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# Human Ribosomal DNA and RNA Polymerase I Fate during UV-induced DNA Repair

Laurianne Daniel

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Laurianne DANIEL

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**Human Ribosomal DNA and RNA  
Polymerase I Fate during UV-induced  
DNA Repair**

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*Face à la roche, le ruisseau l'emporte toujours,  
non pas par la force mais par la persévérance.*  
H. Jackson Brown



*A mes parents, à ma marraine.*





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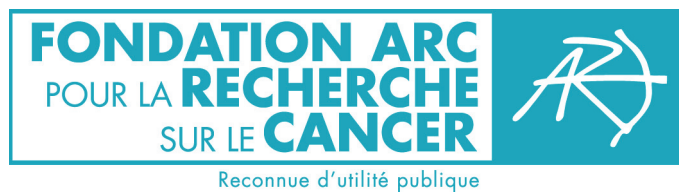
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# SUMMARY



The nucleolus, called the ribosome factory, contains the ribosomal DNA transcribed by the RNA Polymerase I. Half of the ribosomal genes in humans are silent and their transcription represents 60% of total cellular transcription. Ribosomal transcription is also the first and limiting step of the protein synthesis process, i.e. ribosome biogenesis. Moreover, ribosomal defects are involved in several serious diseases such as ribosomopathy, but also in aging and tumorigenesis. Therefore, it is of great importance to study the repair mechanism of this specific part of the genome.

This PhD project focuses on a mechanism which has been poorly investigated until now: the repair of UV-damage in ribosomal genes in humans. On the contrary, the repair mechanism of UV lesions in RNA polymerase II-transcribed genes has been well described and involves the nucleotide excision repair mechanism composed of two separate pathways: the global genome repair which detects the lesion in the whole genome and the transcription-coupled repair which is specific to actively transcribing genes.

Firstly, this project demonstrated the implication of the complete transcription-coupled nucleotide excision repair mechanism to remove bulky adducts on ribosomal DNA in humans, similar to the RNA Polymerase II-transcribed genes. However, a notable behaviour of RNA Polymerase I was observed. Indeed, the latter, in contrary to RNA Polymerase II, accumulated on ribosomal DNA and their displacement at the nucleolar periphery was observed after UV exposure. Furthermore, the return of the RNA Polymerase I was dependent on the complete repair of UV lesions, even those present on or close to silent ribosomal DNA.

Secondly, two proteins were identified, which are not involved in repair, but are required for the return of the RNA Polymerase I into the nucleolus: nuclear actin and myosin I, both of which enhance ribosomal transcription in the nucleolus. Interestingly, a calcium effect was observed on RNA Polymerase I transcription activity, but not on its relocation. Indeed, in the absence of calcium, the ribosomal DNA transcription is inhibited.

In this study, new insights are provided on the UV-damaged ribosomal DNA repair mechanism in humans. As part of this mechanism, a specific relocation of the RNA

## SUMMARY

polymerase I/ribosomal DNA complex during repair has been noted and two proteins required for this displacement identified.

# RÉSUMÉ



Le nucléole, qui contient l'ADN ribosomique transcrit par l'ARN Polymérase I, est le lieu de synthèse des ribosomes. De plus, 50% des gènes ribosomiques sont inactifs et leur transcription, qui représente 60% de la transcription cellulaire, constitue la première étape et non moins limitante de la biogenèse des ribosomes. En outre, une altération qualitative et/ou quantitative des ribosomes a été observée chez des patients atteints de graves maladies telles que les ribosomopathies, mais aussi lors de processus cellulaires tels que le vieillissement ou le développement tumoral. C'est pourquoi il est essentiel d'étudier le mécanisme de réparation des lésions qui apparaissent dans cette partie du génome.

Ce projet de thèse s'articule autour du mécanisme de réparation de l'ADN ribosomique endommagé par les rayons UV. Le mécanisme de réparation des lésions UV a été largement décrit pour les gènes transcrits par l'ARN Polymérase II. Ce mécanisme, la réparation par excision de nucléotides, se divise en deux voies distinctes : la réparation globale du génome qui concerne tout le génome et la réparation couplée à la transcription qui n'a lieu que dans les gènes activement transcrits.

