cells with mutated Rb or p53 have larger nucleoli and transcription is increased (Bywater et al., 2012; Trere et al., 2004). Cancer cells are also characterised by genome instability and the rDNA in cancer cells has been shown to be unstable (Stults et al., 2009).

It is unknown whether the changes in the nucleolus and the rDNA can drive carcinogenesis or whether they are a consequence of cancer. However it is known that c-Myc driven tumours can be suppressed when combined with haplo-insufficiency for a ribosomal protein (Barna et al., 2008). This shows that cancer cells do need the high levels of rDNA transcription to sustain the proliferation rate. These finding have led to the development of potential anti-cancer drug that directly target pol I transcription. The drug CX-5461 prevents the binding of SL1 to the rDNA. Cancer cells treated with CX-5461 die via autophagy, while normal cells tolerate a transient inhibition of rRNA transcription (Drygin et al., 2011). Especially c-Myc driven leukaemia cells with an intact p53 pathway seem highly sensitive to inhibition of rDNA transcription (Bywater et al., 2012).

Apart from directly inhibiting rRNA synthesis, inhibition of the growth factor stimulated pathway can also suppress tumour growth. An example of this is the inhibition of Akt, which decreases rDNA transcription leading to increased levels of apoptosis (Devlin et al., 2013).

Deregulation of ribosome biogenesis can cause cancer. Diamond-Blackfan anaemia, X-linked dyskeratosis congenital and Shwachman-Diamond syndrome all have defects in ribosome biogenesis. These syndromes have cancer susceptibility in common indicating that the deregulation of ribosome biogenesis can be causative (Montanaro et al., 2008). Whether deregulation of ribosome biogenesis driven carcinogenesis is more common or limited to these rare syndromes is unknown. However under normal conditions, p53 expression represses fibrillarin expression. Overexpression of fibrillarin leads to aberrant RNA methylation pattern which alter the translation profile. Proteins like c-Myc will be preferentially translated potentially driving oncogenesis (Marcel et al., 2013).
1.6.1 Does p53 stabilisation depend on nucleolar disruption?

The relation of p53 with the nucleolus is more intricate. One model proposes that the export of p53 is dependent on a functional nucleolus (Boulon et al., 2010; Rubbi and Milner, 2003). All stresses that stabilise p53 also disrupt the nucleolus. Irradiation of the nucleus with ultraviolet radiation (UV) does not stabilise p53 unless a nucleolus is hit (Rubbi and Milner, 2003). Under normal conditions the p53-HMD2 complex interacts with ribosomal proteins (Lindstrom et al., 2007), ‘catching a ride’ on the ribosome to the cytoplasm where p53 is degraded. Disruption of the nucleolus prevents export of p53 and thus its degradation (Rubbi and Milner, 2003). In support of this, inhibition of the proteasome allows p53 to accumulate in the nucleolus (Kruger and Scheer, 2010).

However release of nucleolar proteins like NPM can also inhibit HDM2 providing an additional signal to stabilise p53 (Kurki et al., 2004). And as a consequence of the stabilisation of p53 inhibits rDNA transcription, creating an imbalance between ribosomal proteins and rRNA.

1.7 DNA double strand breaks

High levels of transcription cause a lot of topological stress which can generate DNA double strand breaks (DSBs). Inhibition of the topoisomerases has been shown to induce DSBs in the rDNA and other highly transcribed genes (Govoni et al., 1994). Also exposure to ionising radiation (IR) or certain chemicals like bleomycin and zeocin can cause DSBs. The damage are sensed by the Mre11-Rad50-Nbs1 (MRN) complex, which activates the phosphoinositide 3-kinase (PI3K) related protein kinase (PIKK) Ataxia-telangiectasia mutated (ATM)[Figure 1.13]. The DNA ends are bound by Ku70/Ku80 heterodimer, which activates DNA dependent protein kinase (DNA-PK). Both kinases together with ATM and Rad3-related protein (ATR) phosphorylate a large variety of target proteins including histone variant H2AX which gets phosphorylated at Ser139 (also referred to as γH2AX). Mediator of DNA damage checkpoint protein 1 (MDC1) binds to γH2AX and recruits the E3 ubiquitin ligases Ring finger protein 8 (RNF8) and RNF168 to the DSB. These enzymes ubiquitinated H2A which recruits Tumor suppressor p53-binding protein 1 (53BP1) and Breast cancer type 1 susceptibility protein (BRCA1)(Jackson, 2002).

The abundance of H2AX is very variable between the different cell types. HeLa cells for example have 2.4% H2AX of the total H2A, while SF268 (glioblastoma cells) have 25% (Rogakou et al., 1998).

Repair of these DSBs can be either by error-prone Non-Homologous End Joining (NHEJ) and error-free repair by Homologous Recombination (HR). In mammalian cells DSBs are preferentially repaired by HR in G2/M phase when a sister chromatid is present to serve as a template. In the rest of the cell cycle NHEJ is preferred (Hoeijmakers, 2001). In HR the ends
are resected and the single stranded DNA is initially coated by RPA, which is replaced by Rad51 to facilitate strand invasion (Jackson, 2002).

To date not much is known about the response in the nucleolus to DSBs, but it is known that in mouse cells after IR, ATM can directly or indirectly inhibit RNA polymerase I transcription by silencing an entire nucleolus (Kruhlak et al., 2007). Another report has shown that in human cells DNA-PK silences RNA polymerase I transcription after IR (Calkins et al., 2013).

Large proteome studies looking at proteins that are differentially phosphorylated after DNA damage have identified several proteins involved in RNA polymerase I transcription and processing. These proteins might hold clues to the mechanism of transcriptional inhibition (Beli et al., 2012; Bennetzen et al., 2010; Bensimon et al., 2010; Choi et al., 2012; Matsuoka et al., 2007; Stokes et al., 2007).

**Figure 1.13:** A schematic overview of the DNA double strand breaks (DSBs) response in human cells. A DSB activates the PIKK kinases ATM and/or DNA-PK, while ATR is activated by single stranded DNA (ssDNA). The kinases phosphorylate the histone variant H2AX on Ser139 in the vicinity of the breaks, which recruits other proteins and signal to the repair machinery. Depending on the stage of the cell cycle, breaks can be repaired by non-homologous end joining (NHEJ) or homologous recombination (HR).
DNA damage induces redistribution of many proteins. Nucleolar proteins like DNA topoisomerase 1, p14\textsuperscript{ARF} and NPM leave the nucleolus, while other proteins like PML and HMD2 are recruited to the nucleolus after DNA damage. Both the sequestering of HMD2 in the nucleolus and the release of p14\textsuperscript{ARF} ensure that p53 is stabilised and activates the G1 cell cycle checkpoint (Tembe and Henderson, 2007). Large proteomics studies have showed that some nucleolar proteins are redistributed among the different cellular compartments after treatment with the DNA topoisomerase inhibitor etoposide (Boisvert et al., 2010). These changes in subcellular localisation are mostly independent of p53 (Boisvert and Lamond, 2010). The DNA sensing proteins ATM, ATR and DNA-PK as well as the Mre11, Rad50 and Ku70/80 are present in isolated nucleoli (Andersen et al., 2005). After UV radiation, many proteins change subnuclear localisation. In contrast, IR does not redistribute proteins (Moore et al., 2011). Stresses like heat shock and acidosis have been shown to induce expression of non-coding RNAs from the intergenic spacer. Each type of stress induces a specific RNA, which sequesters a specific subset of proteins to the nucleolus (Audas et al., 2012).

1.8 The rDNA has roles beyond encoding the ribosomal genes

Apart from encoding ribosomal RNA, it has been suggested that the rDNA has been has other roles as well (Boisvert et al., 2007). The high levels of transcription and repetitive nature make the rDNA vulnerable to inappropriate recombination. The NORs have been thought of as one of the most fragile sites (Kobayashi, 2006). The rDNA is therefore thought of as a sensor of genome instability.

1.8.1 rDNA stability in yeast

Yeast cells have about 150 rDNA copies (Bell et al., 1977; Petes, 1979). However the rDNA arrays can expand or contract, a process which is dependent on replication. Upstream of the 35S sequence is the origin of replication (ARS) (Skryabin et al., 1984). The replication fork moves through the 5S genes in the direction of transcription. However to prevent collisions between the replication machinery and RNA polymerase I transcribing the 35S, a replication fork barrier (RFB) is located at the end of the 35S coding sequence [Figure 1.14](Takeuchi et al., 2003). This RFB is bound by the Fork Blocking 1 (Fob1) protein (Kobayashi, 2003; Kobayashi and Horiuchi, 1996). Blocking of the replication fork creates double strand breaks at the RFB (Weitao et al., 2003), which enables the rDNA array to expand or contract (Kobayashi et al., 1998).

Silent Information Regulator 2 (Sir2) is a NAD-dependent histone deacetylase (Imai et al., 2000). This protein is linked to longevity, which in part is due to its role at the rDNA...
Sir2 induces heterochromatin formation in the intergenic spacer (Fritze et al., 1997). The intergenic spacer also has a non-coding bidirectional E-promoter (Kobayashi et al., 2001).

Cohesin is associated with the intergenic spacer, but not the transcribed region (Johzuka et al., 2006). Sir2 suppresses transcription from the E-promoter, which leads to increased association of cohesin (Kobayashi and Ganley, 2005). Cohesin prevents DSBs at the RFB to be repaired by unequal recombination between sister chromatids (Gottlieb and Esposito, 1989; Kobayashi et al., 2004).

\[\text{Figure 1.14: The rDNA repeat structure of } S. \text{ cerevisiae.} \]

The rDNA repeat has the 35S transcribed by RNA polymerase I and the 5S transcribed by RNA polymerase III. Each repeat has a replication fork barrier (RFB) and a replication origin (ARS). The RFB sequence is bound the Fob1 protein which creates a DSB. When the non-coding promoter (E-pro) is silenced by Sir2, cohesion keeps that sister chromatids together leading to equal sister chromatid recombination (ESCR). The number of rDNA repeats therefore remains the same. If the promoter is transcribed, cohesion is lost leading to unequal sister chromatid recombination (USCR) and the copy number changes. From Kobayashi, T., and Ganley, A.R.D. (2005). Recombination Regulation by Transcription-Induced Cohesin Dissociation in rDNA Repeats. Science 309, 1581-1584. Reprinted with permission from AAAS.
A smaller copy number of rDNA repeats is still sufficient for rRNA transcription due to larger polymerase density in each repeat (French et al., 2003; Sanij et al., 2008). However the DNA is more open and vulnerable to DNA damage. The extra copies are needed to repair against and the high levels of transcription interfere proper repair of DSBs (Ide et al., 2010).

In aging yeast cells, extrachromosomal ribosomal circles (ERCs) accumulate preferentially in mother cells (Sinclair and Guarente, 1997). The formation of ERCs can be suppressed by mutations in DNA repair genes (Park et al., 1999). Also the loss of the Fob1 protein extends the life span and reduces the number of ERCs (Defossez et al., 1999). The ERCs are not a cause of aging, but a consequence of the frequency of replication origin firing (Ganley et al., 2009). Instability in aging cells is not limited to the rDNA, but the entire genome has increased instability (McMurray and Gottschling, 2003). Apart from the ERCs, also damaged proteins have been reported to accumulate preferentially in mother cells. Oxidatively damaged proteins are retained in mother cells and this asymmetric segregation is dependent on Sir2 (Aguilaniu et al., 2003; Erjavec et al., 2007). A link between ERCs and damaged proteins has not been shown yet, however both are linked to aging in yeast and affected by the loss of Sir2.

Structural maintenance of chromosome (Smc) proteins form the cohesion complex (Smc1/3) and condensin (Smc2/4) complexes. The Smc5/6 complex however has a role in DNA damage (De Piccoli et al., 2009). In yeast this complex has been shown to be involved in homologous recombination of DSBs of ribosomal genes (Torres-Rosell et al., 2007). Additionally, during mitosis the Smc5/6 complex is need for proper segregation of rDNA as well as telomeres (Torres-Rosell et al., 2005).

1.8.2 rDNA stability in higher eukaryotes

In humans and mice less is known about rDNA stability in aging organisms. It has been reported that old mice have higher levels of γH2AX indicating genome instability (Wang et al., 2009). Whether these breaks occur in the rDNA is unknown. To date, no extrachromosomal circles of the 45S rDNA have been observed in mammalian cells. Instead it has been hypothesized that the non-canonical rDNA repeats are the human equivalent of the ERCs. The percentage of non-canonical repeats seems to be elevated in the premature aging syndrome Werner (Caburet et al., 2005). Werner (WRN) is a nucleolar helicase which together with PARP1 regulate repair of DNA after oxidative damage (von Kobbe et al., 2003). Apart from oxidative damage, WRN is also involved in DSB repair, explaining the genome instability seen in cells from Werner patients (Karmakar and Bohr, 2005). Major rDNA instability has also been observed in cells from patients with another premature aging syndrome, Blooms syndrome (Killen et al., 2009). Like WRN, Bloom syndrome
protein (BLM) is a nucleolar helicase. BLM associates with topoisomerases to resolve Holliday junctions without cross-over (Wu and Hickson, 2003). Therefore instability is not limited to the rDNA, but affects the entire genome (Chaganti et al., 1974; Luo et al., 2000).

The BLM-topoisomerase complex also prevents the formation of RNA:DNA hybrids, which form due to high transcription levels of the rDNA (Grierson et al., 2013; Grierson et al., 2012). Both WRN and BLM are homologues of the yeast protein slow growth suppressor 1 (Sgs1). Mutations in Sgs1 accelerate aging and lead to nucleolar fragmentation likely caused by increased levels of ERCs (Sinclair and Guarente, 1997; Sinclair et al., 1997). Similar to human cells, in yeast Sgs1-topoisomerase complex can disentangle chromosomes (Cejka et al., 2012; Gangloff et al., 1994).

1.8.3 Heterochromatin is important for rDNA stability.
Mammalian nuclei have two large heterochromatin compartments; perinucleolar heterochromatin and lamina-associated heterochromatin. (Carvalho et al., 2001). The perinucleolar heterochromatin is important for the replication of the inactivated X-chromosome in female cells (Zhang et al., 2007).

The rDNA and heterochromatin are somehow linked. The rDNA copy number can influence heterochromatin. Deletions of the rDNA in Drosophila lead to reactivation of transcription of transposons and repeats in heterochromatin (Paredes and Maggert, 2009). But the heterochromatin also influences the rDNA. H3K9methylation, a histone modification enriched in heterochromatin, is important for repeat stability and nucleolar organisation. Upon loss of Su(var)3-9, a H3K9 methylase, the rDNA becomes unstable leading to the formation of extrachromosomal circular DNA (eccDNA) which are similar to the ERCs in yeast (Peng and Karpen, 2007). This does not only affect the rDNA but also other repetitive DNA sequences such as transposons. These repetitive DNA sequences are normally silent and packed in heterochromatin. These repeats reactivate after depletion of H3K9methylation resulting in genome instability in the heterochromatic region (Peng and Karpen, 2009; Pinheiro et al., 2012). Heterochromatin is also enriched in H3K27methylation and this mark has also been implicated in genome stability. H3K27methylases are found to be important for controlling that repeats are only replicated once (Jacob et al., 2009; Jacob et al., 2010). Apart from histone methylation, DNA methylation is also associated with heterochromatin. Upon loss of the DNA methylase Dnmt1, the nucleolus becomes unstable and falls apart in small fragments. This effect is not restricted to the nucleolus but the entire nuclear architecture is disorganised (Espada et al., 2007). So it is difficult to distinguish whether the effect is due to the loss of rDNA silencing or to the loss of perinucleolar heterochromatin.
1.9 Aims of this thesis

Ribosomal gene transcription is highly regulated by growth factors, nutrient availability, stress signals and the cell cycle. The nucleolus has functions beyond ribosome biogenesis potentially governing the p53 pathway and the cell cycle (Kobayashi, 2008; Visintin and Amon, 2000). But the large rDNA arrays are also vulnerable to illegitimate recombination. The high levels of transcription leave the DNA susceptible to DNA damage.

Initially the focus of this thesis was to target foreign sequences to mark NORs and study their behaviour. To date, it is impossible to follow individual NORs in living cells. Integration of sequences that can visualise the DNA or RNA, would enable the study of the dynamics of individual NOR. To stimulate targeting, double strand breaks were induced at the integration site. Nucleoli showed a marked reorganisation in response to DNA damage. This response and the subsequent repair were investigated in more detail.

Therefore the aims of this thesis were to:

1) Develop an efficient transfection method for primary cells, which does not cause a DNA damage response upon transfection.

2) Explore the feasibility of targeting into the rDNA repeat.

3) Characterise the response of nucleoli to DNA double strand breaks.

4) Characterise the repair of the DNA double strand breaks and the influence of the cell cycle on pathway choice.
Chapter 2

Materials and Methods

2.1 DNA manipulation

2.1.1 Plasmid purification - from small bacterial cultures
Small cultures of 10 milliliter (ml) Luria-Bertani (LB) broth with the appropriate antibiotic were grown overnight at 37 degrees Celsius (°C). Cultures were spun 4000x g (gravity) for 15 minutes (min) at 4°C. Plasmid DNA was isolated using the NucleoSpin® Plasmid kit (Macherey-Nagel). Typically the DNA was eluted in 50-100µl TE (10mM Tris pH8.0, 0.1mM EDTA). Alternatively colonies were streaked out on a large area of the LB agar plate and grown overnight at 37°C. The bacteria were scraped of the LB agar plate with an inoculation loop and directly dissolved in the first buffer of the kit.

2.1.2 Plasmid purification - from the large bacterial cultures
Large cultures of 400ml (high copy plasmids) or 800ml (low copy plasmids and Bacmids) LB broth with the appropriate antibiotic were grown shaking overnight at 37°C. Cultures were spun at 4000x g for 15 min at 4°C. Plasmid DNA was isolated using the NucleoBond® Xtra Maxi Plus kit (Macherey-Nagel). After elution from the column, plasmid DNA was precipitated with an equal volume isopropanol and spun at 4000x g for 15 min at 4°C. Pellets were washed with 70% ethanol and typically dissolved in either 500µl or 1ml TE (10mM Tris pH8.0, 0.1mM EDTA) depending of the size of the pellet. Bacmids were typically resuspended in 100µl TE.

2.1.3 Measurement of concentration and purity of nucleic acids
DNA concentration was determined by Picodrop (Picodrop Limited). High concentration RNA and DNA would be diluted in TE. Pure DNA has a \( \text{A}_{260}/\text{A}_{280} \) ratio around 2.0, while pure RNA has a ratio around 2.2-2.3.

2.1.4 DNA electrophoresis
DNA fragments were routinely run on 1.0% (w/v) Agarose dissolved in 1xTAE (40mM Tris, 20mM Acetic acid, glacial, 1mM EDTA) with 0.5µg/ml ethidium bromide (EtBr) to visualise the DNA. 10x DNA loading dye (40% (w/v) sucrose, 0.25% (w/v) Xylene cyanol (XC)) was added to the DNA. Small DNA fragments (<200bp) were run on a 1.5% (w/v) Agarose in 1xTBE buffer (pH 8.5) (80mM Tris, 80mM Boric acid, 2mM EDTA pH8.0) with 0.5µg/ml EtBr. DNA was loading on TBE gels with 10x TBE loading buffer (10mM Tris pH8, 10mM EDTA pH8, 50% (w/v) sucrose, 0.15% (w/v) Bromophenol Blue).
The gel was run with an appropriated DNA Hyperladder (Bioline) at 100 Volts. The DNA was visualised on UV transilluminator (Gbox imager Syngene) and images were captured using GeneSnap software from Syngene.

DNA bands that were excised for purification, were visualised on a bench top UV transilluminator (Benchtop 3UV™ - UVP) at 302 nanometres (nm) and cut out of the gel with a scalpel. The purification is described in section 2.1.8.

### 2.1.5 Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-N1</td>
<td>Vector purchased from Clonetec</td>
</tr>
<tr>
<td>pBluescript-ires-V5-I-Ppol WT</td>
<td>I-Ppol (pBabe-HA-ER-I-Ppol) was cloned as Ncol-Xbal in pENA (Palmer et al. 1993). Annealed oligo’s (5’-CATGGG TAAGCTATCACCTAACCTCTCCTCGTCAGATTCT ACGCG-3’ and 5’-CATGCGCGTAGAATCGAGACCGAGGA GAGGTTAGGGATAGGCTTACC-3’ encoding the V5tag were dropped in the NcoI site.</td>
</tr>
<tr>
<td>pBluescript-ires-V5-I-Ppol H98A</td>
<td>pBluescript-ires-I-Ppol was mutated with 5’-ACCTGCA CAGCATCGCGCTATGCTTACAATACT-3 and 5’-AGTA TTATGACATAGCGCCGATGCTGTGCAGGT-3’ using the QuikChange Lightning Site-Directed Mutagenesis kit.</td>
</tr>
<tr>
<td>pcDNA3-mAG-Geminin (1-110)</td>
<td>Kind gift from Dr. A. Miyawaki, RIKEN institute; subcloned by Chelly van Vuuren</td>
</tr>
<tr>
<td>pcDNA3-mCherry-Cdt1 (30-120)</td>
<td>Kind gift from Dr. A. Miyawaki, RIKEN institute; subcloned by Chelly van Vuuren</td>
</tr>
<tr>
<td>Tubulin-mCherry</td>
<td>Kind gift from the Sullivan lab.</td>
</tr>
<tr>
<td>pTv1-ENA</td>
<td>The rDNA homology region was amplified with 5’-GCT CGAGGAGAGCCCGACATCGAAG GCTCTAACGGCTGGTCGGTC-3’ from the bacmid AL592188 and was cloned as Xbal/Nhel fusion to XhoI in pBluescript SK II (Clonetec). The I-Ppol site was opened up and annealed oligos 5’- GTCGAGAATTCGAGATCCCTAGATTAA-3’ and 5’- TCTAGAAGATCCGAGATTCGAGTCC ACTTAA-3’ were ligated in. The ENA (Palmer et al., 1993) was cloned in the polylinker as EcoRI-Xbal.</td>
</tr>
<tr>
<td>pTv2-ANE</td>
<td>The homology region was cloned similar to pTv1-ENA. The polylinker was inserted in the antisense orientation. The ENA sequence was cloned as EcoRI-Xbal.</td>
</tr>
<tr>
<td>pTv1-ENA-Xen and pTv2-ANE-Xen</td>
<td>Downstream of the homology region the Xen dimer (Mais et al., 2005) was cloned as Sall-Xhol.</td>
</tr>
<tr>
<td>RPA2-GFP-N1</td>
<td>Kind gift from Dr. Michael Carty.</td>
</tr>
</tbody>
</table>
Chapter 2

<table>
<thead>
<tr>
<th>Construct</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pBluescript-IRES-AsiSI</strong></td>
<td>AsiSI open reading frame was amplified with 5′-GACCA TGGGCGAGTCTATTGATCA-3′ and 5′-GGTCTAGACTC ACAACATCACCTGGTC-3′ from genomic <em>Arthrobacter</em> DNA (NEB) and cloned as NcoI-XbaI in pBluescript-IRES-V5-I-PpoI.</td>
</tr>
<tr>
<td><strong>pUC-SARA</strong></td>
<td>A SalI-EcoRI of the of the human rDNA promoter (-250 to +37) cloned in a pUC vector (NEB®).</td>
</tr>
<tr>
<td><strong>gRNA Empty vector and hCas9 vector</strong></td>
<td>Purchased from Addgene.</td>
</tr>
</tbody>
</table>

Table 2.1: Constructs used in this study.

2.1.6 Restriction digests

For diagnostic digests of screening colonies, 10µl mini-prepped plasmid DNA was diluted with the appropriate buffer and 1µl restriction enzyme, purchased from either New England Biolabs® Inc. (NEB) or Roche. For digests to release DNA fragments for cloning, typically contained 5µg DNA in 50µl with at least 2 Units of enzyme.

2.1.7 Ligation

Ligations contained 30-50ng of plasmid backbone and a 3-5 molar excess of insert together with 1-2µl T4 DNA ligase (NEB®). Reactions were incubated 1h to overnight at room temperature before being transformed.

2.1.8 Purification of DNA

DNA from either polymerase chain reactions (PCRs) or cut-out bands from agarose gels was isolated with the NucleoSpin® Extract II (Macherey-Nagel) according to the manufacturer’s instructions.

Alternatively, DNA was be precipitated with ethanol. 2.5-3 Volumes 100% ethanol were added and the sample was incubated in -20°C for 30min. It was spun at 16100x g for 15 min at 4°C. The pellet was washed in 70% ethanol and spun again at 16100x g for 15min at 4°C. The pellet was air-dried for 2-5min to remove any residual ethanol, before it was resuspended in an appropriate buffer.