Premièrement, nous avons démontré, de manière similaire à la réparation des gènes transcrits par l'ARN Polymérase II, l'utilisation complète du mécanisme de réparation couplée à la transcription pour éliminer les lésions induisant une distorsion de la double hélice d'ADN. Cependant, nous avons décrit un comportement singulier de l'ARN Polymérase I. En effet, cette dernière, contrairement à l'ARN Polymérase II, reste accrochée à l'ADN ribosomique et leur déplacement simultané à la périphérie du nucléole a été observé après l'induction de lésions UV. De plus, nous avons établi que le retour de l'ARN Polymérase I à l'intérieur du nucléole dépend de la réparation de toutes les lésions, même celles présentes sur ou à proximité des gènes ribosomiques actifs.

Deuxièmement, nous avons identifié deux protéines nécessaires au retour de l'ARN Polymérase I à l'intérieur du nucléole : l'actine et la myosine 1 nucléaires. Ces dernières sont impliquées dans la transcription par l'ARN Polymérase I. Curieusement, nous avons également noté l'influence du calcium sur la transcription par l'ARN Polymérase I mais pas sur la localisation nucléolaire de cette dernière. En effet, l'absence de calcium induit une diminution de la transcription des gènes ribosomiques



Au cours de cette étude, nous avons découvert de nouveaux aspects concernant le mécanisme par lequel l'ADN ribosomique est réparé chez l'homme après une exposition aux rayons UV. Composant à part entière de ce mécanisme, nous avons décrit le déplacement simultané de l'ARN Polymérase I et de l'ADN ribosomique lors de la réparation et nous avons également identifié deux protéines impliquées dans ce déplacement.