2.1.9 Site directed mutagenesis

Mutagenesis was carried out using the QuikChange® Lightning Site-Directed Mutagenesis kit (Stratagene) following the manufacturer’s instructions and transform into DH10B competent cells.
2.1.10 LR reactions
Gateway® entry (pENTR™) plasmids were shuttled into the desired destination (pDest™) vector following the manufacturer’s instructions with the exception that 4µl diluted plasmid and 1µl LR Clonase® (Life Technologies™) were used.

2.1.11 Generation of the CRISPR vectors.
The guide RNA (gRNA) vectors were designed and generated according to instructions provided by the Church lab available on the website of Addgene (http://www.addgene.org/41815/ under Supplemental document).

2.1.12 Genomic DNA isolation -with kit (colony screening targeting experiments)
Genomic DNA (gDNA) was isolated using the standard protocol for cultured cells from the NucleoSpin® Tissue kit (Macherey-Nagel).

2.1.13 Genomic DNA isolation -without kit (Southern blot)
Cells were washed with phosphate buffered saline (PBS) and incubated overnight at 37°C with 20mM Tris-HCl, pH8.0, 2mM EDTA, pH8.0, 0.5%SDS and 0.3mg/ml Proteinase K (Roche). 1/10th NaCH₃CO₂ was added prior to phenol/chloroform/isoamylalcohol (25:24:1) extraction. 500µg RNAse A was added and incubated at 37°C for 30min. After another phenol/chloroform/isoamylalcohol (25:24:1) extraction, the DNA was ethanol precipitated (section 2.1.8) and resuspended in an appropriate volume TE.

2.1.14 Southern Blotting
5µg gDNA was digested and run on a 1% TAE gel. The gel was depurinated in 0.25M HCl, rinsed with deionised H₂O (dH₂O) and incubated in 0.4M NaOH. DNA was transferred to Hybond™-N+ membranes (Amersham™ GE) overnight with 0.4M NaOH. The membrane was washed with 2xSSC (1xSSC [50mM NaCl, 15mM Na₃citrate, pH7.0]). The DIG High Prime DNA Labelling and Detection Starter kit II (Roche) was used according to manufacturer’s instructions for the hybridisation, probe preparation and detection.

2.1.15 Nick translation
1µg of Bacmid DNA or plasmid was labelled with Green dUTP or Red dUTP using Nick Translation kit (Abbott Molecular) according to the manufacturer’s protocol.
2.1.16 DNA sequencing

DNA was sent to Source Biosciences (LifeScience) for standard Sanger sequencing. The sequence tracks were visualised with 4Peaks (mekentosj.com). Sequence contigs were assembled in Strider 1.4.

2.1.17 End point PCR

Both Taq and PFU polymerases were isolated by other people in the McStay lab. For routine detection Taq polymerase was used. When the PCR products were used for subsequent cloning, the high fidelity polymerase PFU was used. Also the gene targeting primers only worked with the PFU polymerase.

A PCR reaction would contain 1x Taq buffer (10mM Tris pH9.0 (25°C), 50mM KCl, 1.5mM MgCl₂, 0.1% (v/v) Triton X-100) or 1x PFU buffer (20mM Tris pH8.7, 2mM MgSO₄, 10mM KCl, 10mM (NH₄)SO₄, 0.1% Triton X-100, 0.1mg/ml nuclease-free BSA) with 200µM dNTPs (Bioline®) and 0.2µM of each primer. To the reaction, 50-100ng genomic DNA (gDNA) or 10-30ng plasmid was added. GC-rich PCR products were amplified in the presence of 1M Betaine (Sigma Aldrich®). Reactions were run in an Eppendorf Mastercycler® gradient (Eppendorf). A typical program would have 5min at 95°C, 25-30 cycles of 30sec at 95°C, 30sec at 50-65°C, 72°C for 1kb/1min (Taq) or 1kb/2min (PFU).

2.1.18 Quantitative PCR

For quantitative PCR (qPCR) the amount of SYBR® GREEN I (Sigma Aldrich®) was optimised to 1:15000 [Figure 2.1]. SYBR® green fluorescence is optimal with a concentration of 2.5mM MgCl₂, therefore additional MgCl₂ was added. A typical PCR reaction has Taq buffer (10mM Tris pH9.0 (25°C), 50mM KCl, 1.5mM MgCl₂, 0.1% (v/v) Triton X-100) with 1mM MgCl₂ added in the presence of 1mM dNTPs (Bioline®), 0.2µM of each primer with 1:15000 SYBR Green I and 2µl Chromatin Immunoprecipitated (ChIP) DNA. The rDNA and DJ were amplified in the presence of 1M Betaine (Sigma Aldrich®). PCRs were run in a total volume of 25µl in an Opticon® 2 DNA engine (MJ Research) with the Opticon® Monitor 3 software. A typical program would have 2min at 95°C, 40 cycles of 15sec at 95°C, 30sec at 50-65°C, 20sec at 72°C after which the plate was read. After the 40 cycles, melting curve was run from 60-95°C. qPCR reactions were always run in triplicate to control for experimental variation.

<table>
<thead>
<tr>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>promoter</td>
<td>TATATCTTTTCCGCTCCGAGTC</td>
</tr>
<tr>
<td>ETS</td>
<td>GCCGGTTTGGATGAGGAGAGA</td>
</tr>
<tr>
<td>18S</td>
<td>CGACGACCCATTCCGAGTCTT</td>
</tr>
<tr>
<td>28Sa</td>
<td>AGTCGGGTTTGGGGATGTC</td>
</tr>
</tbody>
</table>
Table 2.2: List of primers used in PCR

<table>
<thead>
<tr>
<th>28Sb</th>
<th>ACCTGGCGCTAAACCATTGT</th>
<th>GGACAAACCCTTGTCGAGG</th>
</tr>
</thead>
<tbody>
<tr>
<td>19.4kb</td>
<td>GCCCTTCAAGCCCATTTCG</td>
<td>AATGGAGAGAAGCCCAAGGC</td>
</tr>
<tr>
<td>42kb</td>
<td>AGGGGTCTGTTTTCGGCTCC</td>
<td>AGCGGACAGATCCGGCTGGC</td>
</tr>
<tr>
<td>U2</td>
<td>ACGGAGCAAGTCCTATTCC</td>
<td>TCGGCTTGGCTAAGATC</td>
</tr>
<tr>
<td>DJ4.4kb</td>
<td>ACAACGCAGAAAAGCGACCC</td>
<td>GGCAAGCTGGAGATTGACGT</td>
</tr>
<tr>
<td>DJ138kb</td>
<td>TCCGAGCAGACGAGCGAGG</td>
<td>AATCCGACTGCAACGGGCC</td>
</tr>
<tr>
<td>DJ204.1kb</td>
<td>AAGAGAGGCAAGGAGGCTAGGA</td>
<td>GCGAAGGAGGCTAGGAG</td>
</tr>
<tr>
<td>DJ292kb</td>
<td>TGGGGCTAGGGAGAGCTGGGC</td>
<td>TGGGGCTAGGGAGGCTGGG</td>
</tr>
<tr>
<td>Targeting</td>
<td>GGTATGGCTGATTGATCC</td>
<td>CTCACGTTCCTATTGATGG</td>
</tr>
</tbody>
</table>

Figure 2.1: Optimisation of the amount of SYBR Green.

2.2 Bacterial manipulations

2.2.1 Making glycerol stocks

Freshly grown overnight cultures were chilled on ice and glycerol was added to a final concentration of 20%. The bacteria were aliquoted and stored at -80°C.

2.2.2 Making competent cells

A large culture was inoculated from a LB agar plate or a small overnight culture and grown at 37°C until Optical density (OD)_{590nm} was 0.500. Cultures were chilled on ice for 5 min, divided in 50ml aliquots and spun at 3200rpm for 4min at 4°C. Pellets were resuspended in 20ml prechilled TFB 1 (30mM KCH$_3$CO$_2$, 100mM RbCl, 10mM CaCl$_2$·2H$_2$O, 50mM MnCl$_2$·4H$_2$O, 15% glycerol (v/v) pH5.8) and incubated 15min on ice. Suspensions were spun at 3200rpm for 10min at 4°C and pellets were resuspended in 2ml prechilled TFB
II(10mM Mops, 75mM CaCl$_2$·2H$_2$O, 10mM RbCl, 15% glycerol (v/v) pH6.5 with KOH). Cells were incubated 30min on ice and 100µl aliquots were snap frozen and stored at -80°C.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH10B</td>
<td>Standard cloning</td>
</tr>
<tr>
<td>BL21Ai</td>
<td>Expression of recombinant proteins</td>
</tr>
<tr>
<td>TR1</td>
<td>Propagation of Gateway® constructs ccdB resistant</td>
</tr>
</tbody>
</table>

*Table 2.3: Bacterial strains.*

2.2.3 Transformation

100µl Competent *Escherichia coli* competent cells were slowly thawed on ice and 6µl of the ligation reaction or 30 nanograms (ng) of plasmid was added and mixed by tapping. Cells were placed in a 42°C for 1min and placed back on ice for minimally 1min. 1ml of Luria broth (LB) (Melford) was added and cells were grown shaking at 37°C for minimally 40min. Cells were pelleted by spinning at 3000x g for 2min and plated out of LB-agar (Sigma Aldrich®) plates containing the appropriate antibiotic. The plates were incubated at 37°C overnight and colonies for screening were picked the next day. Depending on the downstream application, an appropriate bacterial strain was chosen (table 2.1).

2.3 RNA techniques

2.3.1 T7 in vitro transcription and polyadenylation

IRES-containing plasmids were transcribed with the MEGAscript® T7 kit (Ambion®) yielding an uncapped transcript, while plasmids without an IRES element were transcribed with the mMessenger mMachine T7 kit (Ambion®) for capped transcripts. For both kits, 1µg linearised plasmid was used per reaction according to the manufactures instructions. The RNA was subsequently polyadenylated with the Poly(A) Tailing Kit (Ambion®) according to the manufacturer’s instructions. After the polyadenylation, the RNA was phenol/chloroform/isoamylalcohol (25:24:1) extracted and precipitated with an equal volume of isopropanol. The pellet was washed with 70% ethanol and dissolved in 100µl TE.

2.3.2 mRNA transfection

Cells were seeded at least 24h prior to transfection in a 6-well plate, such that at the day of the transfection the cell density would be approximately 70-80%. The *in vitro* transcribed mRNA was transfected into cells using the TransMessenger™ Transfection Reagent (Qiagen). For I-PpoI, 1µg I-PpoI messenger RNA (mRNA) and 2µl Enhancer R were diluted in Buffer EC-R and incubated for 5 min at room temperature. 2µl TransMessenger™ Transfection Reagent was added and incubated 10min at room temperature. Cells were
washed 3 times with Phosphate Buffer Saline (PBS). The transfection medium is diluted in 900µl serum-free medium (Dulbecco's Modified Eagle Medium (DMEM)/ Nutrient Mixture F-12 Ham, supplemented with sodium bicarbonate) and gently added to the cells. The cells were grown under normal conditions for 4 hours (4h). The transfection medium was removed and cells were washed 3 times with PBS and grown with full medium. For 10 centimetre (cm) plates 3ml transfection medium was prepared, for 15cm plates 5ml transfection medium.

When transfecting the Fucci RNA, 1µg of Cdt1-mCherry and 1µg Geminin-mKO2 was added to 4µl enhancer diluted in Buffer EC-R. To this mixture 4µl of TransMessenger™ Transfection Reagent was added. AsiSI endonuclease was transfected as 2µg RNA with 4µl enhancer and 4µl of TransMessenger™ Transfection Reagent.

2.3.3 RNA isolation
Total RNA was isolated with TriSure™ (Bioline®) from 10cm dishes according to the manufacturer’s instructions.

2.4 Protein techniques
2.4.1 Expression of recombinant proteins in bacteria
Expression plasmids were transformed in BL21Ai and grown overnight. A part of that culture was used to start a new culture, which was grown till OD$_{600nm}$=0.5 at 37°C. Expression was induced 0.2% (w/v) L-arabinose for 2 hours.

Expression of the protein was assessed by taking 1ml aliquod of the induced and uninduced culture and resuspending the pellet in 1x SDS-PAGE loading buffer. This was incubated at 95°C for 10min and sonicated in the Bioruptor® (Diagenode) at power setting High until the sample was no longer viscose.

To assess the solubility of the recombinant protein, 45ml of the induced culture was spun down and resuspended in 2ml of native extraction buffer (0.1M Tris pH8.0, 10% glycerol, 0.5M NaCl, 1%NP-40) supplemented with 1mM Dithiothreitol (DTT) and 0.1mM Phenylmethanesulfonyl fluoride (PMSF). This was sonicated for 3-5times with the Soniprep 150 (MSE) on ice until the sample was no longer viscose. A 100µl aliquod of the mixture was taken as a whole cell lysate. The remainder of the mixture was spun at 15min at 4000xg at 4°C to remove insoluble material. The supernatant was diluted in 2x SDS-PAGE loading buffer.

All samples were run on NuPAGE® Novex® 4-12% Bis-Tris gels (Life Technologies™) using Xcell SureLock™ Mini-cell (Life Technologies™) and NuPAGE® MES running buffer (Invitrogen) following manufacturer’s protocol. Afterwards gels were stained using
Coomassie brilliant blue (PhastGel® BlueR (Sigma Aldrich®), 10% acetic acid, 40% Methanol).

2.4.2 Isolation of recombinant His-tagged protein
Cultures (800ml) were spun down for 15min at 4000xg at 4°C. Pellets were frozen at -80°C overnight. The next day the pellet was resuspended in 25ml native extraction buffer supplemented with 1mM DTT, 0.1mM PMSF and 0.2-0.4mg/ml lysozyme. The mixture was incubated on ice for 30 min before it was sonicated on ice 7 times, 30s ON/30s OFF, with the big probe of the Soniprep 150 (MSE). The lysates were spun for 15min at 4000x g at 4°C to remove insoluble material. The supernatant was incubated with 4ml 50% Nickel Nitrilotriacetic acid (Ni-NTA) Agarose beads (Qiagen) for 30min at 4°C to bind the recombinant protein. The beads were washed with native extraction buffer with 10mM imidazole pH8. Proteins were eluted with 200mM imidazole in native extraction buffer and collected in 1ml fractions.

The 9ml of eluted protein solution was dialysed in a Slide-A-Lyser 3.5K™ (ThermoFisher™) in 400ml Column buffer 100 (CB100 [25mM HEPES pH 7.9, 100mM KCl, 1mM DTT, 0.1mM EDTA, 20% (v/v) glycerol]) at 4°C for 2 days, changing the buffer 4 times in total. This dialysed protein was aliquoted and stored at -80°C or sent off for immunisation.

2.4.3 Measuring protein concentration
Protein concentration was measured using 1:5 diluted Bradford solution (BioRad) in water and 1-3µl of the protein samples. The absorption was measured at OD_{600nm} in a Colorimeter model 45 (Fisher). Using a standard curve the protein concentration was estimated.

2.4.4 Harvesting protein samples
Cells were scrapped in PBS and spun down 1000x g for 5min at 4°C. The pellet was washed once with PBS before the cells were resuspended in an appropriate volume SDS-PAGE loading buffer (62.5mM Tris pH6.8, 10% glycerol, 2% SDS, 5% β-mercapto-ethanol, 1.25% (w/v) Bromophenol Blue). The samples were boiled at 95°C for 10min and sonicated 2-3x 30sec ON/30sec OFF in a Bioruptor® water bath sonicator (Diagenode) at power setting High.

2.4.5 Western blot
Proteins were separated on NuPAGE® Novex® 4-12% Bis-Tris gels (Life Technologies™) using an XCell® SureLock Mini-cell (Invitrogen). Per lane an equivalent of 6-7.5 x 10^5 cells
were loaded. The gel was run in NuPAGE® MES SDS Running Buffer according to the manufacturer’s instructions. Proteins were stained with Coomassie brilliant blue (PhastGel® BlueR (Sigma Aldrich®), 10% acetic acid, 40% Methanol). Alternatively, the gels were transferred to HybondTM-ECLTM nitrocellulose membranes (Amersham) using XCell® II blot module (Invitrogen) and NuPAGE® transfer buffer (Invitrogen) according to the manufacturer’s instructions. For proteins up to ~100kDa (kilo Dalton), the gel was transferred for 1.5h. To get optimal transfer of large proteins (>100kDa) the gel was transferred for 2h. To assess efficient transfer, membranes were stained with Ponseau S (0.1% (w/v) Ponseau S (Sigma Aldrich®), 5% acetic acid) for 15-20min. The membrane was destained in dH2O. The membranes were routinely blocked in 5% (w/v) non-fat Milk (Marvel) in PBS for 1h at room temperature. Primary antibodies (table2.1) were diluted in 5%Milk/PBS and incubated rotating at 4°C overnight. The next day, the membrane was rinsed in PBS before incubation with an appropriate secondary horse radish peroxidase (HRP)-conjugated antibody (α-Sheep-Sigma Aldrich®, α-Mouse and α-Rabbit both Jackson ImmunoResearch).

Several antibodies (indicated with * in table 2.1) required the membrane to be blocked in 5% Bovine serum albumin (BSA) in TBST (20mM Tris, 150mM NaCl, 0.1% Tween-20). The antibodies were also diluted in 5% BSA/TBST and the membrane was rinsed in TBST. For the Chk2 antibodies (indicated with ¥ in table 2.1) the membrane was blocked with 5% Milk/TBST. The antibodies were diluted in 5%Milk/TBST and rinses between the primary and secondary antibody were in TBST.

Antibodies were detected using Western Lightning® Plus-ECL (PerkinElmer). For quantitative Western blot, blots were incubated with WesternBright™ Quantum™ (MyBio), whose chemiluminesce has a better linear signal. A few antibodies only produced faint bands with the regular ECL and therefore they were imaged again with WesternBright™ Sirius™ (MyBio) which best suited for faint signals. All reagents were used according to their manufactures instructions.

Images were captured with CCD Syngene G-Box XT16 camera and images captured using GeneSnap software (Syngene). Signals were quantification using GeneTools software (Syngene).

### 2.4.5 List of antibodies

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* For Western blot: 5% BSA in TBST
¥ For Western blot: 5% Milk in TBST

**Table 2.4:** List of antibodies used for immunofluorescence and/or Western blot. For each antibody it is indicated at which dilution it was used in immunofluorescence and on Western blot.
2.5 Cell biology

2.5.1 Maintenance of cell lines
hTERT-RPE1 cells were maintained in DMEM/ Nutrient Mixture F-12 Ham (Sigma Aldrich®) supplemented with Penicillin/Streptomycin (P/S)(Sigma Aldrich®), 2mM L-Glutamine (Sigma Aldrich®), 10% (v/v) Fetal Bovine Serum (FBS) and 0.25% (v/v) sodium bicarbonate (Sigma Aldrich®). 1BR3, CCD-1079Sk and BJ primary cells were grown in DMEM/ Nutrient Mixture F-12 Ham (Sigma Aldrich®) supplemented with P/S, 2mM L-Glutamine (Sigma Aldrich®), 10% FBS and non-essential amino acids (Sigma Aldrich®).
HT1080 and Hela were maintained in DMEM GlutaMAX™ (Life Technologies™) with P/S (Sigma Aldrich®) en 10% FBS (Serotec). All cells were grown in plastic flasks or dishes (Sarstedt), which were incubated at 37°C with 5% CO₂.
When cells neared confluence, they were split by incubation in 0.25% Trypsin (Sigma Aldrich®) supplemented with 1mM EDTA, pH8.0 until >95% of the cells were detached from the flask. The trypsin was quenched with medium and cells were spun 1200 revolutions per minute (rpm) for 5min before seeding them out with a 1:5 to 1:10 ratio.

2.5.2 Liquid nitrogen stocks: freezing down and thawing out
Cells were trypsinized and spun down at 1200rpm for 5 minutes. Pellets were resuspended in an appropriate volume of 90% FBS, 10% DMSO (Cell culture grade- Sigma Aldrich®). Aliquots were frozen in Nunc™ cryovials in a Nalgene® Cryobox™ at -80°C overnight.
The next days the vials were transferred to the liquid nitrogen tanks for long term storage. To thaw cells were quickly warmed and diluted in medium. Cells were spun 1200rpm for 5min before seeding them out. The next day cells were rinsed with PBS to remove any residual DMSO and given fresh media.

2.5.3 DNA transfection- calcium phosphate
Calcium phosphate transfection was used for both transient transfections and for generating stable cell lines. In both cases, cells were grown in a 6 well plate and transfected when ~50% confluent. 2.5µg Plasmid was diluted with 11µl 2M CaCl₂ up to a final volume of 90µl with sterile H₂O. The DNA-phosphate mixture was added drop wise to 90µl 2xHBS (16 mg/ml NaCl, 1.5mM Na₂HPO₄, 13 mg/ml HEPES pH7.0). The mixture was added to the cells immediately and incubated with the cells overnight.
For making stable cell lines, after an overnight transfection, the cells were split to a 15 cm dish. Cells were grown with the appropriate antibiotic (1 mg/ml G418, 3 µg/ml puromycin or 10 µg/ml blasticidinS (all from Melford)) for about 2 weeks till colonies formed. 6 to 12 colonies were picked with cloning disks (Sigma) and transferred to 6 well plates.
2.5.4 DNA transfection-TransIT®

7.5µl TransIT®-LT1 Transfection reagent (Mirus) and 1µg plasmid were diluted in 250µl OptiMEM® (Gibco). The transfection mixture was incubated for 30min at room temperature before it was added to the cells. The transfection media was incubated with the cells overnight. 1 Hour before fixation, the cells were washed three times in PBS and fresh medium was added.

2.5.5 Inhibitors and other chemicals

KU55933 (SelleckChem) and NU7441 (Axon Medchem) were added to the medium to a final concentration of 10µM. Caffeine (Sigma Aldrich®) was directly dissolved in medium to a final concentration of 15mM. Cells were treated with imatinib (Enzo® Life Sciences) at 1µM and 5µM. 4-Hydroxytamoxifen (Sigma Aldrich®) was added to a final concentration of 1µM.

2.5.6 Immunofluorescence

Cells were grown on ethanol cleaned coverslips in 6 well plates. The coverslips for seeding HT1080 cells were coated with poly-L-Lysine for 30min, after which the coverslips were rinsed with PBS before seeding. Primary cells were grown on glass without any coating.

Cells were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for 10min and permeabilised with 0.5% (w/v) Saponin and 0.5% (v/v) Triton X-100/PBS for 10min. Primary antibodies were diluted in 1% (w/v) Bovine serum albumin (BSA)/PBS and incubated in a humidity box at 37°C for 1 hour. The secondary antibodies (Jackson ImmunoResearch) were incubated for 40min.

The coverslips were mounted in VectorShield® (Vector Laboratories) with or without DAPI. When the cells were pre-extracted, the coverslips were incubated 10min on ice in ice-cold 0.5% (v/v) Triton X-100/PBS before fixating with 4% PFA/PBS. Coverslips were processed further as described above.

2.5.7 Metaphase spreads

Cells (T75 flask) were treated 0.1ug/ml Demecolcine (Sigma Aldrich®) for 1h and harvested by mitotic shake-off. Cells were spun at 1700rpm for 7min and the pellet was resuspended in prewarmed 75mM KCl (37°C). Cells were allowed to swell at 37°C for 10 min. At the end of the incubation, 2-3 drops of ice-cold methanol/acetic acid (3:1) were added before spinning at 1700rpm for 7min. The pellet was resuspended in methanol/acetic acid (3:1) and spun again at 1700rpm for 7min. The pellet was resuspended in an appropriate volume methanol/acetic acid (3:1). Mitotic cells were dropped using a Pasteur pipette from a height onto Superfrost® slides (Fisher) and air-dried before hybridisation.
2.5.8 *Fluorescent In Situ Hybridisation (FISH) of metaphase spreads*

The DNA probe containing 50ng nick translated probe, 2.5µl Hybloc Cot-1 DNA (Applied Genetics Laboratories) and 5µl Herring Sperm, was ethanol precipitated (section 2.1.8) and resuspended in Hybrisol® VII (Qbiogene). The metaphase slide was dehydrated for 2min in 70% Ethanol, 2min in 90% Ethanol and 2min in 100% Ethanol and air-dried. The probe was applied to the slide and the coverslip was glued on with rubber cement (Marabu-Fixogum). The probe and slide were denatured together at 70°C for 3min and hybridised overnight in a humidity box at 37°C.

The next day the slide was washed in 0.4% SSC/0.3% NP-40 at 74°C for 2min and in 2xSSC/0.1% NP-40 for 5min at room temperature. The slide was allowed to air-dry and mounted in VectorShield® with DAPI (Vector Laboratories).

2.5.9 *3D ImmunoFISH*

Cells were grown on Superfrost® Plus microscopic slides (VWR®). Slides were fixed in 4%PFA/PBS for 10min and permeabilised in 0.5% Saponin (w/v) and 0.5% Triton X-100 (v/v)/PBS for 10min. Slides were incubated in 20% glycerol/PBS for 2h before snapfreezing them in liquid nitrogen. Slides were stored at -80°C.