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# LIST OF ABBREVIATIONS





6-4PP: 6-4 Pyrimidine-pyrimidone Photoproducts

9-1-1: Rad9-Rad1-Hus1

$\gamma$ H2AX: phosphorylated-H2AX

## **A**

ACT $\beta$ :  $\beta$ -Actin

a-NHEJ: alternative NHEJ

AP: apurinic/apyrimidinic

ARC: Association pour la Recherche sur le Cancer

ATM: Ataxia Telangiectasia Mutated

ATP: Adenosine-Tri-Phosphate

ATPase: Adenosine-Tri-Phosphatase

ATR: ATM-Rad3-related

ATRIP: ATR Interacting Protein

## **B**

BER: Base Excision Repair

BLM: Bloom syndrome helicase

BRCA1: BReast Cancer 1

## **C**

Ca<sup>++</sup>: Calcium

CAK: CDK-Activating Kinase

CE: Core Element

CETN2: Centrin2

ChIP: Chromatin ImmunoPrecipitation

Chk1: Checkpoint kinase 1

Chk2: Checkpoint kinase 2

c-NHEJ: canonical NHEJ

COFS: Cerebro-Occulo-Facial-Skeletal

CPD: Cyclobutane-Pyrimidine Dimers

CS: Cockayne Syndrome

CSA: Cockayne Syndrome group A protein

CSB: Cockayne Syndrome group B protein

CTD: C-Terminal Domain

CtBP: C-terminal Binding Protein

CtIP: CtBP-Interacting Protein

## **D**

DAPI: 4',6-diamidino-2-phenylindole

DBA: Diamond-Blackfan Anemia

DDB1: DNA Damage-Binding protein 1

DDB2: DNA Damage-Binding protein 2

DDR: DNA Damage Response

DJ: Distal Junction

DFC: Dense Fibrillar Component

DMEM: Dulbecco's Modified Eagle's Medium

DNA: DesoxyriboNucleic Acid

DNA-PKcs: DNA-dependent Protein Kinase catalytic subunit

DNA pol  $\delta$ : DNA polymerase  $\delta$

DSB: Double-Strand Break

dsDNA: double-stranded DNA

## **E**

EdU: 5-Ethynyl-2'-deoxyuridine

EDTA: Ethylene-Diamine-Tetraacetic Acid

EGFP: Enhanced GFP

EGTA: Ethylene Glycol Tetraacetic Acid

ERCC1: Excision Repair Cross-Complementation Group 1

ES: Embryonic Stem

ETS: External Transcribed Spacer

EXO1: Exonuclease 1

## **F**

FACT: Facilitates Chromatin Transcription

F-actin: Filamentous actin

FAT: FRAP-ATM-TRRAP

FATC: FAT carboxy-terminal

FBL: Fibrillarin

FC: Fibrillar Center

FRAP: Fluorescent Recovery After Photo-bleaching

## **G**

G-actin: Globular actin

GAR: Glycine-Arginine Rich

GC: Granular Component

GFP: Green Fluorescent Protein

GG-NER: Global Genome Nucleotide Excision Repair

GSR: Genomic Stress Response

## **H**

H2AX: Histone H2A.X

HEAT: Huntington-Elongation factor 3-protein phosphatase 2A-TOR1

HEPES: 4-(2-HydroxyEthyl)-1-piperazineEthaneSulfonic acid

hHR23B: human RAD23 Homolog B

HMG: High-Mobility-Group

HMGN1: High Mobility Group Nucleosome binding domain 1

HR: Homologous Recombination

HRP: HorseRadish Peroxidase

## **I**

IF: ImmunoFluorescence

IGS: IntraGenic Spacer

IP: ImmunoPrecipitation

IR: Ionizing Radiation

ITS: Internal Transcribed Spacer

## **J-K**

## **L**

LacO: LacOperon

LacR: LacRepressor

LIF: Leukemia Inhibitory Factor

LIG1: DNA ligase 1

LIG3: DNA ligase 3

LIG4: DNA ligase 4

## **M**

Mag1: 3-Methyladenine DNA glycosylase 1

MDC1: Mediator of DNA Damage Checkpoint 1

miRNA: microRNA

Mlh1: MutL homolog 1

Mlh2: MutL homolog 2

Mlh3: MutL homolog 3

MMR: MisMatch Repair

Mms2: Methyl methanesulfonate sensitivity

Mre11: Meiotic recombination protein 11

MRN: MRE11, RAD50 and NBS1

mRNA: messenger RNA

MRX: Mre11; Rad50; Xrs2

Msh1: MutS homolog 1

Msh2: MutS homolog 2

Msh3: MutS homolog 3

Msh4: MutS homolog 4

Msh5: MutS homolog 5

Msh6: MutS homolog 6

MTase: MethylTransferase

## **N**

NBS1: Nijmegen Breakage Syndrome 1

NER: Nucleotide Excision Repair

NHEJ: Non-Homologous End-Joining

NMI: Nuclear Myosin I

Nop1: Nucleolar protein 1

NOR: Nuclear Organizer Region

Ntg1: endonuclease three-like glycosylase 1

Ntg2: endonuclease three-like glycosylase 2

NT: Untreated

NTIS: Non-Transcribed Intergenic Spacers

## **O**

Ogg1: 8-OxoGuanine Glycosylase 1

## **P**

PAF49: RNAP1-Associated Factor of 49kDa

PAF67: RNAP1-Associated Factor of 67kDa

PARP1: Poly [ADP-Ribose] Polymerase 1

PBS: Phosphate-Buffered Saline

PBS+: PBS; 0.5%BSA; 0.15% glycin

PBS-T: PBS-Tween

PCNA: Proliferating Cell Nuclear Antigen  
PIC: Pre-Initiation Complex  
PIKK: Phosphoinositide 3-Kinase-related Kinase  
PJ: Proximal Junctions  
Pms1: Postmeiotic segregation protein 1  
PNB: Pre-Nuclear Body  
POLR1C: RNA Polymerase I subunit C  
POLR1D: RNA Polymerase I subunit D  
POLR1E: RNA Polymerase I subunit E  
PRMT1: Protein Arginine MethylTransferase 1  
PRMT5: Protein Arginine MethylTransferase 5  
PRR: Post-Replication Repair  
PTRF: Pol1 and Transcript-Release Factor  
PVDF: PolyVinylidene Difluoride Membrane

## **Q**

qPCR: quantitative Polymerase Chain Reaction

## **R**

RAD1: Radiation sensitive protein 1  
RAD3: Radiation sensitive protein 3  
Rad4: Radiation sensitive protein 4  
Rad5: Radiation sensitive protein 5  
RAD9: Radiation sensitive protein 9  
RAD17: Radiation sensitive protein 17



RAD23: Radiation sensitive protein 23  
Rad26: Radiation sensitive protein 26  
Rad28: Radiation sensitive protein 28  
Rad50: Radiation sensitive protein 50  
RAD51: Radiation sensitive protein 51  
RAD52: Radiation sensitive protein 52  
rDNA: ribosomal DNA  
RFC: Replication Factor C  
RFC2: Replication Factor C Subunit 2  
RIPA: RadioimmunoPrecipitation Assay  
RNA: Ribonucleic Acid  
RNAP1: RNA Polymerase I  
RNAP1 $\alpha$ : elongation form of RNAP1  
RNAP1 $\beta$ : initiation form of RNAP1  
RNAP2: RNA polymerase II  
RNAP3: RNA polymerase III  
RPA: Replication Protein A  
RPA-ssDNA: RPA-coated single-stranded DNA  
RPA194: RNA Polymerase I 194 KDa Subunit  
ROS: Reactive Oxygen Species  
RR: Rad17-Rfc2-5  
RRN3: RNA polymerase I-specific transcription initiation factor  
rRNA: ribosomal RNA