Slides were thawed out in PBS at room temperature. Cells were stained for γH2AX antibody before the hybridisation. Both primary and secondary antibody were diluted in 1% BSA and incubated at 37°C in a humidity box for 1h. The antibodies were fixed with 2%PFA/PBS.

Cells were depurinated in 0.1M HCl for 5 min and equilibrated in 50% deionised formamide/2xSSC for 15min in humidity chamber at 37°C.

The DNA probe contained 50ng nick translated probe, 2.5µl Hybloc™ Cot-1® DNA (Applied Genetics Laboratories) and 5µl Herring Sperm. This was ethanol precipitated and resuspended in Hybrisol® VII (Qbiogene). The probe was then applied to slide and sealed with rubber cement (Marabu-Fixogum) and denatured at 73°C for 12mins. DNA was hybridized to the slides at 37°C in a humidity box. The next day slides were washed with 3x5min in 50% formamide/2xSSC at 42°C and 3x5min in 0.1xSSC at 60°C. The slides were rinsed in PBS and stained with antibody as outlined in section 2.5.9.

2.5.10 *RNA FISH*

In RNA FISH experiments, the protocol of 3D-immuno DNA FISH was generally followed with the exception that the probe is denatured separately for 5min at 73°C. The slide is not depurinated and not denatured. The probe was hybridized overnight and washed like stated in section 2.5.9.
2.5.11 BrUTP incorporation assay

Cells grown on coverslips were rinsed in PBS before permeabilisation with 0.05% (v/v) Triton X-100/PBS for 2 min at room temperature. Coverslips were incubated 15 min at 37°C in transcription buffer (100mM KCl, 50mM Tris-HCl at pH 7.4, 5mM MgCl2, 0.5mM EGTA, 5 U/mL RNasin® (Promega) supplemented with 0.5mM ATP, GTP, CTP, 0.2mM BrUTP (Sigma Aldrich®) and 50µg/ml α-amanitin. Cells were fixed in 2% (w/v) PFA/PBS 10min at room temperature and permeabilised with 0.1% Triton X-100. BrUTP was stained with anti-BrdUTP antibody (Roche) (Mais et al., 2005) as indicated in section 2.5.6.

2.5.12 Click-IT chemistry (DNA and RNA)

Cells grown on coverslips were incubated with 1mM 5-Ethynyl Uridine (EU) (Life Technologies™) for 2h or 10µM 5-ethynyl-2'-deoxyuridine (EdU) (Life Technologies™) for 30min. Coverslips were fixed with 4% (w/v) PFA/PBS and permeabilised with 0.5% (w/v) Saponin, 0.5% (v/v) Triton X-100 /PBS. Sites of synthesis were visualised with the Click-IT RNA Alexa® Fluor 488 and Alexa® 594 Imaging Kit (Life Technologies™) according to manufacturer’s instructions. Afterwards staining with antibodies was done as described in section 2.5.6.

2.5.13 Fixed cell imaging

Images were captured and merged using a Photometric® Coolsnap HQ camera and Volocity 6 imaging software (PerkinElmer) with a 63x Plan Apochromat Zeiss objective mounted on a Zeiss Axioplan2 imaging microscope. Typically 30-40 Z-stacks from the bottom of a cell to the top were taken. For metaphase slides, 10 stacked images were taken. The images were deconvoluted by iterative restoration in Volocity 6 with a confidence limit at 95% and a maximum number of iteration of 50. The contrast was enhanced to clarify the image. Snapshots were taken of the enhanced focus (all z-stacked images compiled on top of each other).

2.5.14 Nucleolar isolation ([http://lamondlab.com/f7nucleolarprotocol.htm](http://lamondlab.com/f7nucleolarprotocol.htm))

20 Dishes (Ø15cm) of RPE1 cells at 70-80% confluent were scrapped in PBS and spun at 1200rpm for 5min at 4°C. After 2 PBS washes, cells were resuspended in Buffer A (10mM HEPES pH7.9, 10mM KCl, 1.5mM MgCl2, Complete protease inhibitor (tablets –Roche). Nuclei were released by homogenised in a Douncer tissue homogenizer (Wheaton Scientific) with the “A”-pestle. When >90% nuclei were release, cells were spun at 1200rpm for 5 min at 4°C. The pellet was resuspended in S1 (0.25M Sucrose, 10mM MgCl2, Complete protease inhibitor) and layered over S2 (0.35M Sucrose, 0.5mM MgCl2, Complete protease inhibitor). Nuclei were pelleted by spinning at 1430x g for 5min at 4°C and resuspended in S2.
Nucleoli were released by sonication with a Soniprep 150 (MSE) at 10sec ON/ 10sec OFF on ice until nuclei could no longer be detected under a light microscope. After sonication, nucleoli were purified by layering them on S3 (0.88M Sucrose, 0.5mM MgCl$_2$, Complete protease inhibitor) and spinning at 3000x g for 10min at 4°C. The pellet was resuspended in 100µL 1x SDS-PAGE sample loading buffer (62.5mM Tris pH6.8, 10% glycerol, 2% SDS, 5% β-mercapto-ethanol, 1.25% (w/v) Bromophenol Blue). The sample was boiled at 95°C for 10min and stored at -20°C.

2.5.15 *Live cell imaging*

Cells were seeded in uncoated Glass bottom dishes (MatTek P35G-1.5-10-C). Cells were transfected in the dish and 3 hour afterwards the transfection media was taken off and replaced by fresh media supplemented with 20mM HEPES (pH7.2). In a 37°C chamber, dishes were imaged with a DeltaVision Core (Applied Precision) controlling an interline charge-coupled device camera (Coolsnap HQ2;Roper) mounted on an inverted microscope (IX-71; Olympus). Images were taken with the 63x oil objective at appropriate intervals either with the extended focus setting. Images were deconvolved (95% confidence) and maximum intensity projected using Volocity 6.0 (PerkinElmer).

2.5.16 *Nuclear ChIP*

Cells were prepared according to the manufacturer’s instruction in the HighCell ChIP kit (Diagenode). The cells were sonicated in icy water (<4°C) in TPX tubes (Diagenode) for 25 cycle of 30sec ON/ 30sec OFF in a Bioruptor® water bath sonicator (Diagenode) with powersetting High. The water was refreshed every 5 cycles to prevent heating of the samples. These conditions result in fragments of 200-1000bp in length. The equivalent of 10$^7$ cells was used per immunoprecipitation (IP).

2.5.17 *In vitro transcription assay*

Hela cell nuclear extract (HNE)(CilBiotech, Belgium) in CB100 buffer (25mM HEPES pH 7.9, 100mM KCl, 1mM DTT, 0.1mM EDTA, 20% (v/v) glycerol) was diluted 4 fold into the same buffer. Transcription reactions contained 20µl of diluted HNE combined with 20µl of transcription buffer (25mM HEPES pH 7.9, 80mM KCl, 12mM MgCl2, 1mM DTT, 100µg/ml α-amanitin, 1mM nucleotide triphosphates and 20µg/ml template DNA). The final reaction conditions were: 25mM HEPES pH 7.5, 90mM KCl, 6mM MgCl2, 10% glycerol, 1mM DTT, 50µg/ml α-amanitin, 0.5mM NTPs and 10µg/ml pUC-SARA DNA. Reactions were incubated at 37°C for 30min. The reaction was stopped by adding 360µl *in vitro* Stop solution (50mM Tris pH8.0, 0.15M NaCl, 0.3M Na Acetate, 0.1% (w/v) SDS, 6mM EDTA, 100µg/ml *E. coli* RNA) and extracted by phenol/chloroform/isoamylalcohol (25:24:1). The
RNA was ethanol precipitated and the pellet was washed with 70% (v/v) ethanol. The pellet was resuspended in 27µl H₂O after which the RNA was used in an S1 assay (section 2.5.18).

2.5.18 S1 nuclease assay
The S1 probes were HYPUR gel-purified 60-mer oligonucleotide supplied by MWG, the transcription (5’-TTGGGCGCCCGGTTATGCTGACACGCTGTCCCTGCGACCTGTCGCTGGAGAGGTTG-3’') and A’-cleavage probe (5’- CGGACCGGCCGGAAGA GCACGACGTACCACATCGATCAGAAGACGCCCCCAGGAGC-3’'). Oligonucleotides (200 ng) were 5’ end-labeled with γ-³²P ATP by T4 polynucleotide kinase. Prior to labelling, the probe was boiled to denature any secondary structures.

10-µg sample of RNA is resuspended in 27µl of H₂O together with 3µl of 10× Hybridisation buffer (3M NaCl, 0.1M Tris pH 7.9, 10mM EDTA) and 1µl (2ng) of labelled probe. The reactions were incubated for 3 h at 65°C and then placed on ice. To each reaction, 270µl chilled S1 nuclease buffer (1mM ZnSO₄, 30mM Na acetate pH 5.4, 50mM NaCl) containing 50 units of S1 nuclease (Worthington) was added incubated for 30 min at 37°C. The S1 nuclease digestion was stopped by the addition of 50µl of 5M ammonium acetate, 5µl of 0.5M EDTA, and 10µl of 10% SDS. The RNA/DNA hybrids were ethanol precipitated and resuspended in 6µl of loading buffer (80% deionised formamide, 0.01% xylene cyanol and bromophenol blue dyes in 1×TBE). Samples were run on 8% denaturing (7M urea) polyacrylamide gels run in 1×TBE, and radioactivity was visualised and quantified using a Fuji scanner (FujiFilm FLA-5100).
Chapter 3

RNA transfection, a high efficiency transfection method that causes minimal genomic stress on primary cells.

3.1 Introduction

Traditional DNA transfection methods are highly efficient in most cancer cell lines, but in primary cells the efficiency is low. Additionally transient DNA transfection itself has been shown to induce a γH2AX response. Different lipid-based transfection methods induce a DNA damage response and change gene expression (Igoucheva et al., 2006; Jacobsen et al., 2009). And this is not limited to chemical transfection methods, but viral transfections have also been shown to induce a γH2AX response (Schwartz et al., 2009) and upregulate the expression of DNA repair genes (Smith et al., 2006).

Apart from DNA, RNA can also be transfected. Messenger RNA (mRNA) can be efficiently transfected by electroporation in leukemia cell lines with minimal cell death (Boissel et al., 2009). Alternatively, RNA can be transfected with lipid based methods and is effective in many different cell types (Malone et al., 1989) including primary mesenchymal stem cells (Ryser et al., 2008).

Eukaryotic mRNAs have 5’ and 3’ untranslated regions (UTRs) flanking the coding sequence. The 5’end of the RNA has a modified base, a methylated Guanine called m^7G cap. The 3’end of the RNA is polyadenylated (polyA-tail), which is bound by the poly(A)-binding protein (PABP). The m^7G cap is bound by eukaryotic initiation factor 4F (eIF4F), which interacts with PABP circularizing the mRNA and this recruits the ribosome. Not all mRNAs depend on an m^7G cap for translation. Secondary structures called internal ribosomal entry sites (IRESs) in the 5’UTR of an mRNA can also direct recruitment the ribosome circumventing the requirement for a cap (Spriggs et al., 2010).

If one wishes to study the DNA damage response and subsequent repair, DSBs can be induced at random loci using IR. However to study and compare the response at specific loci or look at the sequential recruitment of factors, DSBs need to be induced at specific sites in all cells reproducibly. Routinely this is done by expressing restriction enzymes or homing endonucleases in human cells (Berkovich et al., 2007; Iacovoni et al., 2010; Massip et al., 2010; Pankotai et al., 2012). The advantage of enzymes is that the site of the DSB can be predicted thus enabling the study of the recruitment of proteins to the site of damage at different time points.
However with homing endonucleases, it is not possible to create stable cell lines because transcriptional regulation is not tight enough and any leakage of expression is selected against (Pankotai et al., 2012). Therefore these homing endonucleases are introduced into cells with viruses (Berkovich et al., 2008).

In this chapter I describe the effects of DNA transfection in human primary cells. Messenger RNA transfection is a better alternative for transfecting primary cells. It is more efficient and does not cause a γH2AX response. Furthermore the expression after a DNA transfection requires cells to progress through mitosis before the DNA can be incorporated in the nucleus. Messenger RNA transfection on the other hand can be expressed in cells independent cell cycle stage.

### 3.2 Results

#### 3.2.1 DNA transfection cause γH2AX response

Routinely cells are transfected with DNA with lipid-based transfection reagents. The liposomes are thought to fuse with the cytoplasmic membrane, releasing the DNA into the cytoplasm (Kim and Eberwine, 2010). The efficiency of a lipid-based transfection method was tested in a telomerase immortalised retinal pigment epithelial cell line, hTert-RPE1 (RPE1). These cells are not transformed and have a normal karyotype (ATCC: CRL-4000). When RPE1 cells are transfected with a plasmid encoding GFP, 34% of the cells expressed GFP 24h after the transfection [Figure 3.1]. To examine the effect of the DNA transfection, the cells were stained with γH2AX antibody. In line with previous publications, the RPE1 cells that were expressing GFP show a robust γH2AX response. This response is not unique to RPE1 cells. When primary fibroblasts (1BR3, CCD and BJ) are transfected with the same lipid based transfection reagent, they show a similar γH2AX response [Figure 3.2].

An alternative method to transfact is by calcium phosphate. Calcium phosphate forms a precipitate with DNA which is taken up by the cells (Kim and Eberwine, 2010). The RPE1 cell line and the primary cells were transfected with a GFP plasmid and stained for γH2AX [Figure 3.3]. In RPE1 cells, the GFP transfected cells show increased γH2AX level. However this response is not seen in the primary BJ and CCD cells. The efficiency of calcium phosphate in these cells is very low. In RPE1 cells the efficiency is about 8%, while the efficiency in BJ and CCD cells is below 1%. Repeated attempts to transfect 1BR3 cells by calcium phosphate were unsuccessful.
Figure 3.1: Lipid based DNA transfection in the primary cell-line RPE1 cells causes a γH2AX response. RPE1 cells were transfected with a GFP plasmid. 24 Hours after the transfection cells were fixed. The GFP-expressing cells have a robust γH2AX response, while cells in the absence of the transfection complexes have low levels of γH2AX.

Figure 3.2: The γH2AX response in DNA transfected primary cells. Similarly to RPE1 cells, other primary fibroblast cells also show a γH2AX response 24h after lipid based DNA transfection.
Calcium phosphate transfection does not cause a H2AX response. Calcium phosphate and DNA form a precipitate that the cells take up. 24 Hours after the transfection cells were fixed and stained for H2AX. The H2AX response does not seem to be activated in calcium phosphate transfected primary cells.

3.2.2 Primary cells are efficiently transfected by RNA, without inducing a H2AX response.

Alternatively, RNA can be transfected into cells. To determine the efficiency and the consequences of RNA transfection, we choose to transflect the Fucci RNA’s. The fluorescent ubiquitination-based cell cycle indicator (Fucci) system consists of mCherry-Cdt1 (30-120) and mAG-Geminin (1-110). Cdt1 is a licensing factor expressed during G1 and early S phase. Geminin on the other hand is expressed from S-phase to G2/M phase. Cells appear red in G1, yellow in S-phase and green in G2-M-phase (Sakaue-Sawano et al., 2008) [Figure 3.4A]. Both mCherry-Cdt1 and mAG-Geminin were cloned in pcDNA3 vector, which has a T7 promoter [Figure 3.4B]. The Fucci RNA’s were transcribed from linearized vectors from the T7 promoter in vitro with a cap analog [m’G(5’)ppp(5’)G]. Subsequently RNAs were polyadenylated in vitro. To check the integrity of the RNAs, aliquods were run on agarose gel. The RNAs should migrate in a defined band and after polyadenylation the band shifted upwards.
**Figure 3.4**: Schematic of the synthesis of the Fucci RNAs. A) mCherry-Cdt1 expression labels G1 cells red. In S-phase both mCherry-Cdt1 and mAG-Geminin are expressed labelling them yellow, while G2 cells only express mAG-Geminin. B) Both mCherry-Cdt1 and mAG-Geminin are cloned in pcDNA3 vector. The vectors were linearized and Cdt1 and Geminin were transcribed in vitro from the T7 promoter. Afterwards the RNA was polyadenylated in vitro. The RNA was run on a 1% agarose gel to check the integrity of the RNA and that polyadenylation reaction increases the size of the run. Figure from Invitrogen-Premo™ FUCCI Cell cycle sensor.

Normally these proteins are stably expressed in cells. However primary cells have a limited number of passages before the cells senesce making stable cell lines in primary cells very difficult.

To assess the efficiency of the RNA transfection in the primary cells, both RNAs were cotransfected. 24 Hours after transfection the cells were fixed and the number of fluorescent cells was counted [Figure 3.5]. In contrast to the DNA transfections, 51-64% of the cells were transfected with RNA.

To see if RNA transfection causes a similar γH2AX response as DNA transfection, all primary cells were transfected with mCherry-Cdt1 mRNA and stained for γH2AX [Figure 3.6]. In all cell types, transfected cells do not show increased levels of γH2AX. Low levels of γH2AX are present in both transfected and untransfected cells.
Figure 3.5: mRNA transfection of all the primary cell lines with the Fucci RNAs. 1µg mCherry-Cdt1 (30-120) and 1µg mAG-Geminin (1-110) were cotransfected in the different cell lines. 51-64% of the cells expressed either mCherry-Cdt1 or mAG-Geminin or both. Quantitation allows us to estimate the cell cycle distribution of the RPE1 cell line and the 1BR3, CCD and BJ cells.

The Fucci system shows the cell cycle distribution. Therefore the Cdt1+, Geminin+ and double positive cells were counted [Figure 3.5]. The RPE1 cells show a larger percentage of cells in S and G2/M phase compared to the other primary cells. This is consistent with a doubling time of about 24h (Lei and Erikson, 2008). The other primary cells have a longer cell cycle (estimated 36-40h) and the quantification of the Cdt1+, Geminin+ and double positive cells reflects that.
Primary cells transfected with the mCherry-Cdt1 mRNA do not show a γH2AX response. Cells were transfected with an in vitro transcribed RNA with a cap analog and polyadenylated in vitro. 24 Hours after transfection the cells were fixed and stained with a γH2AX. Equally low levels of γH2AX are present in both transfected and untransfected cells.

The distribution of the Cdt1+, Geminin+ and double positive cells after RNA transfection was compared to RPE1 cells stably expressing mCherry-Cdt1 or mAG-Geminin [Table 3.1; Figure 6.7B; Figure 6.9B]. The profile of the stably expressing mCherry-Cdt1 cells show 43% in G1, 35% in S and 22% in G2-phase, while mAG-Geminin expressing cells have 50% in G1, 33% in S and 18% in G2 phase (Chapter 6). The RNA transfected cells on the other hand show 64% in G, 16% in S-phase and 20% in G2. The RNA transfected cells have a lower percentage of cells in S-phase. During RNA transfection cells are serum starved for 4 hours which could potentially alter the cell cycle profile.

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
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<th>G2/M</th>
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<tr>
<td>mCherry-Cdt1 stable</td>
<td>43%</td>
<td>35%</td>
<td>22%</td>
</tr>
<tr>
<td>mAG-Geminin stable</td>
<td>50%</td>
<td>33%</td>
<td>18%</td>
</tr>
<tr>
<td>Transfected Fucci RNAs</td>
<td>64%</td>
<td>16%</td>
<td>20%</td>
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Table 3.1: Comparison of the Fucci RNAs with stable RPE1 cell lines expressing either mCherry-Cdt1 or mAG-Geminin. Quantification of the Cdt1+ (G1), Cdt1+/Geminin+ (S) and Geminin (G2) after transfection with the Fucci RNAs. The stable cell lines are characterized in more detail in Chapter 6.
To assess whether the mCherry-Cdt1 and mAG-Geminin are properly regulated, CCD cells were transfected with both Fucci RNAs. 24 Hours after the transfection, mAG-Geminin positive cells were selected and watched for 10h [Figure 3.7]. 8 Out of the 9 mAG-Geminin positive cells that were imaged on the live-cell microscope successfully complete mitosis and expressed mCherry-Cdt1 in both daughter cells. One of the mAG-Geminin positive cells changed to Cdt1 expression without mitosis. Therefore it is likely that in the majority of the cells the Fucci RNAs are properly regulated although only 9 cells were watched.

![Figure 3.7: Live cell imaging of CCD cells transfected with the Fucci RNA's. CCD cells were transfected and 24h after the transfection, cell were imaged for 10h. 8 of the 9 imaged cells successful complete mitosis like the shown cell. One mAG-Geminin expressing cell changed to mCherry-Cdt1 expression without going through mitosis.](image)

3.2.3 Expression after DNA transfection is limited to G1 cells, while the expression after RNA transfection is independent of the cell cycle.

It has long been speculated that translocation of DNA from the cytoplasm to the nucleus mainly occurs after breakdown of the nuclear membrane in mitosis (Kim and Eberwine, 2010). To investigate this hypothesis, the mCherry-Cdt1 RPE1 stable cell line was transfected by calcium phosphate with a GFP plasmid. The cell cycle distribution of the GFP expressing cells was quantified. 24 Hours after the transfection, the majority (93%) of the GFP expressing cells also expressed mCherry-Cdt1 [Figure 3.8]. This indicates that after a DNA transfection, the protein is not expressed until after mitosis and it requires the breakdown of the nuclear membrane in mitosis to be incorporated to the nucleus. It also explains why calcium phosphate is effective in highly proliferative cells, but ineffective in slow proliferating cells.
Figure 3.8: A GFP plasmid transfected by calcium phosphate in stably expressing mCherry-Cdt1 RPE1 cells is preferentially expressed in G1 cells. At 24 hours after transfection the cells were fixed. Quantification of the GFP expressing cells showed that it is preferentially expressed in G1 cells. N=100 cells.

Taking it all together DNA transfections seem to be expressed preferentially in G1, while RNA transfections are expressed in cells independent of the cell cycle stage. With RNA transfection, proteins can be efficiently expressed in primary cells without causing a γH2AX response.

Another potential use of RNA transfections is to induce DSBs at specific sites. DNA transfection would cause a background response at 24h after the transfection. Moreover the enzyme will be preferential expressed in G1 cells. This means that the damage is specifically induced in a subpopulation of cells.

The AsiSI restriction enzyme has an 8bp recognition sequence and cuts about once per Mb. It has previously been used to study induce damage at specific sites (Iacovoni et al., 2010).

Figure 3.9: The synthesis of AsiSI mRNA. The open reading frame of AsiSI was fused with the EMCV IRES element and clones into pBluescript. This vector has a T7 promoter from with the ORF was transcribed in vitro. The RNA was subsequently polyadenylated in vitro.
The AsiSI open reading frame (ORF) was amplified from the *Arthrobacter* genomic DNA. The AsiSI was fused to an Encephalomyocarditis virus (EMCV) IRES element [Figure 3.9]. The RNA was transcribed *in vitro* in the absence of a cap analog relying on the IRES element for efficient translation. When IRES-AsiSI mRNA was transfected, RPE1 cells showed many γH2AX foci scattered throughout nucleus [Figure 3.10].

In summary, DNA transfection has a low efficiency in primary cells and causes a γH2AX response. RNA transfections however have higher transfection efficiencies in primary cells and do not evoke a γH2AX response. In addition, expression after DNA transfection requires the cell to go through mitosis, while proteins can be expressed after RNA transfection throughout the cell cycle. RNA transfections can be used to transfect fluorescently tagged proteins (Fucci RNAs) as well as a method to induced DNA damage at specific sites (AsiSI).

### 3.3 Discussion

There are various ways to induce exogenous proteins into cells. However primary cells are notoriously difficult to transfect by chemical methods. In this chapter, DNA was transfected by lipid-based method and calcium phosphate. The lipid-based method was moderately effective in primary cells, but the cells had a pannuclear γH2AX response. Alternatively cells can be transfected by calcium phosphate. It does cause a moderate γH2AX response in RPE1, but it had low transfection efficiency. In my personal experience, transfections with calcium phosphate can introduce large quantities of DNA into a few cells.

The low efficiency can be explained by the need for the nuclear membrane to break down during mitosis before the DNA can be incorporated to the nucleus. The latter is needed for its expression and therefore the expression after DNA transfection is limited to G1 cells at 24h. RNA transfection on the other hand does not need translocation to the nucleus and can immediately be translated in the cytoplasm. Therefore RNA transfections can express proteins throughout the cell cycle.

Eukaryotic mRNAs typically have a cap and polyA tail. The Fucci RNAs were *in vitro* transcribed in the presence of a cap analog and polyadenylated. However not all RNAs rely on the cap for efficient translation. Alternatively the presence of an IRES element can also stimulate translation as demonstrated with the AsiSI mRNA. These elements are shown to be able to substitute for the cap structure after RNA transfection.
Figure 3.10: The expression of the restriction enzyme AsiSI. RPE1 cells were transfected with the restriction enzyme AsiSI mRNA. The uncapped RNA is fused to an EMCV IRES element. The RNA is in vitro transcribed and polyadenylated. Certain cells, which are presumably transfected, show a massive increase in γH2AX, while other cells do not.
The polyA tail affects the translation efficiency and RNA stability (Elango et al., 2005). Therefore the length of the polyA tail is a potential way to regulate the translation of the transfected RNA. Transfecting RNAs without a polyA tail limits the expression. At early times the expression is similar, however at 24h there is a clear difference between the RNA transfected with and without the polyA tail (Chapter 6).

The live cell movies show that the polyadenylated Fucci RNA can be expressed in the cells for at least 48h. The cell in Figure 3.7 switched from mAG-Geminin expression to mCherry-Cdt1 expression 32 hours after transfection.

Of particular interest to this thesis, RNA transfection can also be used to induce DNA damage at specific sites. Transfection of a plasmid encoding a restriction enzyme would preferentially be expressed in G1 hence studies would be looking in a specific subpopulation. Enzymes transfected by RNA would allow studies in of all stages of the cell cycle.

The expression of AsiSI shows that RNA transfection can be used to express a restriction enzyme. Another enzyme that has been used to induce DNA damage at specific site is I-PpoI. A protocol for expressing this enzyme relies on transduction with retroviruses (Berkovich et al., 2008). However viral transductions have also been shown to induce a γH2AX response (Schwartz et al., 2009). Therefore RNA transfection might be a better alternative. AsiSI transfected cells show very distinct γH2AX foci instead of a pannuclear staining. The defined DNA damage sites enable ChIP experiments looking at the recruitment of proteins to the site of damage over time.

Alternatively, RNA transfection can also be used to express proteins that might be involved in the DNA damage response. To clearly see the difference between before and after damage the method of transfection should not induce DNA damage by itself. Because DNA transfection does induce a γH2AX response, RNA transfection is preferred. Whether the response after DNA transfection is limited to a γH2AX response or whether downstream proteins are also affected, has not been addressed in this study. But I have shown that RNA transfection is a better alternative to DNA transfection for primary cells. RNA transfection does not induce a γH2AX response, has higher efficiency in primary cells and can transfect all stages of the cell cycle.

In summary, RNA can not only be used to express fluorescent fusion proteins, but also restriction enzymes to induce DNA damage at specific sites. RNAs with a cap analog and IRES element have been demonstrated to be successfully expressed and the presence and length of the polyA tail provide a potential way to regulate the stability of the RNA.
Chapter 4

Gene targeting into the rDNA repeat.

4.1 Introduction

In human cells, multiple NORs can be involved in the formation of nucleoli while other NORs can be transcriptionally silent. These silent NORs tend to be dissociated from the nucleolus. However the high sequence similarity between the acrocentric short arms makes it difficult to identify and study the behaviour of individual NORs (Floutsakou et al., 2013). With long arm probes it is possible to identify individual chromosomes that are closely associated with nucleoli in fixed cells. However in living cells, it is impossible to study the behaviour of individual NORs through the cell cycle or upon varying nutrient conditions. Tracking both rDNA and rRNA will answer intriguing research questions. Which chromosomes are active and associated with nucleoli? Is the activity status passed on through mitosis? Are there conditions where the activity status of an NOR can change? For individual repeats, does transcription fluctuate during the cell cycle and are there treatments that can alter the transcriptional activity of the repeat? Is the activity status of individual repeats propagated through mitosis?

4.1.1 Strategies to track the localisation of sequences in vivo

Tracking sequences in living cells can be at the RNA or DNA level. Sequences that are normally not found in the genome can be integrated and used to follow specific loci in vivo. Labelling of rDNA will allow us to follow the location of the NOR throughout the cell cycle and is independent of the transcriptional activity of the NOR. It will not be affect by the transcription silencing of the rDNA in mitosis (Klein and Grummt, 1999)or silencing of the NOR. Sequences that are commonly used are the Tet operator (TetO) and the Lac operator (LacO) (Robinett et al., 1996). These sequences are bound by respectively the Tet-Repressor (TetR) and Lac-Inducer (LacI) proteins fused to a fluorescent protein to visualise a locus in the living cell. They can either be integrated randomly in the genome or targeted to a specific locus. In yeast the latter approach has been used to follow the rDNA specifically through mitosis, meiosis and after DNA damage (Harrison et al., 2009; Li et al., 2011a; Miyazaki and Kobayashi, 2011; Torres-Rosell et al., 2007).

Labelled rRNA will reflect the transcriptional activity of endogenous promoters and how that might fluctuate throughout the cell cycle, upon stress and after mitosis in daughter cells. Depending on the integration site in the rDNA repeat, it will be possible to see different precursors of the ribosomal RNA. There are 2 methods to visualise RNA in living cells both depending on RNA hairpin structures. The bacterial phage MS2 coat protein fused to GFP
recognises the MS2 RNA hairpins, which is especially suited to follow RNA transport in the cytoplasm (Bertrand et al., 1998). Recently a second method was developed. Spinach is a small secondary structure in the RNA. The hairpin will bind the non-fluorescent molecule DFHB that upon binding will fluoresce (Paige et al., 2011).

4.1.2 Gene targeting

Whether sequences will be tracked at RNA or DNA levels, all strategies require targeted integration of a sequence at a specific site. Sequences can be targeted to a specific locus via homology directed repair, but this requires large homology arms and it has a low efficiency. Homologous recombination was first used to modify mouse embryonic stem cells. Cells would be screened and positive clones would then be implanted in a blastocyst to generate chimeric mice. By breeding heterozygous and homozygous mice could be made (Capecchi, 1989; Gossler et al., 1986; Koller and Smithies, 1992).

Homologous recombination can also modify human cells but spontaneous homology directed repair is a rare event. The fibrosarcoma cell line used in this chapter, HT1080 typically has a rate of 1 event per 10^6 -10^7 cells (Ganguly et al., 1994; Itzhaki and Porter, 1991; Porter and Itzhaki, 1993; Thyagarajan et al., 1995). Homologous recombination has been used to target the 5’ETS of the rDNA repeat with this targeting strategy, but the efficiency was low (Wen et al., 2008).

DNA double strand breaks at the target site can greatly enhance the efficiency. It was first reported that the homing endonuclease I-SceI from Saccharomyces cerevisiae induced double strand breaks and stimulate HR even in extrachromosomal DNA both in yeast and mammalian cells (Choulika et al., 1995; Rouet et al., 1994; Sargent et al., 1997). However it is not possible to design I-SceI to target new sequences.

Zinc finger nucleases (ZFNs) are artificial endonucleases that usually have 3-4 zinc finger domains which each recognise a specific triplet of nucleotides giving each domain a recognition sequence of 9-12bp [Figure 4.1]. The zinc finger modules are fused to a FokI nuclease domain, which cleaves sequence-independent (Smith et al., 2000). Due to the requirement for heterodimerization and the large binding sequence (18-24bp) it is possible to target breaks to a specific locus (Miller et al., 2007; Szczepk et al., 2007). The rate of gene targeting of the ZFNs is comparable to I-SceI, but the ZFNs can in theory be designed to target anywhere in the genome (Bibikova et al., 2003; Porteus and Baltimore, 2003).
Figure 4.1: Structure of zinc finger nucleases. Individual zinc finger domains are fused to each other and the FokI endonuclease domain that can only be activated upon heterodimerisation.

ZFNs have successfully been used to correct mutations in stem cell lines and primary T-cells (Lombardo et al., 2007; Urnov et al., 2005). Sequences that have been targeted can be single base pair change to entire expression cassettes without selective pressure with good efficiencies (Moehle et al., 2007).

ZFNs can be purchased from Sigma Aldrich, but are very expensive. Therefore for these pilot experiments, I utilised a homing endonuclease I-PpoI.

4.1.3 The homing endonuclease I-PpoI

The homing endonuclease I-PpoI is encoded in the intron PpLSU3, which is inserted in the rDNA of the slime mold Physarum polycephalum at the end of each 26S/28S rRNA gene (Muscarella et al., 1990). The intron encodes the I-PpoI endonuclease ORF in the 5’half and a ribozyme in the 3’half, which catalyses the self-splicing of the intron and ensures that the intron is removed from the pre-rRNA (Lin and Vogt, 1998, 2000; Muscarella et al., 1990; Ruoff et al., 1992). The I-PpoI protein forms homodimers and cleaves a 15bp recognition sequence (CTCTCTTAAGGTAGC) leaving a 4bp 3’-overhang (Ellison and Vogt, 1993; Lowery et al., 1992; Wittmayer and Raines, 1996). The site is conserved in the human 28S sequence. In contrast to restriction enzymes, at high enzyme concentrations I-PpoI can tolerate some degeneracy at certain positions in its recognition sequence albeit much lower cleavage efficiencies (Argast et al., 1998; Wittmayer et al., 1998).

Crystal structures of I-PpoI in complex with DNA revealed several amino acids to be important for binding and catalysis (Mannino et al., 1999). Histidine 98 was important for cleavage of the phosphate bond in the DNA and mutation into an Alanine renders the enzyme catalytically dead (Eklund et al., 2007; Flick et al., 1998; Galburt et al., 1999; Mannino et al., 1999).

I-PpoI can be expressed in human cells and introduce double strand breaks (Berkovich et al., 2007; Monnat Jr et al., 1999; Oka et al., 2011; Pankotai et al., 2012).

This chapter demonstrates the feasibility of targeting of heterologous sequences in the rDNA repeat and shows that the targeting can be stimulated with the addition of RNA pol I regulatory elements.
4.2 Results

4.2.1 mRNA transfection to induce double-strand breaks specifically in the rDNA with the homing endonuclease I-PpoI

Constitutive expression of I-PpoI in human cells is lethal as has been shown in yeast (data not shown) (Lin and Vogt, 2000). Transient DNA transfection however induces a background γH2AX reaction [Chapter 3]. Therefore to get transient high expression without the risk of integration, I-PpoI was transfected as mRNA.

Experiments in this chapter were carried out with the previously published construct, HA-ER-I-PpoI (Berkovich et al., 2007). In the absence of oestrogen, the oestrogen receptor (ER) will force localisation of I-PpoI in the cytoplasm. Nuclear translocation was induced by the addition of tamoxifen. However when cells were transfected with HA-ER-I-PpoI and stained for γH2AX as a marker for DNA damage, it was observed that even in the absence of tamoxifen, I-PpoI causes DSBs in the rDNA [Figure 4.2].

Charcoal stripping of serum can remove oestrogen from FBS (Peck and Clark, 1977) and phenol-red has been reported to oestrogen mimic (Berthois et al., 1986). However charcoal stripped serum and phenol-red free medium did not improve the number and size of the foci in the absence of tamoxifen [data not shown].

For these reasons, the I-PpoI construct was redesigned to study the DNA damage response in the rDNA (Chapters 5 and 6), but the targeting experiments were carried out using this HA-ER fusion construct.

The I-PpoI recognition sequence in the rDNA is not unique in the human genome. Besides the I-PpoI site in the rDNA repeat, elsewhere in the genome the DAB1 and ERC2 sites have been used in studies (Berkovich et al., 2007; Pankotai et al., 2012). To identify all potential sites, the recognition sequence was used in Basic Local Alignment Search Tool (BLAST) searches against the Human Genome (built GRCh37-hg19). All sequences that had an I-PpoI site are depicted in figure 4.3. Except for the DAB1 and ERC2 sites, all other sites have a high degree of sequence homology to the rDNA. These are likely rDNA pseudogenes that have been randomly integrated after reverse transcription throughout evolution (Arnold et al., 1987).
Figure 4.2: The HA-ER-I-Ppol mRNA is able to induce DNA damage in hTert-RPE1 cells in the presence and absence of tamoxifen. hTert-RPE1 cells were either mock transfected or transfected with HA-ER-I-Ppol. At 6 hours after the start of the transfection, cells were fixed and stained with γH2AX as a DNA damage marker. The cells shown are representative of all transfected cells. In the tamoxifen treated cells UBF forms a few large UBF caps, while in the ethanol treated cells the caps are smaller and more numerous. The γH2AX foci adjacent to the UBF caps in the ethanol treated cells are smaller and fainter, while in the presence of tamoxifen the foci are larger and brighter.

These same sites and 3 additionally contigs which have not been mapped to the human genome, were also identified by Pankotai and colleagues (2012). The high sequence similarity around the I-Ppol site in the contigs indicates that these unlisted contigs are also likely to be pseudogenes.

4.2.2 Targeting is stimulated by the presence of double strand breaks in the rDNA repeat.

The targeting plasmid contains 1.5kb of the 28S rDNA from around the I-Ppol site. A previously described cassette of an EMCV IRES fused to a Neomycin resistance gene (NeoR) and a SV40 polyA-signal (ENA) (Palmer et al., 1993) was cloned into the I-Ppol site, destroying the site. The targeting plasmid does not have a mammalian promoter and will not be transcribed, unless it is integrated in a transcribed region. The targeting plasmid was also constructed with the IRES and NeoR ORF in the antisense orientation (pTv2-ANE) to estimate levels of NeoR resistance arising for random integration (Figure 4.4A).
Figure 4.3: I-PpoI restriction sites in the human genome identified by BLAST searches aligned around the I-PpoI site. The sequence surrounding the I-PpoI site in the rDNA differs significantly from the other sites. The rDNA are in exons. Chr-Chromosome.

<table>
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<th>Location</th>
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<th>Chromosome</th>
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The sequence surrounding the I-PpoI site in the rDNA differs significantly from the other sites. None of the I-PpoI sites outside of the rDNA are in exons.
Figure 4.4: Initial targeting experiments into the rDNA repeat. A) The targeting strategy. The plasmid contained 1.5kb of the 28S rDNA around the I-Ppol site. In the cleavage site of the plasmid an EMCV IRES element with a Neomycin resistance gene followed by a SV40 polyA-signal (ENA) HT1080 cells were transfected with the I-Ppol mRNA followed by plasmid transfection (pTv1-ENA: sense orientation; pTv2-ANE: antisense). Next day, G418 selection was applied and the resulting colonies were analysed by PCR 1-2 weeks later. B) The number of colonies per condition. C) 1-3 Colonies of each condition were analysed for targeted integrated with PCR. One of the primers (red arrow) was located at the end of the Neo<sup>R</sup> gene, while the other primer was located downstream of the homology region (grey area) in the 28S. None of the colonies in the absence of targeted DNA damage (vector only) shows the targeted integration in the rDNA. 1 out of 3 colonies in the presence of low levels of DNA damage without tamoxifen and all three colonies in the presence of high levels of DNA damage were positive for targeted integration.

Orientation should not affect the rate of random integrations, while only the integrated sense plasmid can be translated into a functional Neomycin transferase.

HT1080 cells (10<sup>5</sup>) were transfected with 2µg HA-ER-I-Ppol mRNA and 3h later with the targeting plasmids [Figure 4.4A]. The next day selection with G418 was applied and the resulting colonies were analysed by PCR for integration 1-2 weeks later. In general it was noted that 24-48h after the mRNA transfection, many cells died due to the toxicity of I-Ppol to cells. Both the targeting vectors resulted in 20 colonies in the absence of targeted DNA damage [Figure 4.4B]. When cells were transfected with the plasmids and I-Ppol mRNA (+I-Ppol mRNA), the vector in the sense orientation gave 3 colonies, while in the other orientation no colonies grew. When HT1080 were transfected with the plasmids and the I-Ppol mRNA and also treated with tamoxifen (+tamoxifen), the vector in the sense orientation resulted in 6 colonies. The antisense vector resulted in 1 colony.

From each condition 1-3 random colonies were selected for analysis by PCR. One of the primers was located at the end of the Neo<sup>R</sup> gene and the other primer downstream of the...
homology region present in the targeting vectors [Figure 4.4C]. Only a correctly targeted integration would yield a PCR product. When the vectors were transfected alone, none of the colonies had a correctly targeted ENA sequence in the rDNA. Without the tamoxifen 1 of the 3 colonies tested positive for targeted integration, while 3 of the 3 colonies tested positive with tamoxifen. For the antisense vector none of the colonies yielded a PCR product.

To verify that the PCR product specifically detects targeted integration in the rDNA, the PCR products from each of the 3 “+tamoxifen” colonies were cloned and sequenced. The sequence shows part of the Simian vacuolating virus 40 (SV40) polyadenylation signal fused to the rDNA beyond the 3’-homology region. The sequencing showed that all PCR products were from sequences that had correctly integrated via homology directed repair and were not randomly integrated [Figure 4.5]. One of the sequenced clones had a small deletion in a region that corresponds to variable region 9. These variable regions vary greatly between species and there is even variation between repeats within the same species [Figure 4.6] (Gonzalez et al., 1985; Gonzalez et al., 1988; Maden et al., 1987). Individuals have different levels of variants and even within an individual person, different tissues will express different levels of the variants (Kuo et al., 1996; Leffers and Andersen, 1993). The sequence difference between clone 6 and the consensus rDNA repeat is likely an rDNA variant.

**CLUSTAL 2.1 multiple sequence alignment: Forward sequencing**

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**SV40 polyA signal**

**rDNA homology region**
Figure 4.5: Multiple sequence alignment of the cloned PCR products of three of the clones of the ++pool (with RNA transfection and with tamoxifen).
The variable regions have been correlated with secondary structure features of ribosomal RNAs under EM (Wakeman and Maden, 1989). The V8 region is thought to form a double looped structure [Figure 4.6].

The integrations were not visible with FISH on metaphase chromosomes, so the integration sites could not be assigned to specific chromosomes. A single integration does not generate enough fluorescence to be visible.

![Figure 4.6: A schematic representation of the 28S rRNA with the variable regions and the corresponding secondary structure features that have been observed by EM. The homology region is indicated with the blue line. The double loop structure is thought to be formed by the sequences of the V8 region and is depicted in green. Figure adapted from Wakeman and Maden, 1989.](image)

4.2.3 Targeting is enhanced by the inclusion of UBF binding site arrays

To improve the targeting efficiency, elements that will help to localise the plasmid to the nucleolus were added on. Unpublished results indicate that certain DNA elements have the ability to localise a plasmid to certain part of the nucleus. For example, RNA polymerase I regulatory elements have a tendency to integrate near the NORs. The pseudoNORs, which consisted of *Xenopus* enhancer (Xen) elements, have been shown to bind human UBF. In the pseudo-NOR cell lines, 4 out of 8 clones had the arrays integrated in or near NORs (Mais et al., 2005). The hypothesis is that either the elements bind UBF and are therefore dragged to the nucleolus or that the DNA sequence itself stimulates localisation to the nucleolus where it can bind UBF. Either way it would be expected that Xen elements added to the targeting plasmids at the 3’end of the homology region, would improve the targeting efficiency by increasing the local concentration in the nucleolus.

HT1080 cells (10^5) were first transfected with the targeting plasmids to ensure their localisation in the nucleolus. 24h later the cells were transfected with 1µg HA-ER-I-PpoI mRNA and the next day selection was applied [Figure 4.7A]. The quantity of RNA was lowered to reduce cell death due to toxicity of I-PpoI expression.
Figure 4.7: Xen elements improve targeting into the rDNA repeats. A) The targeting strategy. HT1080 cells were transfected with the targeting plasmids containing Xen elements (red blocks) after the homology region the day prior to transfection with the I-Ppol mRNA. The next day, cells were selected with G418 and the stable pools were analysed. B) Number of colonies for the different targeting conditions of the first and second experiment. C) PCR analysis for targeted integration in the pools of G418 resistant cell.

The estimated number of pTv1-ENA-Xen colonies was about 10-fold higher than pTv2-ANE-Xen [Figure 4.7B]. Compared to the first experiment, numbers of surviving colonies was significantly higher (3 vs. an estimated ~150 colonies in pTv1+RNA+tamoxifen). This indicates that the addition of the Xen elements can significantly improve integration efficiency or the expression of the NeoR gene.

Genomic DNA from the pools was tested with the primers at the 3’integration site. All the pTv1-ENA-Xen pools showed targeting although the levels differed [Figure 4.7C]. To confirm the integration into the NOR, metaphase chromosomes were hybridised with the rDNA probe and a probe against the inserted sequence, ENA. Individual metaphase spreads showed different intensity of ENA signal. Metaphases with robust signal presumably have multiple integrations into the rDNA repeats. The pools of cells which were transfected with the plasmid only (--pool) were compared with the pool with were transfected with the plasmid and HA-ER-I-Ppol mRNA in the presence of tamoxifen (++pool).

10% of the metaphase spreads of the HT1080 cells without DSBs had detectable ENA signal at the NOR, while with DSBs 50% of the spreads had detectable ENA signal at the NOR [Figure 4.8A]. About 20-25% of the spreads had ENA localised elsewhere in the genome irrespective of DNA damage.
To estimate what percentage of the integrations was random, metaphase spreads were hybridised with Xen and the rDNA [Figure 4.8B]. Without mRNA transfection, 25% of the metaphases have detectable Xen colocalizing with rDNA, while this decreases to 7% with DSBs. The levels of metaphases without any detectable Xen signal is stable, while in the presence of DSBs the number of random integration outside of the NORs increases from 0% to 20%.

**RNA FISH of the ++pool confirmed that the sequence was transcribed in the nucleolus and thus is presumably transcribed by RNA polymerase I [Figure 4.9A]. Other cells in the same pool do not show any ENA staining showing the specificity of the ENA probe.**

To assess how efficiently the ENA RNA is translated into protein, whole cell lysates of the three pools were blotted for the neomycin phosphotransferase. This showed that the protein was expressed, but at low levels compared to an unrelated G418- resistant cell line, where expression of Neo\(^R\) is driven by the RNA polymerase II SV-40 promoter [Figure 4.9B].
RNA FISH shows that ENA is transcribed in the nucleolus (kindly done by Alice Grob). Not all cells of the ++pool have a visible ENA signal indicating that the probe is not detecting an endogenous RNA. Western blot shows that ENA RNA is not efficiently translated into protein. “–pool”, only targeting plasmid transfected; “+pool”, targeting plasmid and RNA transfected; “++”pool, targeting plasmid and RNA transfected and tamoxifen added.

In summary, targeting foreign sequences to the rDNA repeats is possible and it is stimulated by DSBs at the target site. The addition of *Xenopus* enhancer elements to which UBF binds with high affinity, increased the number of colonies and the number of correct integrations.

### 4.3 Discussion

From both a basic research and a translational research perspective, targeting in the rDNA would be interesting, therefore I explored whether it is possible and what influence DNA damage at this target site has on the efficiency. These pilot experiments utilised an endonuclease that cleaves the 28S rDNA in *Physarum polycephalum*. The results presented here demonstrate that it is possible to target foreign sequencing into the rDNA repeat and that in agreement with previous studies, the presence of DSBs at the integration site stimulates integration by homology directed repair (Bibikova et al., 2003; Choulika et al., 1995; Porteus and Baltimore, 2003; Rouet et al., 1994; Sargent et al., 1997). Sequencing of the PCR products confirmed that the ENA sequence was targeted into the rDNA correctly via homology directed repair.
The addition of UBF binding sites (Xen elements) on the targeting plasmid improved the targeting efficiency several fold. This might be explained by the tendency of this plasmid to localise to the nuclear compartment where UBF is normally localised. The plasmid would have an increased local concentration which stimulates homology directed repair. Additionally in the first experiment both the RNA and DNA were transfected almost simultaneously. I-PpoI would start to be expressed while the plasmid was transfected. This might have decreased the targeting efficiency because the plasmid was not in the vicinity of the DSB. Also in the first experiment 2ug of I-PpoI mRNA was transfected, which was decreased to 1ug in the second targeting experiment due to the toxicity of I-PpoI. Therefore in the second attempt the DNA was transfected first to enable the plasmid to localise to the nucleolus and the amount of transfected RNA was reduced.

**Figure 4.10: A model for Xen-driven integration versus homology directed integration in the presence of DSBs.** In the absence of DSBs the Xen elements facilitate the formation of big concatenates which will integrate randomly in the NOR. However DSBs stimulate homology directed repair which is more dependent on the sequence around the break site.

Metaphase spreads of the same pool of HT1080 cells in the absence of DSBs, the ENA probe was detected in 10% of spreads at the NORs and 25% with the Xen probe. This indicates that the ENA signal is probably largely due to random integration. Integrations are likely due to the presence of the Xen elements and to not depend on homology. Therefore these integrations are unlikely to be by HR [Figure 4.10]. The PCR showed low levels of the targeting specific PCR product under these conditions. It is thought that the Xen elements help to form big concatenates in cells. These then integrate in a single event into the rDNA hence the high levels of Xen in the NORs. This will integrate some ENA correctly which
explains the PCR product and the ENA signal on 10% of the metaphase spreads. But the integrations in these events is thought to be dependent on the Xen elements.

When DSBs were induced at the target site, 50% of spreads had ENA signal at the NORs, but the Xen signal decreased to 7%, indicating that the number of homology directed repair events is increased. There are multiple smaller integrations which are dependent on the homology arms because of the DSBs. This translates in less Xen integrating in the NORs and more ENA being correctly targeted to the rDNA. The presence of the DSBs makes that integrations are more dependent on the homology sequence, rather than the Xen elements.