**S**

Sae2: Sporulation in the absence of spo eleven

SDS-PAGE: Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis

SEM: Standard Error of the Mean

siMock : Non-targeting siRNA

siRNA: small interfering RNA

SL1: Selective Factor1

snoRNP: small nucleolar ribonucleoprotein

snRNA: small nuclear RNA

SSA: Single-Strand Annealing

ssDNA: single-stranded DNA

SSPE: Saline-Sodium Phosphate-EDTA

## T

TAF: TBP-Associated Factors

TBP: TATA-box-Binding Protein

TC-NER: Transcription-Coupled Nucleotide Excision Repair

TCS: Treacher Collin Syndrome

TdT: Terminal deoxynucleotidyl Transferase

TEL2: Telomere Maintenance 2

TFIIH: Transcription Factor II H

TFIIS: Transcription Factor II S

TIP60: Tat Interactive Protein, 60kDa

TOPBP1: Topoisomerase II Binding Protein 1

tRNA: transfer RNA

TTD: TrichoThioDystrophy

TTDA: TrichoThioDystrophy group A protein

TTDN1: TrichoThioDystrophy Nonphotosensitive 1

TTF-1: Termination Transcription Factor 1

TWISTNB: TWIST NeighBour

## **U**

Ubc13: Ubiquitin-conjugating protein 13

UBF: Upstream Binding Factor

UCE: Upstream Control Element

UDS: Unscheduled DNA Synthesis

Ung1: Uracil DNA N-glycosylase 1

USP7: Ubiquitin Specific Peptidase 7

UV: Ultra-Violet

UV-DDB: UltraViolet radiation-DNA Damage-Binding protein

UVSS: UV-sensitive syndrome

UVSSA: UV Stimulated Scaffold protein A

## **V**

## **W**

WT: Wild Type

## **X**

XAB2: XPA Binding Protein 2

XFEPS: XPF-ERCC1 progeroid syndrome

XLf: XRCC4-Like Factor

XP: Xeroderma Pigmentosum

XPA: Xeroderma Pigmentosum, complementation group A

XPB: Xeroderma Pigmentosum, complementation group B

XPC: Xeroderma Pigmentosum, complementation group C

XPD: Xeroderma Pigmentosum, complementation group D

XPE: Xeroderma Pigmentosum, complementation group E

XPF: Xeroderma Pigmentosum, complementation group F

XPG: Xeroderma Pigmentosum, complementation group G

XPV: Xeroderma Pigmentosum Variant

XRCC4: X-Ray Repair Cross Complementing 4

Xrs2: X-Ray Sensitive protein 2

## **Y**

Yku70p: Yeast ku70 protein

Yku80p: Yeast ku80 protein

## **Z**



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# INTRODUCTION



## **.1. The nucleolus: structure and functions**

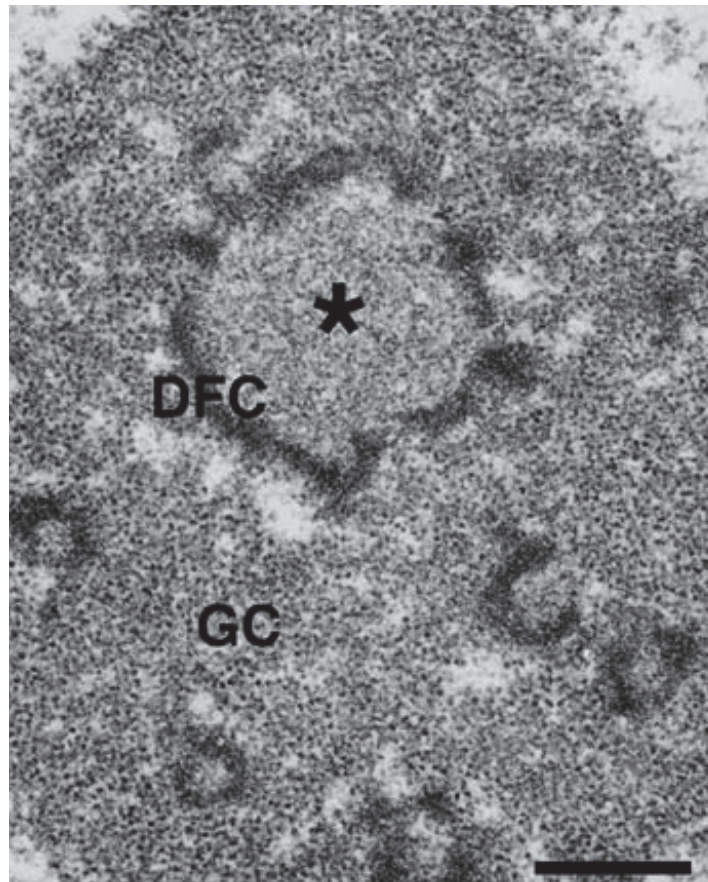
### **.1.1. Organization of the nucleolar region**