RNA polymerase I has been reported to be able to transcribe protein-coding sequences integrated in the rDNA. These uncapped sequences can be translated into protein (Lin and Vogt, 2000). Additionally IRES elements can be used to enhance the translation efficiency of the uncapped RNA polymerase I transcripts (Palmer et al., 1993). However integration into the highly structured rRNA might interfere with the correct folding or recognition of the IRES element explaining the poor translation efficiency in our system. The targeting site is in a region of the 28S with a highly conserved secondary structure (Clark et al., 1984; Gorski et al., 1987). A different target site might yield better protein expression. The study from Wen et al. target the beginning of the 18S and got efficient protein expression similar to what had been seen in the Palmer study.

In summary, targeting foreign sequences to the rDNA repeats is feasible. DSBs at the target site stimulate integration by homology directed repair. The addition of *Xenopus* enhancer elements to the targeting plasmid can improve the targeting efficiency.
Chapter 5

Characterisation of rDNA damage sensing and transcriptional inhibition in the nucleolus.

5.1 Introduction
In the previous chapter I described the response of nucleoli to DNA damage. Upon expression of the homing endonuclease I-PpoI which cleaves preferentially in the nucleolus, the nucleoli segregate to form nucleolar caps. A similar response of nucleoli has been documented to DSBs induced by IR in mouse cells (Kruhlak et al., 2007). However there is no detailed study into the sensing of DSBs in nucleoli and whether the mechanisms that operate in the rest of the nucleus also apply in the nucleolus. Experiments in this chapter describe the recognition of the DSB and the response of nucleoli. The subsequent repair and cell cycle signalling will be described in the next chapter.

5.1.1 The DNA damage response
The initial response to DSBs involves changes in the local chromatin environment. This leads to the activation of kinases and the signal is amplified to induce cell cycle arrest and recruitment of the repair machinery. Many factors are retained at the site of damage forming foci. Other factors such as the checkpoint kinases Chk1 and Chk2 are activated at the site of damage and spread from there through the entire nucleus (Lukas et al., 2003).

5.1.2 Double strand breaks distorts the chromatin which is sensed by the PIK kinases.
A DNA double strand break causes a change in the chromatin environment. In proximity to the DSB, the chromatin decondenses (Kruhlak et al., 2006). The ATP-dependent chromatin remodelling complex INO80 has been reported to be recruited to the site of damage aiding in repair and possibly decondensation of the chromatin (Morrison et al., 2004; van Attikum et al., 2004)

The distortion of the local chromatin environment is sensed by c-Abl and Tip60 [Figure 5.1]. Under normal conditions the acetyltransferase Tip60 is present in multiple complexes; the chromatin modifying complex Tip60 complex (Sapountzi et al., 2006) and in separate constitutive complexes with DNA-PK (Jiang et al., 2006) and the ATM (Sun et al., 2005). In addition, Tip60 has been reported to interact with and acetylate UBF (Halkidou et al., 2004). It is unknown in which complex Tip60 interacts with UBF.

c-Abl, a tyrosine kinase constantly phosphorylates Tip60 on Tyr44, which is quickly dephosphorylated under normal conditions. A double strand breaks will distort the chromatin exposing trimethylated histone H3 lysine 9 (H3K9me3). The phosphorylated tyrosine on
Tip60 enhances binding of Tip60 to H3K9me3 (Kaidi and Jackson, 2013). H3K9me3 has been shown to be present on both active and inactive rDNA repeats (Yuan et al., 2007). The substrate specificity of Tip60 after binding to H3K9me3, shifts from acetylating chromatin to ATM, where Lys3016 on ATM gets acetylated by Tip60 (Sun et al., 2005; Sun et al., 2007).

![Diagram of Tip60 and ATM activation](image)

**Figure 5.1: Activation of ATM.** Chromatin changes lead to the activation of ATM. Under normal conditions the histone modification H3K9me3 is masked by chromodomain containing proteins. Under these conditions, the phosphorylation of c-Abl on Tip60 is rapidly turned over. A DSB causes the chromatin in the vicinity to relax and H3K9me3 becomes exposed. Phosphorylated Tip60 has increased affinity for H3K9me3 and upon binding the phosphorylated form is stabilised. The activity of Tip60 is then redirected from chromatin to ATM, which gets acetylated on lysine 3016 (Kaidi and Jackson, 2013; Sun et al., 2005; Sun et al., 2007).

5.1.3 **The chromatin around the break site is modified to recruit repair factors.** Exposed DNA ends are vulnerable to exonucleases that can remove nucleotides and hence information is lost. Therefore the ends are quickly bound by the Ku70-Ku80 heterodimer to protect them. Another complex that is recruited is the MRN complex consisting of Mre11, Rad50 and Nijmegen breakage syndrome 1 (Nbs1). The crystal structure of this complex revealed that it can bind to both sides of the break and keep them in close proximity of each other (Lammens et al., 2011; Williams et al., 2008).
ATM, after being acetylated by Tip60 and upon interacting with MRN complex, phosphorylates itself (Lee and Paull, 2004; Sun et al., 2007). This autophosphorylation on Ser1981 triggers the dimer to disassociate into active monomers (Lee and Paull, 2005). ATM can then phosphorylate a wide range of targets (Matsuoka et al., 2007; Stokes et al., 2007). One of these targets is the histone variant H2AX on Ser139, also referred to γH2AX (Rogakou et al., 1998)[Figure 5.2].

Figure 5.2: Modification of the local chromatin to facilitate recruitment of repair factors. ATM is recruited to the DSB phosphorylating H2AX on S139 also called γH2AX. This phosphorylation can spread up to 1Mb away from the break site (Rogakou et al., 1999). γH2AX phosphorylation recruits MDC1. Upon recruitment to the break site, MDC1 gets phosphorylated by ATM, which recruits the E3 ubiquitin ligases RNF8 and RNF168. They ubiquitinate H2A which recruits 53BP1 and BRCA1 (Jackson, 2002; Lavin, 2008; Thompson, 2012).

All 3 PIK kinases ATM, DNA-PK and ATR have been shown to phosphorylate H2AX (An et al., 2010; Burma et al., 2001; Stiff et al., 2004; Ward and Chen, 2001). They phosphorylate the consensus sequence [S/T]Q (Downs et al., 2000; Kim et al., 1999).
Phosphorylation of H2AX is known to spread 1 megabase either site of the break (Iacovoni et al., 2010; Rogakou et al., 1999; Shroff et al., 2004). Phosphorylation of H2AX is important for the recruitment of repair factors to the site of damage (Paull et al., 2000). Nbs1 has a fork head associated (FHA) and a breast cancer susceptibility protein 1(BRCA1) C-terminal (BRCT) domain which interacts with γH2AX recruiting the MRN complex to the break site (Kobayashi et al., 2002; Tauchi et al., 2001). Similarly, MDC1 also interacts with γH2AX via a BRCT domain. Both of these proteins are substrates for ATM. MDC1 interacts in an ATM phosphorylation dependent way with the RING finger proteins RNF8 and RNF 168. The E3 ubiquitin ligases ubiquitinate H2A, recruiting the p53 binding protein 1 (53BP1) and BRCA1 (Jackson, 2002; Lavin, 2008; Mattioli et al., 2012; Rappold et al., 2001; Schultz et al., 2000; Thompson, 2012).

The free DNA ends are bound by the heterodimer Ku70 and Ku80. The Ku70/80 complex recruits the catalytic subunit of DNA-PKcs to the break site and activates it (Smith and Jackson, 1999). DNA repair has slow and a fast component in mammalian cells. The fast repair is non-homologous end joining (NHEJ), while if that fails, breaks are repaired by homologous recombination (Frank-Vaillant and Marcand, 2002). The interplay between ATM and DNA-PK is thought to play a role in the decision making. DNA-PK gets phosphorylated by DNA-PK itself and by ATM. This is thought be important for the release of DNA-PK from DNA freeing up the ends for the HR machinery (Convery et al., 2005; Martin et al., 2012; Shrivastav et al., 2009). Both ATM and DNA-PK interact directly with c-Abl prior to damage. After IR, the interaction with the kinases is essential for high c-Abl kinase activity during the DNA damage response. c-Abl also participates in the DNA damage response. DNA-PK is phosphorylated by c-Abl and renders it unable to bind DNA (Kharbanda et al., 1997; Shafman et al., 1997).

5.1.4 DNA double strand breaks silences transcription in the vicinity of the break site

Transcription can potentially interfere when the DNA breaks in or near a gene. Not only will the gene not be properly transcribed but the polymerase itself could also interfere with the recruitment of repair proteins. Therefore it is important that the gene in the close proximity of a DSB is silenced and cleared of polymerases.

Both ATM and DNA-PK have been reported to silence RNA polymerase II transcription around the site of damage. ATM indirectly induces H2A ubiquitination which silences RNA polymerase II transcription (Shanbhag et al., 2010). Unlike ATM, DNA-PK has been reported to directly phosphorylate RNA polymerase II (Pankotai et al., 2012).
Phosphorylation of H2AX spreads up to a megabase either site of the breaks. However γH2AX does not spread evenly. It does not spread over active genes and transcription away from the break is not affected by γH2AX. Only genes that are directly cut within the gene body are silenced (Iacovoni et al., 2010). Cohesin prevents spreading of γH2AX onto transcribed genes in the vicinity. Possibly chromosome looping prevents the spread over active genes (Caron et al., 2012).

To date it is unclear whether the mechanisms that operate in the nucleus also work in the nucleolus. Nucleoli do have certain feature that set them apart from the rest of the genome. The rDNA array in yeast is susceptible to illegitimate recombination resulting in ERCs (Kobayashi, 2006). In humans this might be evidenced by rearranged rDNA repeats (Caburet et al., 2005). The origin of the non-canonical repeats is unknown, but one hypothesis is that they have arisen after faulty repair after DNA double strand breaks (DSBs). This is supported by the finding that in cells from Werner patients the percentage of rearranged rDNA repeats are elevated (Caburet et al., 2005).

The rDNA is very highly transcribed, which creates topological stress which if not properly resolved, can cause DSBs (Govoni et al., 1994; Miller and Beatty, 1969).

Another aspect of DSB repair is that the nucleolus is surrounded by heterochromatin separating it from the rest of the nucleoplasm. Proteins involved in the initial response have been found in the nucleolus, however many proteins involved in the later stages of repair are not (Ahmad et al., 2009; Andersen et al., 2005). For these reasons the DNA double strand breaks response in nucleoli might be different from the rest of the nucleus.

Studies in mouse cells have shown that ATM inhibits RNA polymerase I by an unknown mechanism. It interferes with the formation of the pre-initiation complex and leads to premature termination of elongating polymerases (Kruhlak et al., 2007). This is contrast to a study in human cells that showed that IR inhibits transcription in a DNA-PK dependent manner (Calkins et al., 2013). From in vitro studies it is also known that DNA-PK can inhibit RNA polymerase I (Michaelidis and Grummt, 2002). In in vitro transcription reactions SL1 gets phosphorylated by DNA-PK by blocking formation of the pre-initiation complex (Kuhn et al., 1995; Labhart, 1995).

Recently large phospho-proteomic studies identified proteins that are differentially phosphorylated upon DNA damage have revealed clues towards a potential mechanism (Beli et al., 2012; Bennetzen et al., 2010; Bensimon et al., 2010; Matsuoka et al., 2007; Stokes et al., 2007). Several nucleolar proteins involved in transcription and processing of rRNA are either directly or indirectly phosphorylated.
The nucleolar database shows that ATM, ATR and DNA-PK are found in nucleoli as well as Mre11, Rad50, XRCC1 (X-ray repair cross-complementing protein 1), PARP1, BLM and WRN (Ahmad et al., 2009; Andersen et al., 2005). Several repair proteins have also been reported to localise to the nucleolar caps after DSBs. These include 53BP1, MDC1 and NBS1 (Berkovich et al., 2007; Foltankova et al., 2013; Oka et al., 2011). Additionally RNF8 and BRCA1 have been reported to be nucleolar prior to damage. After damage both proteins exit the nucleolus and form foci at break sites in the nucleus (Guerra-Rebollo et al., 2012).

Experiments in this chapter describe the response of nucleoli to DNA damage and investigate which kinase is responsible for this response. Ongoing transcription is inhibited when DSBs are induced in the rDNA repeat. The kinase responsible for this inhibition is already present in nucleoli prior to damage and is not recruited from the nucleoplasm.

![Figure 5.3: The schematic of the method of transfection. I-PpoI is fused to an EMCV IRES element and in vitro transcribed from a T7 promoter. The transcript is in vitro polyadenylated and the mature transcript is transfected in hTERT-RPE1 cells.](image)

### 5.2 Results

During the targeting experiments, an interesting response of nucleoli to DNA damage was observed [Chapter 4]. In this chapter that response was studied in depth, but for these studies HA-ER-I-PpoI construct was redesigned. The ER tag did not provide tight enough regulation to create cell lines. Even in the absence of tamoxifen, DSBs were induced [Figure 4.2]. The I-PpoI ORF was cloned into a vector with an IRES element and V5 tag, which was in vitro transcribed and polyadenylated [Figure 5.3]. The mRNA was transfected into hTert-immortalised RPE1 cells. Cancer cells often have mutations in the DNA damage pathway (Hanahan and Weinberg, 2011). Therefore I used hTert-RPE1 cells and primary skin fibroblast, both of which are not transformed and have a normal karyotype. They should give a more accurate representation of the DNA damage response in human tissue. After optimisation of the ratios of RNA to transfection reagent for RPE1 cells, a time course was carried out to determine the earliest time point where maximum DSB response was achieved. At 4 hours after the start of the transfection, a few cells have γH2AX foci around the nucleoli. This further increases to 70-80% of the cells at 6 hours and stays stable over at
least 24 hours after the start of the transfection [data not shown]. In order to study the early events of recognition of the DNA double strand breaks and its effects on transcription, 6 hours was chosen as a fixed time point at which all other experiments were carried out unless otherwise stated.

To be certain that the effects are due to the catalytic activity of I-PpoI, a catalytically dead enzyme was made by introducing a point mutation at histidine 98. The H98A point mutation has been reported to render the enzyme catalytically dead (Eklund et al., 2007; Flick et al., 1998; Galburt et al., 1999; Mannino et al., 1999). To verify that the wild-type I-PpoI can cleave the rDNA in vivo and to estimate the levels of cleavage as well as confirming that the H98A mutant is catalytically dead, genomic DNA from transfected cells was isolated. The DNA was digested with NcoI and transferred to a membrane for Southern blotting. The blot was hybridised with a probe against DNA adjacent to the I-PpoI site [Figure 5.4A].

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**Figure 5.4**: I-PpoI can induce double strand breaks in the rDNA in vivo. A) Genomic DNA from transfected RPE1 cells was isolated 6h after transfection and digested with NcoI. The Southern blot was hybridised with an rDNA probe from sequences adjacent to the I-PpoI site. In vivo cleavage of the rDNA is observed with the wild-type I-PpoI enzyme while the point mutation renders the enzyme catalytically dead. B) Western blot analysis of whole cell lysates shows a robust increase of γH2AX levels with expression of the V5 tagged wild-type enzyme, while the V5 tagged mutant enzyme does not have the same effect. Upon expression of the wild-type enzyme the checkpoint proteins p53, Chk1 and Chk2 are phosphorylated indicative of a cell cycle arrest at G1 and G2/M (Ahn et al., 2000; Melchionna et al., 2000; Shieh et al., 1997; Zhao and Piwnica-Worms, 2001).
Cleavage of the rDNA was only observed in cells transfected with the wild-type enzyme, while cells transfected with the mutant showed no cleavage. Quantification of the band intensity gave an estimate of 20% of the rDNA repeats being cut in wild-type I-PpoI transfected cells. With 600-700 copies per diploid genome this translates to an average of 120-140 double stand breaks in the rDNA per cell at any given time (Sakai et al., 1995; Schmickel, 1973; Stults et al., 2008).

To verify that the expression of I-PpoI could induce a characteristic DNA damage response, whole cell lysates were blotted for γH2AX, an early marker of DNA damage and proteins involved in checkpoint activation; Chk1, Chk2 and p53. Wild-type I-PpoI induces a robust γH2AX response as well as phosphorylation of ATM at S1981. Activation of the various checkpoints was confirmed by blotting for phosphorylation of p53-S10, Chk1-S317 and Chk2-T68 [Figure 5.4B](Ahn et al., 2000; Chan et al., 2000; Gatei et al., 2003; Melchionna et al., 2000; Shieh et al., 1997; Zhao and Piwnica-Worms, 2001). The catalytically dead enzyme however does not induce any of the phosphorylations indicating that the catalytic activity of I-PpoI is needed to induce a DSB response. Chk2 levels are reduced upon transfection because the transfection media does not contain serum. Chk2 levels have been reported to drop upon serum starvation (Tominaga et al., 1999). Both wild-type and mutant I-PpoI are V5 tagged to monitor expression levels of the enzymes.

5.2.1 RPE1 cells form nucleolar caps upon DSBs in nucleoli

To assess the effect of the double strand breaks on nucleolar morphology, cells were stained with UBF as a nucleolar marker and V5 to identify transfected RPE1 cells [Figure 5.5A]. As shown in the previous chapter, wild-type I-PpoI transfected cells have altered nucleolar morphology; nucleolar caps have formed at the nucleolar periphery. (Kruhlak et al., 2007). The H98A mutant does not induce this alteration in nucleolar morphology. The reorganisation of the nucleolus is associated with large γH2AX foci around the nucleoli in wild-type transfected cells. Cells transfected with the mutant I-PpoI do not have an increase of γH2AX levels above background. This response is not restricted to hTert-immortalised RPE1 cells, but normal primary skin fibroblasts respond similarly [Figure 5.5B]. Other proteins involved in the transcription and processing of nascent ribosomal RNA transcripts also localise to the caps [Figure 5.6]. These include the RNA polymerase subunit Paf49, RNN3, Treacle and Fibrillarin.
Figure 5.5: Expression of I-Ppol causes the formation of nucleolar caps. A) The expression of wild-type I-Ppol in RPE1 cells induces the formation of nucleolar caps at the nucleolar periphery and large $\gamma$H2AX foci around the nucleoli. The catalytically dead mutant enzyme does not induce a rearrangement of the nucleolar structure nor does it induce $\gamma$H2AX foci. B) This response is not only observed in RPE1 cells but also in primary human skin fibroblasts. This response is seen in all transfected cells.

Figure 5.6: Other proteins involved in transcription and processing of the ribosomal genes also localise to the caps. The RNA polymerase I subunit Paf49 and RNN3 are part of the pre-initiation complex, Treacle and Fibrillarin are associated with 2'-O-methylation of the nascent transcripts by the BoxC/D snoRNPs (Gonzales et al., 2005; Shav-Tal et al., 2005).
The formation of the caps containing both rRNA transcription and processing factors is reminiscent of the caps formed upon inhibition of RNA polymerase I by low doses of ActD. Higher concentrations of ActD which will inhibit all transcription can induce γH2AX (Mischo et al., 2005). However upon treatment with 100 ng/ml ActD, a concentration that will mainly affect RNA polymerase I transcription, γH2AX levels are not elevated above background levels [Figure 5.7]. I-Ppol also inhibits transcription, but the caps are surrounded by large γH2AX foci. Therefore transcriptional inhibition of RNA polymerase I by itself does not induce a γH2AX response.

3D- ImmunoFISH was performed to prove that the γH2AX foci at the nucleolar periphery contain the rDNA, Cells were stained for γH2AX and hybridised with a probe against the intergenic spacer of the rDNA repeat. The rDNA is covered by γH2AX in I-Ppol transfected cells [Figure 5.8A].

Besides the γH2AX foci around the nucleolus, there are smaller γH2AX foci elsewhere in the nucleus, which could be any of the other target sites [Figure 4.3]. The DAB1 locus has previously be studied in detail (Berkovich et al., 2007). The DAB1 gene is located on p-arm of chromosome 1 as indicated in the ideogram of Figure 5.8. To determine if the DAB1 target site is often cleaved in RPE1 cells, cells were stained for γH2AX and hybridised with the bacmid AL391826 spanning the DAB1 locus. [Figure 5.8B].

Despite examining numerous cells, none of the cells had co-localisation of the bacmid with γH2AX. It is unknown whether DAB1 is expressed in RPE1 cells. Therefore it is possible that the DAB1 site is packed in heterochromatin and inaccessible for the enzyme. Another possibility is that the I-Ppol site is not intact in hTert-RPE1 however there are no polymorphisms known in the I-Ppol recognition site in the DAB1 gene (NCBI- genome database).
Figure 5.8: I-Ppol cleaves the rDNA in vivo, while the DAB1 gene is not targeted. A) 3D-immunoFISH reveals that the γH2AX foci cover the rDNA. B) In RPE1 cells, one of the other I-Ppol sites in the DAB1 gene is not cleaved. The bacmid AL391826 from chromosome 1 was hybridised to I-Ppol transfected cells. The white arrow heads point to the DAB1 locus. The ideogram on the right indicates where on chromosome 1 the DAB1 is located.
Figure 5.9: Nucleolar caps form adjacent to sequences distal of the NOR and contain 1 or more individual NORs. Cells were hybridised with a distal junction (DJ) probe and stained with UBF. The DJ is anchored in the perinucleolar heterochromatin (Floutsakou et al., 2013). Nucleolar caps that form upon I-Ppol transfection, form adjacent to the DJ sequences. Similar caps are formed upon treatment with 100 ng/ml Actinomycin D for 1h. The caps contain either individual or multiple NORs.

Other work in the lab has identified sequences distal to the NORs which are conserved among all acrocentrics (Floutsakou et al., 2013). In RPE1 cells 9 of the 10 NORs have rDNA repeats and in most cells all NORs are associated with the nucleolus (Chelly van Vuuren). To investigate where the caps form relative to these distal sequences, cells were stained with UBF and hybridised with the distal junction sequences (DJ) [Figure 5.9]. The caps form adjacent to these sequences. Each individual caps contains 1 or multiple NORs. This is similar to the caps that form after ActD treatment. The DJ sequence is thought to anchor the rDNA in the heterochromatin enable the formation of the caps.
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**Figure 5.10: DNA double strand breaks induced by CRISPR show a similar response as I-Ppol transfected cells.** A) The Cas9 protein binds a guide RNA which is complementary to the DNA. The nuclease induces a double strand break at this specific sequence. Three guide RNAs were designed targeting different regions of the rDNA repeat. B) DNA transfection with the Cas9 protein and the empty guide RNA vector did not induce a γH2AX response. The targeted guide RNAs all show a large γH2AX response mainly around nucleoli. CRISPR pictures from Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. (2012). A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science 337, 816-821. Reprinted with permission from AAAS.

There are alternative methods to induce DNA damage at specific sites. In genome editing DSBs at the target site can be induced in ZFNs, however recently the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system has been described. It consists of the CRISPR associated protein 9 (Cas9) nuclease which is guided by a specific highly structured RNA [Figure 5.10A]. The guide RNA (gRNA) has 20 nucleotides complementary to DNA, which targets the Cas9 nuclease to create DSBs at a specific site (Cong et al., 2013; Mali et al., 2013b). Three gRNAs were designed to the rDNA repeat. Guide RNA 1 and 2 are located in the 5’-ETS before and after the primary cleavage site respectively. The third gRNA is outside of the transcribed region in the intergenic spacer. DNA transfections of Cas9 together with the guide RNAs shows that the CRISPR system can induce large γH2AX foci at the nucleolar periphery [Figure 5.10B]. All 3 gRNAs show a similar response indicating that the response is not restricted to the transcribed region.
5.2.2 DNA damage in nucleoli induces transcription inhibition

DNA double strand breaks are known to silence RNA polymerase II transcription in close proximity of the break site. To verify that upon I-PpoI transcription the transcription is silenced, cells were permeabilised and incubated in a transcription buffer containing the nucleotide analogue BrUTP (5-Bromouridine 5’-triphosphate) and 50μg/ml α-amanitin for 15 minutes. Cells were then fixed and stained with a BrUTP antibody to detect transcription. Cells with a normal punctuated UBF staining have a robust incorporation of BrUTP indicative of untransfected cells [Figure 5.11]. However cells with UBF caps have no incorporation of BrUTP. Assuming the UBF caps only form in transfected cells, this indicates that I-PpoI induced DSBs cause transcriptional inhibition. Cells transfected with mutant I-Ppol have a similar UBF staining to untransfected cells and a robust incorporation of BrUTP.

The BrUTP incorporation assay is an in vitro assay. To look in vivo, transcription was visualised using click chemistry (Cappella et al., 2008; Kliszczak et al., 2011; Salic and Mitchison, 2008). RPE1 cells were incubated with the nucleotide analogue EU. After permeabilisation a fluorophore reacts with an alkyne group on the incorporated EU enabling visualisation of global transcription levels. Normal cells have a staining throughout the nucleus with the nucleoli lighting up more brightly compared to the surrounding nucleoplasm [Figure 5.12A]. These brightly staining areas colocalise with UBF and Paf49 staining, validating that these bright areas are indeed nucleoli. The preferential incorporations of nucleotide analogues is similar to the preferential incorporation of FU in nucleoli (Casafont et al., 2006).

Figure 5.11: BrUTP incorporation assay shows that double strand breaks inhibits RNA polymerase I transcription. BrUTP incorporation assay in the presence of 50μg/ml α-amanitin and stained with UBF to identify cells with caps.
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Figure 5.12: Double strand breaks inhibit RNA polymerase I transcription. A) Normal RPE1 cells were exposed to the nucleotide analogue EU and stained for the nucleolar proteins UBF and the RNA polymerase I subunit Paf49. B) RPE1 cells transfected with either wild-type or mutant I-Ppol in the presence of nucleotide analogue EU and stained with V5 to identify transfected cells. Cells transfected with the wild-type enzyme have a loss of nucleolar transcription while cells transfected with the mutant have no loss of nucleolar transcription.

RPE1 cells were transfected with wild-type and mutant I-Ppol and incubated with EU. After the click reaction, the cells were stained for V5 expression to identify transfected cells. RPE1 cells transfected with wild-type I-Ppol have a similar intensity of staining in the nucleoplasm as untransfected cells. However, the nucleoli have no EU staining indicating transcription in the nucleolus is inhibited. Cells transfected with the mutant I-Ppol have a similar staining pattern as untransfected cells.
5.2.3 *ATM is the signaling kinase*

DNA double strand breaks can be sensed by either ATM or DNA-PK. To investigate which kinase is responsible for the signalling upon I-PpoI transfection, cells were transfected in the presence of increasing amounts of caffeine. With increasing concentrations of caffeine, the levels of γH2AX decreased [Figure 5.13A]. Caffeine inhibits both ATM and ATR, while DNA-PK is not affected at these concentrations (Blasina et al., 1999; Hall-Jackson et al., 1999; Sarkaria et al., 1999; Zhou et al., 2000). ATR responds to single stranded DNA (ssDNA) and is only implicated as a secondary kinase in the DNA double strand breaks response (Jazayeri et al., 2006). To confirm ATM is the sensing kinase, cells were incubated with more specific inhibitors for the kinases. KU-55933 is a selective ATM inhibitor (Hickson et al., 2004) while NU-7441 is a selective DNA-PK inhibitor (Leahy et al., 2004). The ATM inhibitor decreased γH2AX levels, while cells treated with DMSO or the DNA-PK inhibitor, have similar γH2AX levels as untreated cells [Figure 5.13B]. Southern blots with genomic DNA from RPE1 cells transfected with wild-type I-PpoI showed that the inhibitors do not affect the ability of I-PpoI to cleave the DNA [Figure 5.13C]. This supports the idea that the lack of γH2AX is due to ATM inhibition and not due to an effect on creating of the DSBs. The intensity of the band in the presence of the ATM inhibitor is lower. This is possibly due to increased levels of apoptotic/ necrotic cells. RPE1 cells were transfected with I-PpoI and treated with either DMSO or the ATM inhibitor. 6 Hours after the start of the transfection, cells were stained with Trypan Blue. This dye is excluded from living cell but stains dead cells blue due to the loss of plasma membrane integrity (Tait and Green, 2010). In the presence of the ATM inhibitor, there were more cells that are stained [Figure 5.13D]. Increased cell death can explain the lower levels of the cleaved rDNA product on the Southern blot. Inhibiting ATM will therefore not only prevent phosphorylation of H2AX, but prevent activation of checkpoints, which can lead to increased cell death (Yao et al., 1996; Zhou et al., 2000) [Figure 5.13D].
Figure 5.13: ATM is the signalling kinase upon I-Ppol transfection. A) Cells were treated with increasing concentrations of caffeine. Whole cell lysates showed decreased γH2AX response with increasing caffeine concentrations. B) Cells were treated with specific inhibitors against ATM (10µM KU-55933) and DNA-PK (10µM NU-7441). C) Southern blot of genomic DNA harvested at 6h after transfection in the presence of the various PIKK inhibitors. D) Cells were transfected in the presence of 10µM KU-55933 (ATM) and at 6h stained with Trypan Blue to assess cell viability. Living cells exclude the dye, while necrotic or apoptotic cells have no longer an intact membrane allowing the dye to enter the cells (Tait and Green, 2010). The number of blue cells is increased in the presence of the ATM inhibitor compared to the DMSO control, indicative of increased cell death when ATM is inhibited.

To investigate the role of ATM in nucleolar segregation, RPE1 cells were transfected with wild-type I-Ppol and stained with V5 and UBF antibodies. Nucleolar morphology of 100 random cells (transfected and untransfected) was scored to be normal, partially segregated or completely segregated [Figure 5.14A]. In RPE1 cells transfected with I-Ppol, 74% of the cells have segregated nucleoli, 3% have partially segregated nucleoli and 23% have a normal UBF staining. RPE1 cells transfected with I-Ppol and treated with DMSO showed a similar distribution; 75% of nucleoli segregated, 3% with partially segregated nucleoli and 23% with normal UBF staining. The DNA-PK inhibitor (NU7441) also does not affect the nucleolar morphology; 66% of nucleoli segregated, 4% partially segregated and 30% with normal morphology.
staining. The ATM inhibitor (KU55933) however shows 18% of nucleoli segregated, 16% partially segregated and 65% with normal UBF staining. The lack of nucleolar segregation is not due to lower transfection efficiencies as the staining of V5 positive cells was not affected by the inhibitors [Figure 5.14B]. Under all condition between 45-50% of the cells has V5-staining. Low expression of V5-I-Ppol can cause DSBs in the rDNA, but detection with the V5 antibody is not possible. This explains why 70% of nucleoli are segregated, but only 45-50% of cells show detectable V5-staining.

**Figure 5.14:** ATM is responsible for nucleolar segregation upon DNA damage. A) The nucleolar morphology of 100 random cells (transfected and untransfected) was divided in 3 categories; normal, partially segregated or completely segregated. Examples of each of the categories are shown with UBF staining (red) representing the nucleolar morphology. Only the ATM inhibitor (KU55933) can prevent nucleolar segregation. B) The appearance of largely normal nucleolar morphology is not due to decreased transfection efficiencies. The levels of V5 positive cells stay stable in the presence of all inhibitors, indicating that the transfection efficiency is not affected by the presence of the inhibitors. The graph shows the average of 3 separate experiments.
Figure 5.15: ATM inhibits RNA polymerase I transcription after V5- I-PpoI transfection. A) RPE1 cells were transfected with I-PpoI and incubated with EU in the presence of the ATM inhibitor KU55933. Quantification of 100 V5-positive cells shows that in cells incubated with DMSO, nucleolar transcription is inhibited, while with the ATM inhibitor KU55933 nucleolar transcription is not inhibited. B) BrUTP incorporation assay in the presence of 50μg/ml α-amanitin in the presence of the ATM inhibitor (KU55933). In cells treated with DMSO 45.8% of cells showed BrUTP incorporation while 98.4% in the presence of the ATM inhibitor has BrUTP incorporation. N≥100.
The nucleolar caps after I-PpoI transfection are reminiscent the caps formed upon transcriptional inhibiting of RNA polymerase I by ActD. The normal UBF staining of the nucleoli in the presence of the ATM inhibitor might be due to a failure to inhibit transcription, indicating that the segregation is a consequence of the inhibition of transcription rather than a direct consequence of activation of ATM.

To verify that ATM inhibits RNA polymerase I transcription in RPE1 cells after V5-I-PpoI transfection, cells were incubated with EU in the presence of the ATM inhibitor KU55933 or DMSO [Figure 5.15A]. In V5 positive cells treated with DMSO, nucleolar transcription is inhibited 98% of cells, while only 1% of cells has inhibited nucleolar transcription in the presence of the ATM inhibitor. The inhibition of RNA polymerase I transcription was confirmed with a BrUTP incorporation assay in the presence of α-amanitin [Figure 5.15B]. 45.8% of all cells (transfected and untransfected) showed BrUTP incorporation when cells were treated with DMSO, while 98.4% of cells had BrUTP incorporation in the presence of the ATM inhibitor KU55933. Taking it all together, ATM activity is responsible for the transcriptional inhibition which causes nucleolar segregation and γH2AX phosphorylation.

5.2.4 Prior to DNA damage ATM is already localized in the nucleolus

To better understand the role ATM plays in the recognition of DSBs in the rDNA repeat, cells transfected with wild-type I-Ppol were stained with an ATM antibody. The kinase has previously been shown to form foci at the site of damage (Andegeko et al., 2001). In all transfected cells ATM is present at the nucleolar caps [Figure 5.16A]. Because this can either be active or inactive ATM, staining with a phosphorylated ATM at S1981 revealed that the ATM localised at the caps is the active kinase [Figure 5.16B]. Although the experiments with the inhibitors suggests that DNA-PK activity is not required for inhibition of RNA polymerase I transcription and γH2AX phosphorylation, the kinase might still localise to the nucleolar caps. Therefore cells transfected with I-Ppol were also stained for DNA-PK. Although the kinase seems to localise to the nucleolus before DNA damage, it is excluded from the nucleolus after damage [Figure 5.16C]. Unlike ATM, DNA-PK does not seem to accumulate at the nucleolar caps.
Figure 5.16: ATM, not DNA-PK is localised at the nucleolar caps. A) RPE1 cells transfected with I-Ppol were stained for ATM to investigate its localisation at the nucleolar caps. B) Autophosphorylated ATM (S1981) localises to the caps. C) RPE1 cells were stained with DNA-PK. In untransfected cells DNA-PK is nuclear with a similar intensity staining in nucleoli. In transfected cells, DNA-PK gets excluded from nucleoli, but does not appear to be recruited to caps.

In untransfected cells, it is unclear whether ATM localises to nucleoli or not. Due to the density of the nucleolus or masking of the antigen, some antibodies have difficulties staining proteins in nucleoli. An example of that is the TATA box binding protein TBP, which is part of the SL1 complex (Jordan et al., 1996). Upon pre-extraction of the cells, nucleolar staining is revealed. Therefore normal untreated RPE1 cells were pre-extracted with 0.5% Triton for 10 minutes on ice before fixation. After pre-extraction, the ATM antibody does stain nucleoli [Figure 5.17A]. This means that ATM is already present in nucleoli prior to DNA damage and the ATM at the caps is likely not recruited from the nucleoplasm but co-segregated with the rDNA to the caps.
Figure 5.17: Pre-extraction reveals nucleolar ATM. A) Normal RPE1 cells were extracted with 0.5% Triton on ice for 10 minutes before fixation. Staining of the cells with the ATM antibody afterwards showed staining of nucleoli colocalizing with UBF. B) Tip60, which has previously also been reported to be involved in rDNA transcription, also localises to nucleoli (Halkidou et al., 2004).

Tip60 has previously been reported to be involved in rDNA transcription (Halkidou et al., 2004). Because Tip60 is also important for ATM activation, pre-extracted cells were also stained for Tip60 [Figure 5.17B]. Tip60 is like ATM localised to the nucleoli.

Figure 5.18: Treatment with the c-Abl inhibitor, imatinib reduces γH2AX levels, but does not interfere with nucleolar segregation. Imatinib was added at the start of the transfection. γH2AX levels are affected but not completely inhibited. Nucleoli are still able to segregate in the presence of the imatinib.
To further investigate the role of Tip60 and c-Abl in the nucleolar DNA damage response, cells were treated with the c-Abl inhibitor imatinib [Figure 5.18]. By inhibiting c-Abl the binding affinity of Tip60 to H3K9me3 is reduced which prevents ATM activation (Kaidi and Jackson, 2013). At 6h the RPE1 cells were fixed and stained for γH2AX and UBF. In the presence of 1µM and 5µM imatinib nucleoli still segregate into caps. The γH2AX foci do seems smaller and the effect is concentration dependent. However the γH2AX foci were not completely inhibited. This is in contradiction with the previous publication were 1µM imatinib prevented ATM activation and the subsequent γH2AX response. This might indicate that c-Abl mediated activation of ATM is redundant in the nucleolus.

Chromatin immunoprecipitation shows a direct interaction between the rDNA and ATM. Cells were cross-linked and the fragmented chromatin was precipitated with antibodies against UBF, the RNA polymerase I subunit RPA43 and ATM. UBF binds across the rDNA repeat as previously shown (O'Sullivan et al., 2002)[Figure 5.19]. RNA polymerase I subunit RPA43 shows a similar enrichment all over the transcribed region of the rDNA and on the promoter region (42kb). Both UBF and RPA43 however do not show binding to the sequences adjacent to the NORs (DJ) or to the U2 genes. ATM shows a similar pattern as RPA43 and UBF; enrichment over the rDNA repeat, but not on sequences adjacent to NOR or the snRNA U2 genes.

![Figure 5.19: Nuclear ChIP shows ATM at the rDNA repeat, but not at the adjacent distal junction (DJ) sequence. UBF and the RNA polymerase I subunit RPA43 are enriched all over the rDNA transcribed region, while it is not present at the sequences distal to the NORs. ATM follows the same pattern showing a similar enrichment as RPA43.](image)
It is possible to physically isolate nucleoli. To verify the immunofluorescence and nuclear ChIP data, nucleoli were isolated and on Western blot stained for ATM and DNA-PK [Figure 5.20]. The band intensities were quantified. UBF levels were set at 100% and it was calculated to estimate which % of the total cellular protein resided in the nucleoli. ATM and DNA-PK are both found in nucleoli at low levels. This is in agreement with the nucleolar protein database (Ahmad et al., 2009; Andersen et al., 2005). Therefore it is likely that ATM is intrinsic to nucleoli and is not recruited from the nucleoplasm.

Figure 5.20: Cell fractionation shows that both ATM and DNA-PK are present in the nucleolus. The band intensities were used to calculate which fraction of the total cellular pool of protein was in the nucleolus setting UBF levels at 100%. Tubulin is a cytoplasmic protein showing the quality of the nucleolar preparation.

In summary, the DSBs in the rDNA cause transcriptional inhibition which triggers nucleolar segregation separating the rDNA in caps containing individual NORs. This is dependent on ATM phosphorylation of the transcription and processing machinery. ATM is not recruited from the nucleoplasm, but is already present in nucleoli prior to DNA damage.

5.3 Discussion

Nucleoli are isolated both chromosomally on metaphase chromosomes and physically in the interphase nucleus. I investigated if the response to DNA damage in nucleoli differs from the rest of the nucleus. To investigate this I utilised I-Ppol to induce DNA double strand breaks preferentially in the rDNA repeat. The rDNA damage response is dependent on ATM kinase activity. ATM has also previously been shown to inhibit RNA polymerase I transcription in mouse cells after IR (Kruhlak et al., 2007). In human cells RNA polymerase I transcription is reported to be inhibited by DNA-PK after IR (Calkins et al., 2013). On the other hand, it has also been reported that IR does not a response in nucleoli (Al-Baker et al., 2004; Moore et al., 2011). This might be explained by the differences in cell type or species; human (Moore et al., 2011) or mouse (Kruhlak et al., 2007). The heterochromatin surrounding the nucleolus
in human cells might prevent IR from causing DNA in the rDNA. However the expression of I-PpoI circumvents this problem.

In the nucleus Tip60 acetylates ATM triggering its activation. Chromatin changes in the vicinity of the break stabilise a phosphorylation of c-Abl on Tip60. This alters its substrate specificity from chromatin to ATM. By inhibiting c-Abl with imatinib, the activation of ATM can be prevented (Kaidi and Jackson, 2013). However after I-PpoI expressing a concentration (1µM) that was shown to strongly inhibit the γH2AX response after IR does not inhibit this response to the same extent after I-PpoI expression. This might indicate that the response in nucleoli does not rely on stabilisation of the c-Abl phosphorylation on Tip60 as much as the rest of the nucleus.

Tip60 has been shown to interact with and acetylate UBF (Halkidou et al., 2004). Disruption of this interaction might also important for Tip60 to activate ATM. Alternatively the high number of DSBs in close proximity of each other triggers enough residual kinase activity to activate Tip60. I have not investigated whether ATM is acetylated by Tip60 after I-PpoI expression. However ATM is autophosphorylated and the acetylation is essential for that (Sun et al., 2005).

Nucleolar segregation induced by UV and IR radiation, induces changes in the nucleolar proteome (Al-Baker et al., 2004; Moore et al., 2011). The transcriptional inhibition after irradiation is dependent on DNA-PK and PARP1 (Calkins et al., 2013). PARP1 is a nucleolar protein, which is released from nucleoli when RNA polymerase I transcription is inhibited (Meder et al., 2005). PARP1 can also be recruited to sites of damage and silence RNA polymerase II transcription (Chou et al., 2010). Hence it is possible that PARP1 might also play a role in addition to ATM in silencing rDNA transcription.

DNA-PK has been shown to in vitro to phosphorylate SL1 and thereby silencing RNA polymerase I transcription (Kuhn et al., 1995; Labhart, 1995; Michaelidis and Grummt, 2002). DNA-PK and ATM have the same consensus sequence, therefore it is possible that the site on TAF110 identified in the phospho-proteomic studies is responsible for the inhibition. This might be the mechanism for DNA-PK induced transcriptional inhibition after UV.

### 5.3.1 Possible phosphorylation targets of ATM

Large proteomics studies after DNA damage have identified numerous proteins that are differentially phosphorylated upon DNA damage (Beli et al., 2012; Bennetzen et al., 2010; Bensimon et al., 2010; Choi et al., 2012; Matsuoka et al., 2007; Stokes et al., 2007). Table 5.1 lists the DNA damage induced phosphorylations of the transcription machinery. Comparing the DNA damage induced phosphorylations with mitotic phosphorylations shows large similarity between the proteins that are phosphorylated [Table 1.1].
TAF110 has 2 residues which are differentially phosphorylated after DNA damage. During mitosis the same region is responsible for mitotic silencing (T852)(Heix et al., 1998). As well an antibody against this region of the protein strongly inhibits in vitro transcription (Appendix 1). Taking this all together suggests that this region is important for transcription. The N-terminus of UBF contains a dimerization domain and a putative SANT domain. The latter is characterised by 3 conserved Trp residues. Mutation of either of these Trp residues abolished the localisation of UBF in nucleoli [Doctoral thesis Dr. Christine Colleran]. The first tryptophan is right beside the potential DNA damage induced phosphorylation. Therefore the phosphorylation might disrupt interactions with chromatin or other proteins. The effect of the phosphorylation on Treacle, TTF1 and UTP14A is less clear. TTF1 is important for the activity status of rDNA repeats, recruiting both the silencing complex NoRC and transcriptional activator G9a (Santoro and Grummt, 2005; Yuan et al., 2007). It is possible that posttranslational modifications control these interactions. Treacle interacts both with UBF and the 2'-O-methylase complex linking transcription and modification of the pre-rRNA (Gonzales et al., 2005; Valdez et al., 2004). It is possible that the phosphorylation after DNA damage disrupts or alters these interactions. UTP14A is part of the SSU processome, which cleaves the primary cleavage site (Dragon et al., 2002; Hu et al., 2011). UTP14A also interacts with p53 and promotes its degradation under normal conditions (Hu et al., 2011). Whether this interaction might be disrupted after DNA damage is unknown.

5.3.2 Movement of DSBs

DSBs in the rDNA cause nucleolar segregation collapsing the rDNA in caps which contain individual NORs. This response after DSBs in the nucleolus has been shown before (Kruhlak et al., 2007; Oka et al., 2011). In yeast double strands breaks also move to the periphery of the nucleolus. In contrast to mammalian cells, in yeast only the DSBs moves, while the rest

<table>
<thead>
<tr>
<th>Protein</th>
<th>Phosphorylated residue*</th>
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*Peptides from Stokes and Matsuoka only.*

**Table 5.1: Differentially phosphorylated protein after DNA damage.** The residues and the sequences are from the Stokes and Matsuoka papers only.
of the rDNA stays in the nucleolus and the nucleolus does not segregate (Torres-Rosell et al., 2007). However the yeast nucleolus is different from the mammalian nucleolus. During mitosis yeast rDNA is not silenced, while transcription ceases in mammalian cells (Klein and Grummt, 1999). Therefore the segregation of the nucleolus might have evolved in mammalian cells, just as the silencing through mitosis.

Movement of DSBs is not unique to the nucleolus. After IR, DSBs have a tendency to cluster together (Aten et al., 2004). The movement of DSBs is also facilitates repair by homologous recombination. The movement enables pairing with a homologous sequence (Dion et al., 2012; Gandhi et al., 2012; Mine-Hattab and Rothstein, 2012).

In *Drosophila*, DSBs in heterochromatin move to the periphery of the heterochromatin to be repaired. Both ATM and ATR are required for this movement (Chiolo et al., 2011). This is similar to the movement upon I-Ppol expression in human cells, which is also dependent on ATM. I have not assessed the involvement of ATR, however when ATM is inhibited both segregation and γH2AX signalling are largely inhibited.

The segregation of the nucleolus is a consequence of the transcriptional inhibition and not of γH2AX directly. Chemical inhibition of transcription with low dose ActD causes segregation of the nucleolus independent of γH2AX signalling. In mouse cells, DSBs cause transcription inhibition in an ATM dependent manner (Kruhlak et al., 2007). Similarly in human cells the transcriptional inhibition is dependent on ATM. Inhibition of ATM also inhibits the segregation of nucleoli. Therefore the model is that ATM signalling silences transcription and the inhibition of transcription causes the nucleoli to segregate.

In summary, the sensing of double strand breaks in the nucleolus causes transcriptional inhibition and subsequent segregation of nucleoli. This is dependent on the activity of ATM, which is constitutively present in nucleoli. The activation of ATM is possibly triggered by Tip60, which is also in nucleoli. Large proteomics studies have identified numerous proteins that are differentially phosphorylated after DNA damage. Among these are factors involved in ribosomal gene transcription and processing. This gives a plausible model for ATM induced transcriptional inhibition.
Chapter 6

rDNA damage repair by homologous recombination is cell cycle independent

6.1 Introduction

DNA double strand breaks need to be repaired for the cell to survive. There are 2 major pathways by which a DSB can be repaired; NHEJ and HR. These two pathways have different kinetics. Repair by NHEJ is fast, but error-prone, while repair by HR is slow and error-free (Fishman-Lobell et al., 1992). DNA ends are bound by Ku70-Ku80 complex, which protects the ends. This transient stability of the ends allows NHEJ to precede HR (Frank-Vaillant and Marcand, 2002). However when NHEJ does not repair the break quickly, the ends are resected by exo- and endonucleases. Once the ends are resected the cell is committing to repair by HR. The sister chromatid acts as a template for repair (Huertas, 2010; Symington and Gautier, 2011).

6.1.1 Non-homologous end joining

The Ku70-Ku80 complex has high affinity for DNA double strand ends. The catalytic subunit of DNA-PK is recruited to the ends by the Ku70-Ku80 complex. Dimerisation of DNA-PK keeps the DNA ends together and activates its kinase activity. DNA-PK is autophosphorylated and it phosphorylates the nuclease Artemis. Conformational changes recruit XRCC4 and DNA ligase 4. The ends can be processed by Artemis before XRCC4-Ligase 4 complex ligates the ends back together (Jackson, 2002; Thompson, 2012).

Figure 6.1: Non-homologous end joining. The Ku70-Ku80 complex recruits the catalytic subunit DNA-PKcs to the break site. After dimerization DNA-PK is autophosphorylated and conformational changes recruit Artemis, XRCC4 and DNA ligase 4. The Artemis nuclease can process the ends, before DNA ligase 4 ligases the ends (Jackson, 2002; Thompson, 2012).
6.1.2 Homologous recombination

In homologous recombination, resection generates large stretches of ssDNA which can invade into a homologous sequence for repair. Resection is initiated after the recruitment of BRCA1 and CtBP-interacting protein (CtIP) to the breaks (Escribano-Díaz et al., 2013). Both proteins interact with the MRN complex and CtIP stimulates Mre11 nuclease activity (Sartori et al., 2007; Zhong et al., 1999). Mre11 has both endo- and 3’-5’exonuclease activity (Paull and Gellert, 1998). Mre11 creates nicks which are substrates for the exonucleases. Mre11 resects in the 3’-5’ direction, while Exo1 resects in a 5’-3’ direction (García et al., 2011). The Ku70-Ku80 complex only has high affinity for double strand ends and therefore is released when Mre11 resection reaches the ends. The ssDNA is bound by the replication protein A (RPA) heterotrimer, which is replaced by Rad51 prior to strand invasion (Lisby et al., 2004). The strand invades into a homologous sequences forming D-loop. This allows Rad54 dependent DNA synthesis. The D-loop can either be resolved with or without crossing over (Thompson, 2012).

![Diagram of homologous recombination](image)

**Figure 6.2: Homologous recombination.** CtIP interacts with Mre11 and stimulates the Mre11 nuclease activity (Sartori et al., 2007). Nicks induces by the endonuclease activity of Mre11 creates ends for Exo1 to resect in 5’-3’ direction, while Mre11 resect in the 3’-5’ direction (García et al., 2011). This releases the Ku70-Ku80 complex from the ends. The single strand DNA is bound by the RPA complex. RPA is exchanged for Rad51 before strand invasion (Thompson, 2012).
6.1.3 Cell cycle regulation of repair

Double strand breaks repair pathway choice is influenced by the stage of the cell cycle. In G1, DSBs are mainly repaired by NHEJ, while cells in S/G2 repair by homologous recombination (Aylon et al., 2004; Lisby et al., 2003). A crucial step in the choice between these 2 pathways is whether or not to resect the ends. Once the ends are resected, the repair is committed to HR (Huertas, 2010; Symington and Gautier, 2011). Cdk1 kinase activity is required for resection of the DNA ends and recruitment RPA and Rad51 (Aylon et al., 2004; Ira et al., 2004).

The initiation of resection is regulated by the interplay between 53BP1-RIF1 and BRCA1-CtIP (Chapman et al., 2012). In G1, 53BP1-RIF1 is bound, which inhibits resection and promotes repair by NHEJ (Callen et al., 2013; Chapman et al., 2013). During S and G2, BRCA1 and CtIP interact in a cdk-phosphorylation dependent manner (Escribano-Díaz et al., 2013). Additionally, protein expression of CtIP and Rad52 is cell cycle regulated. Both proteins have low protein expression in G1, which increases during S-phase (Chen et al., 1997; Limbo et al., 2007).

6.1.4 Checkpoint signaling

The cell has multiple checkpoints to halt cell cycle progression to enable the cell to detect and repair DNA damage. The G1 checkpoint depends on the tumour suppressor p53. ATM and ATR both phosphorylate p53 at Ser15, which prevents p53 degradation (Banin, 1998; Canman, 1998). High p53 levels induce expression of p53 responsive genes. One of these genes is p21, a cdk inhibitor, which suppresses the kinase activity of cyclinE- and cyclinA-cdk2. This prevents cells from entering S-phase (Abraham, 2001; Yao et al., 1996).

Two kinases are central to G2/M checkpoint; Chk1 and Chk2 (Gatei et al., 2003). Both are phosphorylated by ATM and ATR. Chk1 is phosphorylated on Ser317 and Ser345, which activates its kinase activity (Gatei et al., 2003; Zhao and Piwnica-Worms, 2001), while Chk2 is phosphorylated Thr68 (Ahn et al., 2000; Melchionna et al., 2000; Tominaga et al., 1999). Both kinases can phosphorylate the phosphatases cdc25A and cdc25C (Brown et al., 1999; Falck et al., 2001; Furnari et al., 1997). This phosphorylation targets cdc25A for degradation and sequesters cdc25C in the cytoplasm (Brown et al., 1999; Peng et al., 1997; Yao et al., 1996). The phosphatases are needed to activate cyclinA-cdk2 and cyclinB-cdc2 (O'Connell et al., 2000; Sanchez, 1997).

Nucleolar proteins also participate in checkpoint signalling. In response to stress, NPM redistributes evenly throughout the nucleus. This stimulates the interaction of NPM with HDM2, which inhibits the E3 ubiquitin ligase activity resulting in stabilisation of p53 (Kurki et al., 2004).
Experiments described in this chapter aim to determine whether DSBs in the rDNA are repaired by NHEJ or HR and whether the pathway choice is influenced by the cell cycle. Additionally, preliminary data indicates that after I-PpoI transfection cells activate cell cycle checkpoints.

6.2 Results
The nucleolar database lists the sensing proteins Mre11, Rad50 and the Ku complex. However, there are no other repair proteins from either NHEJ or HR pathway in the database (Ahmad et al., 2009; Andersen et al., 2005). To get a better understanding which proteins are present in the nucleolus of RPE1 cells, isolated nucleoli were stained on Western blot from various proteins involved in HR (Rad51, Rad52, RPA2) and NHEJ (Ku80) as well the BRCA1 and 53BP1 [Figure 6.3]. Similar to DNA-PK, high levels of Ku80 are present in nucleoli, which is in agreement with the nucleolar database (Ahmad et al., 2009; Andersen et al., 2005). Of the HR factors only Rad52 seems to be in nucleoli, while Rad51 and RPA2 are excluded. BRCA1 has been reported to be nucleolar (Guerra-Rebollo et al., 2012), however in RPE1 cells BRCA1 is not detected in nucleoli. 53BP1 on the other hand can be found in nucleoli.

![Figure 6.3: Cell fractionation shows which repair factors are present in isolated nucleoli.](image)

The NHEJ protein Ku80 is present in nucleoli similar to 53BP1 and Rad52. Rad51, RPA2 and BRCA1 are not present in nucleoli. The band intensities were used to calculate which fraction of the total cellular pool of protein was in the nucleolus setting UBF levels at 100%. Tubulin is a cytoplasmic protein showing the quality of the nucleolar preparation.
Figure 6.4: Repair factors localise to the nucleolar caps, but factors in NHEJ do not. RPE1 cells were transfected with l-Ppol and stained with antibody 6 hours after transfection.
6.2.1 Proteins involved in homologous recombination are recruited to the nucleolar caps.

The presence or absence of repair proteins in the nucleolus does not indicate whether breaks are repaired by HR or NHEJ. To look at the recruitment of these proteins to the caps, RPE1 cells were transfected and at 6 hours after transfection cells were stained for proteins involved in HR and NHEJ [Figure 6.4]. The HR proteins RPA2, Rad51 and Rad52 localise to the caps, similar to 53BP1, while NHEJ factors XRCC4 and Ku80 do not localise to the caps. This suggests that the breaks are repaired by HR and not by NHEJ.

To validate the subcellular fractionation, I choose to examine the subnuclear distribution of RPA2 in more detail. This protein is recruited first to the resected ends and therefore an indicator of ongoing repair by HR. Normal cells were stained for RPA2 which shows that RPA2 is excluded for nucleoli [Figure 6.5A]. It is possible that the epitope is masked similar to ATM and Tip60 [Figure 5.17]. Therefore pre-extracted cells were stained for RPA2 [Figure 6.5B]. This was done in I-Ppol transfected cells to have a positive control. The transfected cells show RPA2 is bound to the chromatin at the nucleolar caps. This is in contrast to untransfected cells, where no RPA2 staining was detected. This demonstrates that RPA2 does not localise to the nucleolus prior to damage and has been recruited from the nucleoplasm to the caps.

![Figure 6.5: RPA2 is excluded from nucleoli. A) Staining with a RPA2 antibody indicated that RPA2 is not present in nucleoli. B) Pre-extracted cells show RPA2 foci at the nucleolar caps. Neither normal nor damaged cells show RPA2 staining in the nucleolus.](image-url)
Figure 6.6: The HR factors Rad51 and RPA2 localise to caps in mCherry-Cdt1 positive cells. RPE1 cells stably expression mCherry-Cdt1, were transfected with I-Ppol and stained for Rad51 (A) or RPA2 (B).

6.2.2 HR factors are recruited independent of the cell cycle stage.

In mammalian cells HR is largely restricted to S- and G2-phase (Aylon et al., 2004; Lisby et al., 2003). Loci outside of the NORs would require the sister chromatid as a template for repair thereby limiting HR to S and G2. But the rDNA does not have this limitation because of the large arrays which are compacted into the nucleolar caps. The repetitive nature of the rDNA could potentially allow repair via homologous recombination independent of the cell cycle. While imaging transfected cells, I noted that a very high percentage of cells showed
foci with HR factors. Thus I determined the cell cycle distribution of the cells with RPA2 and Rad51 foci using the Fucci system. As described in Chapter 3, the Fucci system consists of mCherry-Cdt1 (30-120) and mAG-Geminin (1-110) (Sakaue-Sawano et al., 2008). mCherry-Cdt1 is expressed in G1 till early/mid S-phase, while mAG-Geminin is expressed from S-phase till G2-phase. A stable RPE1 cell line expression mCherry-Cdt1 was generated. After transfection with I-PpoI these mCherry-Cdt1 expressing cells were stained for Rad51 [Figure 6.6A]. The mCherry-Cdt1 (30-120) fragment is enriched in nucleoli and the expression of mCherry-Cdt1 is maximal in G1. Cells with the strongest Cdt1 expression have Rad51 foci at the caps. Similarly the RPE1 mCherry-Cdt1 cells were stained for RPA2 after transfection. [Figure 6.6B]. Cells with highest Cdt1 expression have RPA2 foci.

Figure 6.7: The cell cycle distribution of mCherry-Cdt1 RPE1 cell line. A) RPE1 cells were incubated with the nucleotide analog EdU, which is incorporated in the DNA. B) Quantification of >100 cells shows the cell cycle distribution of the mCherry-Cdt1 cells. This profile was compared with the distribution of Cdt1 positive and negative cells that exhibit Rad51 or RPA2 foci. The early-mid S-phase population that still expressed Cdt1, cannot account for all Cdt1+ cells with Rad51 or RPA2 foci.
In order to quantify the cell cycle distribution of cells with Rad51 and RPA2 foci, it was necessary to determine which percentage of the mCherry-Cdt1 positive cells is in S-phase. The mCherry-Cdt1 RPE1 cells were incubated 30 min in the presence of the nucleotide analog EdU, which will be incorporated in the DNA during S-phase. The cells were fixed and using Click-chemistry, cells in S-phase could be visualised [Figure 6.7A]. This verified that the highest expression of Cdt1 is seen in G1 cells, while early/mid S-phase cells have lower Cdt1 expression. In late S-phase Cdt1 is no longer expressed.

Quantification of >100 cells showed that 43% of the mCherry-Cdt1 RPE1 cells was in G1, 16% in early to mid S-phase, 19% in late S-phase and 22% in G2 or mitosis [Figure 6.7B]. This profile was compared to the profile of the Rad51 and RPA2 positive cells. 46% of the Rad51 positive cells had mCherry-Cdt1 expression. This proves that the S-phase population (16%), which still expresses mCherry-Cdt1 is not sufficient to account for all Rad51 positive cells. Quantification of RPA2 positive cells showed a similar distribution as Rad51 positive cells. This indicates that DSBs in the rDNA are repaired by HR in G1.

To verify these observations, a mAG-Geminin RPE1 cell line was generated. The mAG-Geminin cells were transfected with I-PpoI and stained for RPA2 and Rad51 [Figure 6.8A-B]. Both Rad51 and RPA2 show foci in mAG-Geminin positive and negative cells. mAG-Geminin is expressed from mid-late S-phase until mitosis. To determine which percentage of mAG-Geminin negative cells is in early S-phase, cells were incubated with EdU [Figure 6.9A] and a cell cycle prolife was generated [Figure 6.9B]. In the mAG-Geminin RPE1 cell line 50% of the cells were in G1, 8% in early S-phase, 25% in mid-late S-phase and 18% in G2/M. This prolife was compared with the Geminin positive and negative cells that show Rad51 or RPA2 foci. The early S-phase population (8%) cannot account for the 50-52% of Geminin negative cells with Rad51 or RPA2 foci.
Figure 6.8: The HR factors Rad51 and RPA2 localise to caps in mAG-Geminin negative cells. RPE1 cells stably expression mAG-Geminin, were transfected with I-Ppol and stained for Rad51 (A) or RPA2 (B).
Figure 6.9: The cell cycle distribution of mAG-Geminin RPE1 cell line. A) RPE1 cells were incubated with the nucleotide analog EdU, which is incorporated in the DNA. B) Quantification of >100 cells shows the cell cycle distribution of the mAG-Geminin cells. This profile was compared with the distribution of Geminin positive and negative cells that exhibit Rad51 or RPA2 foci. The early S-phase population that has no Geminin expression cannot account for all Geminin- cells with Rad51 or RPA2 foci.
Figure 6.10: RPA2-GFP/mCherry-Cdt1 double stable cell line confirms recruitment of HR factors in G1. An RPE1 cell line stably expressing both RPA2-GFP and mCherry-Cdt1 was transfected with I-Ppol and fixed. Quantification of the cells with RPA2 foci shows 49% of the cells expressing Cdt1, while 51% does not.

Both the mCherry-Cdt1 and mAG-Geminin cell lines show repair by HR of DSBs in the rDNA in G1 cells. To confirm this independent of antibody staining, a cell line stably expressing both RPA2-GFP and mCherry-Cdt1 was made. The cells were transfected with I-Ppol and the number of mCherry-Cdt1 positive and negative was quantified in the cells that showed RPA2-GFP foci [Figure 6.10]. Of the cells with RPA2-GFP foci, 49% also expressed mCherry-Cdt1, while 51% had no detectable cdt1 expression. This is similar to the antibody staining which showed 44% Cdt1+ and 56% Cdt1- [Figure 6.7].

6.2.3 Checkpoints signaling after DSBs in the rDNA

All the transfections so far have been with a polyadenylated I-Ppol mRNA. These levels of I-Ppol expression cause an average of 120-140 DSBs in the rDNA per cell at any given time [Figure 5.4]. High and prolonged levels I-Ppol expression can continuously cut repeats. Even if the breaks are repaired by HR, the I-Ppol can be cleaved again. This can explain why during the targeting experiments transfection with polyadenylated I-Ppol caused high levels of cell death [data not shown]. By destabilizing the mRNA, expression of the protein can be reduced. I-Ppol mRNA which is not polyadenylated (I-Ppol -polyA tail) can still induce DNA damage (6h), however in contrast to the polyadenylated mRNA (I-Ppol +polyA tail), this damage is largely repaired by 24h [Figure 6.11]. Transfection with I-Ppol -polyA tail shows that cells can repair the DSBs in the rDNA and recover.
Chapter 6

Figure 6.11: The expression of the protein is controlled by the stability of the RNA. RPE1 cells were transfected with the homing endonuclease I-Ppol fused to an IRES element. After the in vitro transcription the –polyA tail RNA was isolated directly while the +polyA tail RNA was in vitro polyadenylated. At 6 h and 24 h the cells were fixed. At 6 h the γH2AX response is the similar, while at 24 h the RPE1 cells transfected with I-Ppol with a polyA tail still have large foci, but in the absence of a polyA tail cells the γH2AX have largely disappeared.

Cells have multiple checkpoints to stop cell cycle progression to adequately detect and repair DNA damage. The G1 checkpoint depends on p53 stabilization, while the G2/M checkpoint relies on the checkpoint kinases Chk1 and Chk2. Figure 5.4 shows that both Chk1 and Chk2 are phosphorylated after I-Ppol transfection.

The nucleolar disruption has been proposed to mediate stabilization of p53 in response to stress. Export and subsequent degradation of p53 is dependent on a functional nucleolus. In addition NPM, which is involved in ribosome assembly and trafficking, leaves the nucleolus in response to stress (Rubbi and Milner, 2003). In the nucleoplasm, NPM can interact with HDM2, which stabilizes p53 (Kurki et al., 2004). To determine the kinetics of the redistribution, RPE1 cells were treated with ActD [Figure 6.12]. Prior to treatment, NPM is exclusively localized to the nucleolus. However RPE1 cells treated with ActD for 1 h show a robust nuclear staining. The nucleolar staining progressively weakens until at 5 h the nucleolar staining is almost indistinguishable from the nucleoplasmic staining. This phenomenon is not common amongst rRNA processing factors. Nop52 stays associated with the nucleolus throughout ActD treatment.
Figure 6.12: Actinomycin D causes translocation of NPM but not Nop52. In normal cells NPM localises exclusively to the nucleoli, however after treatment with 100\text{ng/ml} ActD, NPM shows a nuclear staining. This is not a common phenomenon of processing factors as Nop52 stays nucleolar.
Cells were transfected with I-PpoI to determine if NPM also redistributes when DSBs are induced in the rDNA. After transfection with the polyadenylated I-PpoI, NPM leaves the nucleolus [Figure 6.13]. Similarly NPM becomes nuclear in I-PpoI –polyA tail transfected cells. This shows that although the cells transfected with I-PpoI +polyA tail do not recover, while cells transfected with I-PpoI –polyA tail do, NPM responds similarly to DSBs in the rDNA. These preliminary experiments show that DSBs in the rDNA activates cell cycle checkpoints.

In summary, I have shown that DSBs in the rDNA are repaired by HR and this repair is not restricted to S and G2.

### 6.3 Discussion
Double strand breaks can be repaired by NHEJ or HR. However HR requires a template strand for repair, which usually is the sister chromatid restricting HR to S- and G2. DSBs in
G1 cells are repaired by NHEJ (Fishman-Lobell et al., 1992). However the rDNA genes are organised in large tandem arrays. Adjacent repeats can be used as template to repair by HR independent of the stage of the cell cycle. After I-Ppol transfection, proteins involved in HR are recruited to the caps, while NHEJ factors are not [Figure 6.4]. These foci form at the nucleolar caps in G1, S and G2 phase cells [Figure 6.7&Figure 6.9]. This proves that the rDNA is repaired by HR independent of cell cycle stage. This agrees with previous publications in yeast, where DSBs in the rDNA are also repaired by HR. The damaged rDNA repeat transiently moves to the periphery of the nucleolus, where Rad52 is recruited from the nucleoplasm (Torres-Rosell et al., 2007). This is similar to human cells where repair factors like RPA2 are recruited to the caps from the nucleoplasm. In yeast the recruitment of Rad52 is regulated by the Shu complex (Bernstein et al., 2013). Whether this complex also exists in human cells and whether it regulates recruitment of HR factors to DSBs in the rDNA remains to be investigated.

The balance between HR and NHEJ is regulated by the interplay between 53BP1-RIF1 and BRCA1-CtIP. 53BP1 is recruited to the nucleolar caps, while BRCA1 foci do not appear at the nucleolar caps. Additionally BRCA1 has been reported to be nucleolar prior to DNA damage. After damage it is released from the nucleolus to accumulate at DSBs (Guerra-Rebollo et al., 2012). The cell fractionation however indicates that in RPE1 cells, BRCA1 is not nucleolar [Figure 6.3]. Possibly the localisation of BRCA1 is cell type specific. Nucleolar disruption in response to stress has been suggested to mediate p53 stabilisation. The model proposed by Rubbi and Miller predicts that damaged cells with segregated nucleoli express high levels of p53 and p21, while damaged cells without nucleolar segregation should have low levels p53 and p21.

In summary, the organisation of the rDNA in tandem arrays facilitates repair by HR independent of cell cycle stage. It remains to be determined what characteristic of the rDNA chromatin promotes repair by HR against a neighbouring repeat.
Chapter 7

General discussion and future directions

The nucleolus is a multifunctional subnuclear compartment where ribosome biogenesis takes place. Ribosomal RNA transcription is stimulated by growth factors and down regulated by stress signals. Nucleoli are separated from the rest of the nucleoplasm and have a unique proteome. In this thesis I described how the nucleolus responds to DSBs. In addition I described targeting experiments into the rDNA that used localised DSBs to improve targeting efficiency.

Targeted integration into the rDNA would enable us to follow the location of the rRNA or rDNA in the living cell. However spontaneous targeted integration by homologous recombination is a rare event (Ganguly et al., 1994; Itzhaki and Porter, 1991; Porter and Itzhaki, 1993; Thyagarajan et al., 1995). Promoter-less antibiotic selection markers can improve integration efficiency (Sedivy and Sharp, 1989; Wen et al., 2008). This is further stimulated by DNA double strand breaks at the integration site (Bibikova et al., 2003; Choulika et al., 1995; Lombardo et al., 2007; Porteus and Baltimore, 2003; Sargent et al., 1997; Urnov et al., 2005).

Our targeting construct consists of the homology arms with a Neomycin resistance gene fused to an IRES element inserted in the I-PpoI site. Double strand breaks were induced by expression of the homing endonuclease I-PpoI which cleaves once per rDNA repeat at the end of the 28S. In the initial experiment 3/3 of the G418 resistant colonies that had targeted DNA damage at the integration site, had incorporated the Neo\textsuperscript{R} gene in the rDNA. Sequencing of the rDNA showed that these colonies integrated the NeoR gene via homology-directed repair. The number of colonies was increased by the presence of Xen elements, which we believe help to localise the targeting plasmid to the nucleolus. This theory is currently still under further investigation. The Xen elements also enable large arrays to integrate in the NORs which are visible on metaphase chromosomes.

7.1 5’ETS might provide a better suitable targeting site for protein expression

The RNA of the neomycin resistance gene is transcribed in the nucleolus. Therefore it is likely that RNA polymerase I is the transcribing polymerase. The targeting construct has no promoter and therefore transcription can only be driven by endogenous promoters. But RNA polymerase I transcripts are not capped or polyadenylated. The cap and polyA tail aid in the translation of the RNA into protein. This can be circumvented with an IRES element, a secondary RNA structure that directly recruits the ribosome (Spriggs et al., 2010). Previously it has been shown that with an IRES element protein expression from RNA polymerase I transcripts is possible (Palmer et al., 1993). In our experiments however only low protein
expression was observed compared to Cytomegalovirus (CMV) driven construct. This might be due to the site of integration in the rDNA repeat. The 28S is highly structured and therefore the IRES element might not be able to fold properly or is not recognised amidst the 28S structure (Wakeman and Maden, 1989). For optimal translation an integration site closer to the promoter in 5’ETS might be preferred. Another advantage of the 5’ETS is that once the sequence is cleaved, it is rapidly degraded. By tagging a short lived RNA species it is possible to see fluctuations in transcription rates of an endogenous rDNA promoter. Of the potential sequences that can be used to visualise specific RNAs in living cells, the Spinach hairpin would be better suitable then MS2 arrays, due to its smaller size. One Spinach aptamer is expected to give a high enough fluorescent signal, while many MS2 hairpins (at least 6) are needed to generate a high enough signal (Bertrand et al., 1998; Paige et al., 2011; Strack et al., 2013).

There are also concerns about the effect of binding of the GFP-MS2 proteins. The proteins might affect the endogenous behaviour of the RNA especially because of the nuclear localisation signals on GFP-MS2 (Tyagi, 2009). The Spinach aptamer however does not rely of protein binding, but it fluoresces upon addition of chemical. The hairpin is a small and is therefore unlikely to have an impact on the behaviour of the tagged RNA. Another advantage of the Spinach aptamer is that there is no background fluorescence. With MS2 arrays, stable cell lines with low expression of MS2-GFP protein need to be generated. To follow the location of the DNA, the localisation of the target site is less critical. The intergenic spacer would be best suited, because the binding of fluorescent proteins is not hindered by the transcription machinery. In yeast such arrays have been used to study the localisation of the rDNA through mitosis and meiosis (Harrison et al., 2009; Li et al., 2011a; Miyazaki and Kobayashi, 2011). However due to differences between yeast and human, it will be interesting to visualise the behaviour of individual repeats under normal or stress conditions.

7.2 Zinc fingers nuclease, TALENs and CRISPR

In the gene targeting experiments, I used I-PpoI to induce double strand breaks in the rDNA repeat with reasonable specificity [Chapter 4]. To target the 5’ETS would however require a different method to induce DNA damage at the integration site.

To date there are several methods to induce DNA damage as illustrated in Figure 7.1. Restriction enzymes form homodimer in order to cleave [Figure 7.1A]. Some enzymes have the catalytic site and DNA binding site in the same domain, while in other enzymes the binding domain is separated from the nuclease domain. For genome editing however
restriction enzymes have a limited value. In higher organisms there are too many recognition sites.

Homing endonucleases have larger recognition sites than restriction enzymes and therefore a smaller number of sites in the genomes of higher organisms [Figure 7.1B]. I-Ppol, the enzyme used throughout these studies, belongs to this class. However these enzymes are not easily redirected to a novel sequence, which limits the use of these enzymes (Eklund et al., 2007). Additionally, homing endonucleases can tolerate degeneracy giving off-target effects (Argast et al., 1998; Petek et al., 2010).

The triple-helix forming oligos (TFO) which are covalently attached to a restriction enzyme, can address to a specific site (Eisenschmidt et al., 2005) [Figure 7.1F]. However this strategy requires transfection of the protein-oligo complex.

![Figure 7.1: Different methods for genome editing. A) Restriction enzymes form homodimer to create double strand breaks. Some restriction enzymes have the DNA binding domain and the active site in the same domain, while other enzymes have a separate DNA binding and nuclease domains. B) Homing endonucleases have a larger recognition sequence then regular restriction enzymes. C) The Cas9 nuclease is guided by a structured RNA to cleave specific sequences. D) Zinc finger domains are fused a FokI nuclease domain. Upon dimerization the nuclease domain are activated and create a DSB. E) The repeats of TALENs each recognise a single nucleotide. Like zinc finger nucleases they are fused to a FokI nuclease domain. F) Triple helix forming oligos (TFO). Restriction enzyme with a small oligos covalently linked to it to target it to 1 specific site. Figure from Oost, J.v.d. (2013). New Tool for Genome Surgery. Science 339, 768-770. Reprinted with permission from AAAS.]

Most genome editing studies up till recently used zinc finger nucleases. However recently during the course of our studies, two new methods became available; TALENs and CRISPR [Figure 7.1 C-E](Oost, 2013).

Zinc fingers nucleases are chimeric enzymes, which have zinc fingers as a DNA binding domain while an aspecific FokI domain cleaves the DNA upon dimerization (Kim et al., 1996; Smith et al., 2000). The expression of ZFNs in cells have repeatedly been shown to stimulate homology-directed repair both in human cells and in other organisms (Bibikova et
al., 2003; Bibikova et al., 2001; Lombardo et al., 2007; Moehle et al., 2007; Porteus and Baltimore, 2003; Urnov et al., 2005). Even in human embryonic stem cells ZFNs have been used to successfully edit the genome (Hockemeyer et al., 2009).

But the nucleases exhibit cytotoxicity in cells due to off-target cleavage. This can be suppressed by using redesigned FokI nuclease domains, which only cleave the DNA upon heterodimerisation and prevent homodimerisation which is thought to be responsible for the toxicity (Miller et al., 2007; Szczepé et al., 2007).

Design of the ZFN can also suppress toxicity. ZFNs with 4 fingers have higher DNA binding energy and a better specificity resulting in less off-target cleavage (Cornu et al., 2007; Gabriel et al., 2011; Pattanayak et al., 2011). The use of naturally occurring finger is also better then engineered fingers (Kim et al., 2009; Zhu et al., 2013).

Transcription activator-like effector nucleases (TALENs) are plant transcription factors. They have 30-35 amino acid repeats that are important for DNA recognition. There is an easy code to determine the DNA specificity with each repeat recognising a single nucleotide (Miller et al., 2011). Similar to zinc finger nucleases, TALENs can stimulate genome editing by homology-directed repair (Christian et al., 2010). Alternatively they can also be used to create knock-outs by relying on NHEJ mediated repair in the absence of a template (Bedell et al., 2012; Li et al., 2011b). However TALEN assembly is more laborious because each repeat needs to be assembled in the backbone of the protein in a particular order. Several methods for quick assembly have been described (Reyon et al., 2012; Zhang et al., 2011).

Comparisons between zinc finger nucleases and TALENs, show that TALENs are more effective than zinc fingers and that they have a lower toxicity (Chen et al., 2013; Mussolino et al., 2011; Wood et al., 2011).

Recently a third system was described utilising the CRISPR/Cas9 system from bacteria. Many bacterial species have a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system which functions as an adaptive immune system. The CRISPR associated (Cas) nucleases are guided by RNA sequences to specifically cleave the bound sequences. By altering the RNA sequence, the nuclease can be redirected to different sites (Jinek et al., 2012; Mali et al., 2013b). The system has been successfully used to edit the human genome (Cho et al., 2013; Jinek et al., 2013).

This system allows quick and easy targeting of many different sites, but there are some concerns about the specificity. Mismatches between the DNA sequence and the guide RNA are well tolerated in the 5’half to the 20bp target site, leading to potential off target effects (Fu et al., 2013; Hsu et al., 2013; Mali et al., 2013a; Pattanayak et al., 2013).
One way to overcome this problem is the use of a nickase, which can still stimulate homology directed targeting at a low efficiency, but it eliminates repair by NHEJ (Kim et al., 2012; van Nierop et al., 2009). By mutating one of the nuclease domains Cas9 is converted into a nickase (Cong et al., 2013). By targeting with 2 guide RNAs to opposite strands in close proximity of each other, the nickase can create double strand breaks, but compared to the wild-type Cas9 the specificity is greatly enhanced (Ran et al., 2013).

Due to the ease of the design CRISPR holds great promise. Currently we are implementing this new technology in our lab. Preliminary experiments have shown that 3 guide RNAs targeting the rDNA are active and show a similar response as I-PpoI transfection [Figure 5.10]. The system is currently adapted to an RNA transfection method such that primary cells and cell lines can efficiently be transfected as outlined in Chapter 3. By targeting sequences into the rDNA repeat, we can study the behaviour of the NOR and the marked repeat under normal conditions and under stress conditions, such as serum starvation, transcriptional inhibition or DNA damage.

The applications of CRISPR are not limited to inducing double strand. A nuclease deficient Cas9 can be fused to different functional domains, targeting an activator, repressor or catalytic domain to a specific site altering the local chromatin environment (Gilbert et al., 2013; Qi et al., 2013). Alternatively Cas9 can also be fused to fluorescent proteins to visualise specific DNA loci.

The Cas9 protein used in most studies is just one of a family of Cas proteins with different specificities and substrates. New Cas homologous are characterised and allow visualisation of multiple targets simultaneous (Fonfara et al., 2013). Apart from targeting DNA, Cas variants can also cleave specific RNA sequences. The Cas variant Csy4 cleaves a 28nt target sequence in RNA offering an alternative method for knock-down of proteins (Qi et al., 2012). Another application can be to specifically target and cleave splice variants of a protein.

### 7.3 DNA damage induced in the rDNA repeat is repaired by homologous recombination independent of the stage of the cell cycle

When I-PpoI was transfected into cells, I observed a nucleolar reorganisation and the formation of large γH2AX foci at the nucleolar periphery. I propose the following model to explain the biological relevance of this response [Figure 7.2]. I-PpoI creates a DSB in the rDNA repeat, which activates ATM. ATM phosphorylates RNA polymerase I transcription and processing factors. The transcriptional inhibition causes the formation of the caps, which enable recruitment of repair factors from the nucleoplasm. Each of the caps contains a single
NOR with on average 70 rDNA repeats. The increased local concentration of homologous sequences allows DSBs to be repaired by homologous recombination.

**Figure 7.2: The biological relevance of segregation of nucleoli.** When the DNA is cleaved, the break is sensed by the PIK kinase ATM. The damage induces phosphorylation on proteins involved in rDNA transcription and processing freeing up the repeat for the DNA sensing proteins. The transcriptional inhibition causes the rDNA to form caps at the nucleolar periphery. Each cap is an individual NOR. The caps allow the recruitment of repair factors from the nucleoplasm. Additionally the caps contain a high local concentration of homologous sequences. This promotes repair by HR against a neighbouring repeat.

ATM activation by DSBs in the nucleus is dependent on c-Abl and Tip60 activity (Kaidi and Jackson, 2013; Sun et al., 2005; Sun et al., 2007). While inhibition of c-Abl prevented ATM activation after IR, it did not prevent activation after I-PpoI expression. It is unknown whether c-Abl is present in nucleoli, but Tip60 does localise to nucleoli [Figure 5.17]. This is in agreement with a previous report which states that Tip60 interacts with and acetylates UBF (Halkidou et al., 2004). Whether ATM is acetylated by Tip60 after I-PpoI expression is unknown. Therefore it is unclear whether activation of ATM in the nucleolus relies on Tip60 to the same extent as the rest of the nucleus. It is possible that Tip60’s change in substrate specificity is not governed by c-Abl in the nucleolus. The interaction between UBF and Tip60 might be important for the activation of ATM.

DNA damage has been shown to induce phosphorylation of UBF, TAF\(_{110}\), Treacle, UTP14A and TTF1 [Table 5.1]. Whether these are direct or indirect targets of ATM is
unknown, but all identified sites match the SQ consensus sequence. Kinase assays with fragments of the putative targets can show whether they are direct targets of ATM in vitro. That might provide important clues to what happens in vivo and how transcription and processing are inhibited.

I have shown that transcription is inhibited by DSBs [Figure 5.12] and the inhibition is dependent on ATM activity [Figure 5.15]. This is consistent with previous publication showing that after IR, ATM inhibits RNA polymerase I transcription in mouse cells (Kruhlak et al., 2007). However IR has also been reported not to induce a response in human cells (Moore et al., 2011), while another paper has shown that transcriptional inhibition after IR is dependent on DNA-PK and PARP (Calkins et al., 2013). Inhibition of RNA polymerase II has also been reported to be ATM dependent (Shanbhag et al., 2010), while another publication claims it is DNA-PK dependent (Pankotai et al., 2012). Different cell types might have differential regulation of the interplay between ATM and DNA-PK. Additionally by irradiating cells, DSBs are induced throughout the nucleus. Therefore the effect of DSBs in the nucleus and the effect of DSBs in the nucleolus cannot be separated. By expression of I-Ppol allows us to look at DSBs in the nucleolus in isolation, which might explain the differences.

Phosphorylation by DNA-PK itself and by ATM on DNA-PK are important for the release of DNA-PK from DNA freeing up the ends for the HR machinery (Convery et al., 2005; Martin et al., 2012; Shrivastav et al., 2009). In addition c-Abl also phosphorylates DNA-PK, rendering it unable to bind DNA (Kharbanda et al., 1997; Shafman et al., 1997). The c-Abl inhibitor imatinib did not impair γH2AX signalling [Figure 5.18], however we have not assessed whether the inhibitor alters repair pathway choice. It is possible that the c-Abl induced phosphorylation suppresses repair by NHEJ favouring repair by HR.

PARP1 and PARP2 are both enriched in nucleoli (Meder et al., 2005). After inhibition of RNA polymerase I by ActD, PARP becomes evenly distribution throughout the nucleus (Desnoyers et al., 1996). After DNA damage PARP is recruited to the DSB. This recruits nucleosome remodelling and deacetylase complexes. It is thought that PARP removes nascent RNA from the DNA and silences RNA polymerase II transcription, which is independent of ATM and ATR (Chou et al., 2010). PARP also modifies DNA-PK directly enhancing its activity (Ruscetti et al., 1998). Therefore it is possible that PARP also contributes to RNA polymerase I transcriptional inhibition. In addition it might also play a role in the decision which repair pathway is chosen. Cells can be transfected with I-Ppol in the presence PARP inhibitors. We can use the click-chemistry assay to assess whether transcription is inhibited and by staining the cells with antibodies against HR proteins, whether the preferential repair by HR is affected by PARP inhibitors.
The effect of DSBs on processing of the pre-rRNA has not yet been assessed. Inhibition of processing would prevent the release of processing factors from the nucleolus. However it does create an imbalance with the ribosomal proteins and 5S rRNA which are imported from the nucleoplasm. Alternatively if processing is not inhibited, processing factors will run out of substrate. To distinguish between the two scenarios, RNA can be labelled and chase. If processing is inhibited the labelled RNA in the transfected cells remains in the nucleolus, while if processing continues the labelled RNA will be processed and exported to the cytoplasm.

The inhibition of transcription also leads to a reorganisation of the rDNA into caps. These caps have several advantages. Firstly, each caps contains a single NOR [Figure 5.9] which can prevent repair between different NORs leading to translocations. DNA damage has been shown induce chromosome translocation, inversions, duplications and deletions (Brunet et al., 2009; Lee et al., 2010; Lee et al., 2012). The rearranged rDNA repeats are thought to have arisen from inversions. The percentage of rearranged rDNA repeats in cells before and after damage can be compared. Potentially cells with higher levels of rearranged rDNA repeats might have a proliferative disadvantage or might be less able to cope with stress. Multiple DSBs in the same NOR can also lead to loss or gain of repeats similar to yeast [section 1.8.1]. We can compare the number of rDNA repeats from cells that have recovered from DNA to cells that have never experience DNA damage. By generating isogenic cell lines with different number of rDNA repeats, we can determine what the function of the excess rDNA repeats is.

In addition cells recovered after DNA damage might have Robertsonian translocations, which is one of the common translocation in humans (Hamerton et al., 1972).

Secondly, all the repeats of a single NOR are compacted in a small area. The high local concentration of homologous sequences can stimulate repair via homologous recombination. Also the formation of the caps eliminates the search for a homologous sequence (Dion et al., 2012; Gandhi et al., 2012; Mine-Hattab and Rothstein, 2012).

And lastly, the DSBs relocate to the caps at the periphery of the nucleolus, where they are accessible for repair factors that are excluded from the nucleolus. Proteins involved in the initial sensing of DSBs do localise to the nucleoli, but the proteins in the later stages of repair are excluded from nucleoli (Ahmad et al., 2009; Andersen et al., 2005) [Figure 5.20 and 6.3].

The rDNA is repaired by homologous recombination independent of cell cycle stage [Figure 6.6 and 6.8]. It is possible that all repetitive genes are repaired by HR. Alternatively the unique organisation of the nucleolus might contribute. To distinguish between two possibilities we can analyse the response to DSBs in the U2 small nuclear RNA array or 5S
rDNA array. With the CRISPR system it is possible to specifically induce DSBs in the arrays. These genes are transcribed by RNA polymerase III in the nucleoplasm. If these genes are repaired by HR independent of the cell cycle stage indicating that this is a property of repetitive genes. If not, possibly the protein composition of nucleoli might stimulate HR. It is possible that the exclusion of proteins is active process. In yeast the Smc5-Smc6 complex is required to exclude Rad52 foci from nucleoli. The exclusion dependent on sumoylation and prevents ERC formation (Torres-Rosell et al., 2007).

We have not yet identified a mechanism that prevents NHEJ and promotes HR of DSBs in the rDNA. Large 53BP1 foci overlap with the γH2AX foci [Figure 6.4]. The interplay between 53BP1-RIF1 and BRCA1-CtIP controls the initiation of resection. In G1, 53BP1 is bound, which inhibits resection. During S and G2, BRCA1 displaces 53BP1 and cdk-stimulated CtIP activity initiates resection (Escribano-Díaz et al., 2013). The binding of 53BP1 is disrupted when H4K16 is acetylated by Tip60. Tip60 deficiency inhibits HR, while treatment of cells with the HDAC inhibitor TSA stimulates HR (Tang et al., 2013). So apart from the potential role in activating ATM after nucleolar damage, Tip60 might also control the repair pathway choice for DSBs in the rDNA by modifying the local chromatin environment.

Nucleolar proteins also participate in checkpoint signalling. Under stress conditions, NPM gets evenly redistributed throughout the nucleus. This stimulates the interaction of NPM with HDM2, which inhibits the E3 ubiquitin ligase activity and stabilises p53 (Kurki et al., 2004). In addition, phosphorylated NPM has been reported to be able to override the G2/M checkpoint (Du et al., 2010). Whether the release of NPM from the nucleolus is dependent on ATM kinase activity remains to be determined.

The translocation of NPM during ActD treatment stabilises p53 (Abella et al., 2010). The cell cycle inhibitor p21 is a transcriptional target of p53 and essential to arrest cells in G1 (Cazzalini et al., 2010; Harper et al., 1993). The NPM redistribution and PIKK signalling work together to stabilise p53. Preliminary data shows that the introduction of DSBs in the rDNA releases NPM from the nucleolus [Figure 6.13]. After stabilisation, p53 induces p21 expression. However RPE1 cells have high levels p21 expression in the absence of damage [Figure 7.3]. Treatment of RPE1 cells with ActD for 10h increased the number of cells that expressed p21 and also increased the level of p21 expression in those cells increased. However not all cells express p21. It is possible that this is due to the stage of the cell cycle and only in G1 cells, p21 expression is induced.

Due to this complexity I have not been able to determine whether there are differences in checkpoint activation after transfection with I-Ppol and AsiSI. It is possible that the cell copes differently with DNA damage in the nucleolus. Perhaps DNA damage in the nucleolus
does not induce a potent cell cycle arrest, whereas similar number of DSBs in the nucleoplasm would.

**Figure 7.3:** RPE1 cells have high levels in the p21 expression, which increase after ActD. Even in the absence of stress many cells have p21 expression. The levels of p21 expression and the number of cells expressing increases after treatment with ActD for 10h.

To investigate the activation of checkpoints, I can determine the cell cycle stage with the Fucci system and follow the cells as breaks are induced in the rDNA. By following the cells I can determine whether they move into the next cell cycle stage. The polyadenylation offers a potential way to distinguish better high levels of breaks (+polyA tail) and limited levels (-polyA tail). In figure 6.11, it is shown that cells have recovered 24 hours after transfection with I-PpoI –polyA tail, while cells transfected with I-PpoI +polyA tail still have large γH2AX foci and segregated nucleoli. This difference between recovery after damage and cell death can be exploited to decipher how DNA damage in the nucleolus contributes to the recovery or cell death.

DNA damage in the rDNA can either be an advantage or disadvantage for the recovery of cells. Advantage if damage in the rDNA helps to override checkpoints or a disadvantage is it sensitizes cells to cell death.

Given the associations between the changes in the nucleolus and cancer [section 1.6], we can compare different cell lines to detect if there is a proliferative advantage or disadvantage in karyotypically normal or cancer cells. If we have a better understanding of the relationship
between the nucleolus and cell cycle checkpoints, perhaps this can be exploited in designing new therapeutic strategies.
Appendix 1

Raising a Treacle (TCOF1) antibody in sheep

Treacher Collins syndrome is caused by mutations in the TCOF1 gene. It is an autosomal dominant disorder characterised by craniofacial abnormalities (Sakai and Trainor, 2009). The mutations mainly cause premature stop codons and mislocalisation of the protein (Edwards et al., 1997; Marsh et al., 1998).

TCOF1 encodes Treacle which seems to link transcription with processing by interacting with both UBF and Nop56 (Gonzales et al., 2005; Valdez et al., 2004). Because Treacle is also a potential target of a DNA damaged induced phosphorylation (Matsuoka et al., 2007; Stokes et al., 2007), I decided to raise an antibody against Treacle to aid further study this protein.

The full length size of Treacle has 1488 amino acids (Q13428- Uniprot). For immunisation a fragment was selected based on the domain structure and isoforms. Predictions of the domain analysis reveal that Treacle does not have characterised domains based on the amino acid sequence. The only recognised domain is a LisH domain, which is thought to be involved in microtubule dynamics and cell migration (Emes and Ponting, 2001). The domain prediction is based on an amino acid motif and its function in Treacle has not yet been validated.

The pink boxes indicate internal acidic repeats that make up most of the protein. Each of these repeats contain potential CK II phosphorylation sequences (Dixon et al., 1997). In the Dephoure study, a number of phospho-peptides from Treacle with a CK II consensus sequence was found arguing that the might be in vivo targets (Dephoure et al., 2008).

Treacle has multiple isoforms, but the N-terminus is similar in all isoforms [Figure A1.2]. With the exception of isoform, which lacks the first 60 amino acids, all other isoforms are similar. The amino acids sequences starts to diverse around 200 amino acids. Therefore the first 210 amino acids were chosen for immunisation. All isoforms should be detected by the antibody and it is likely that the structure is preserved.

Figure A1.1: Prediction of the domain structure of Treacle (SMART-smart.embl-heidelberg.de).

Figure A1.2: Prediction of the Treacle isoforms.
CLUSTAL W(1.2.0) multiple sequence alignment

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**Figure A1.2:** A multiple sequence alignment of the N-terminal sequence of all Treacle isoforms.
Methods:
The N-terminus of Treacle was PCR amplified from a cDNA clone using the following primers GCCATGGCCGAGGCCAGGAAGCG and TTACTCATCCTGGAGCTGGAGG. The product was cloned as a NcoI-EcoRV fragment into the Gateway™ entry vector pEntr4. The sequence was verified by sequencing (Source Biosciences). To express the N-terminal fragment, the insert was shuttled into the destination vector pDest17 using the LR clonase (Invitrogen).
pDest17-Treacle N-term was transformed into *E.coli* strain BL21Ai for expression. The His-tagged protein was shown to be soluble [Figure A1.3A] and was be purified using a Ni-NTA column [FigureA1.3B]. The eluted protein was pooled and sent for immunisation in sheep (Scottish National Blood Transfusion Service, Penicuik, Scotland).

Results
To test if the crude sheep sera after immunisation recognised Treacle, the 3rd bleed was tested on Western blot [Figure A1.4A]. A band of the estimated size of ~200kDa for Treacle isoform 1 was detected. To prove that the bands seen on Western blot are Treacle, cells were transfected with V5-tagged Treacle isoform 5. The Treacle antibody recognises the same
band as the V5 antibody proving that it is indeed Treacle that the antibody recognises [Figure A1.4B].

**Figure A1.4: Crude sera recognise band of the correct size on Western blot.** A) The 3\(^{rd}\) bleed was used 1:1000 on a Western blot. Both in nuclei and nucleoli, a band of ~200kDa was seen which is the expected size of Treacle isoform 1. B) To prove that the antibody is seeing the right protein, Treacle isoform 5 (cDNA clone) was V5 tagged and overexpressed. The V5 antibody and the Treacle antibody recognise the same band proving that the antibody does recognise Treacle. Due to the level of high level of expression the endogenous Treacle bands are very faint. Experiment was carried out by Kate Dwyer.

The specificity of the antibody was also demonstrated by immunostaining of Hela cells. Antibodies against fibrillarin were used as a positive control. Fibrillarin stained the same structures, confirming that the sera contain antibodies that recognise a nucleolar protein. In interphase cells, both antibodies show a punctate staining like other FC/DFC proteins. On metaphase chromosomes the Treacle antibody stains NORs while Fibrillarin has a diffuse staining. This is in agreement with previous publications that reported Treacle at mitotic NORs (Gonzales et al., 2005; Lin and Yeh, 2009).

Some antibodies against proteins involved in RNA polymerase I transcription can inhibit *in vitro* transcription. The Taf\(_{110}\) antibody is incubated with the nuclear extract prior to addition of the template DNA. The mixture was incubated at 37\(^{\circ}\)C for 30min to allow rRNA to be synthesized. The RNA is used in an S1 nuclease assay to measure the *in vitro* transcription. Addition of small amounts of the TAF\(_{110}\) antibody strongly inhibits transcription indicating that the antibody probably prevents formation of the preinitiation complex. We tested if Treacle antibody affects *in vitro* transcription similarly to the Taf\(_{110}\)
antibody However the Treacle sera do not seem to inhibit the in vitro transcription, indicating that Treacle is not part of the basal transcription machinery which is in agreement with a role for Treacle linking transcription and processing (Gonzales et al., 2005; Valdez et al., 2004).

**Figure A1.5:** Immunofluorescence with the Treacle sera shows a puncate nucleolar staining colocalizing with Fibrillarin. HeLa cells were fixed with 4% PFA and stained 1:200 with the 3rd bleed of the Treacle sera and Fibrillarin. The Treacle sera give a punctate nucleolar staining similar to Fibrillarin in interphase. In contrast on mitotic chromosome, Treacle stains the NORs, while Fibrillarin has a diffuse staining.

**Figure A1.6:** In vitro transcription reactions are inhibited by Taf110 sera but not by Treacle sera. Experiments carried out by Brian McStay.

**Discussion and future plans**

A Treacle antibody has been raised in sheep and validated. All experiments so far have been with the crude sera. Both on Western and in immunofluorescence, the signal was very bright. If the antibody was purified and directly coupled to a fluorophore, it can be used in conjunction with other sheep antibody like the UBF-Rhodamine coupled antibody. Also the bright signal makes it possible that the antibody is used in 3D-immunoFISH.
To date, no full length image clone of Treacle isoform 1 is available. However recent efforts in the lab have reconstructed this isoform. The full length Treacle can also be expressed in cells to verify that the largest band seen on Western blot is indeed isoform 1.

Treacle has multiple isoforms, but the expression pattern in various cell types and cell lines is unknown. Because of the difference in domains, some isoforms might have isoform specific functions. In addition to that Treacle has many potential phosphorylations (Dixon et al., 1997; Wise et al., 1997). The function of all the phosphorylation sites is unknown. CK II phosphorylation on RNN3 have been shown to be important for the release of the protein from the elongating polymerase. It might be speculated that the phosphorylation sites on Treacle might have a similar function regulating interactions with the transcription and/or processing machinery.

Recently it has also been reported that Treacle recruits RNA polymerase I independent of UBF. Upon siRNA knockdown of Treacle, both the localisation of UBF and RNA polymerase I was disrupted (Lin and Yeh, 2009). This suggests that Treacle functions in the preinitiation complex. To test this, the Treacle antibody was incubated in in vitro transcription reaction. In contrast to Taf110 antibody, a known component of the preinitiation complex, the Treacle antibody had no effect on transcription. It is possible that the N-terminus, which was used as an antigen, is not important for transcription. Indeed the central repeats of Treacle (213-962) have been shown to interact with RNA polymerase I (Lin and Yeh, 2009).

Also the role of the potential ATM phosphorylation site is unknown. By generating a phosphomimetic mutant (S1333E or S1333D) or changing it to an amino acids that can’t be phosphorylated, might elucidate the function of the phosphorylation after DNA damage. It is possible that the phosphorylation is needed to inhibit transcription and segregate nucleoli. Another possibility is that the phosphorylation impairs processing.

In summary, an antibody against Treacle has been generated. The crude sera react strongly on Western blot and in immunofluorescence. Many questions still remain around the exact function of Treacle with respect to transcription and processing and how that is influenced by a potential DNA damage induced phosphorylation. This antibody will be an important tool in further studies.
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