The nucleolus was first described by Felix Fontana as a ball-shaped structure in the nucleus (Fontana, 1781). Since that time, the great advances in microscopy have permitted a deeper understanding of the nucleus' structure. Electron microscopy together with biochemical techniques have allowed the visualization of the nucleolus as a dense fibrillar component (Thiry et al., 1991). The nucleolus is a membraneless structure composed of ribosomal DNAs (rDNAs), ribosomal RNAs (rRNAs), microRNAs (miRNAs) and proteins involved in ribosome biogenesis, chromatin structure, messenger RNAs (mRNAs) metabolism and/or translation (Andersen et al., 2002; Politz et al., 2009; Scherl et al., 2002).

In mammalian cells, the nucleolar compartment consists of 3 different subdivisions: the Fibrillar Center (FC), the Dense Fibrillar Component (DFC) and the Granular Component (GC) (Figure 1; Sirri et al., 2008). FC is of low density and contains the rDNA - in a non-nucleosomal state - that can be transcriptionally active or inactive. DFC, which surrounds the FCs, presents a higher density because DNA is more condensed. Small particles of 15nm in diameter form the Granular Component that envelopes the DFC.

Ribosomal DNA is transcribed by RNA Polymerase I (RNAP1) in the nucleolus (Goodfellow and Zomerdijk, 2012; Russell and Zomerdijk, 2005). Although the mechanism of rDNA transcription may have been well described (see .1.4), some doubts still remain concerning its location within the nucleolus. Several hypotheses have been formulated about the location of the transcription of rDNA. Based on several studies, Huang (2002) established 3 different models for the pre-rRNA synthesis. It could operate either in the FCs, or in the FCs including the FCs/DFC border, or in the DFC together with the DFC/FCs zone (Huang, 2002). Even though no solid agreement on the specific site of RNAP1 transcription has yet emerged, research groups have agreed that rDNA transcription does not occur inside the FCs but rather takes place at the FC/DFC border (Boisvert et al., 2007; Derenzini et al., 2006). Indeed, the inner part of the FCs contains untranscribed "open" rDNA. The DFC

seems to be involved in early rRNA maturation, whereas GC is involved with late rRNA (Grob and McStay, 2014).



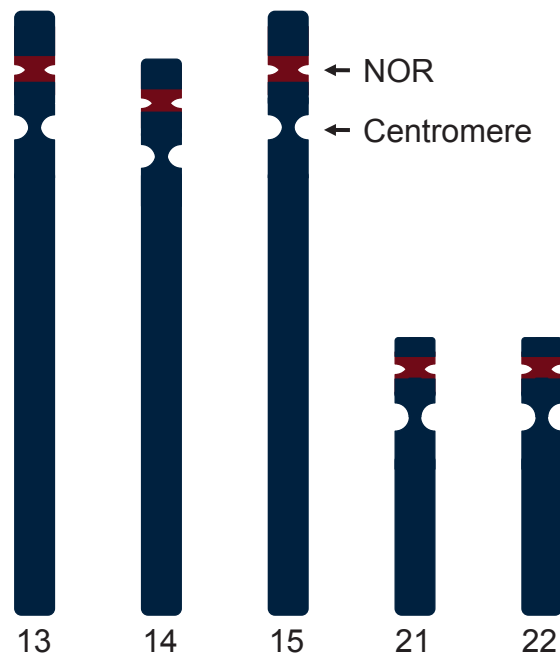
**Figure 1: Organisation of the nucleolus in human cells.**

*Via electron microscopy, the organisation of the nucleolus is observable. Here, the three nucleolar components of the nucleolus can be identified. Fibrillar Centers (FCs) of different sizes are visible, the larger one is indicated by an asterisk \*. Dense Fibrillar Component (DFC) surrounds the FC and is embedded in the Granular Component (GC). Scale bar: 0.5 $\mu$ m. Sirri et al., 2008*

The nucleolus, as described above, is only visible during interphase. In real, the nuclear envelope disappears when the cell enters mitosis, as does the nucleolus. Throughout mitosis, the nucleus releases its contents in order to be equally distributed to the daughter cells. At the end of mitosis, the nucleus reassembles and the nucleolus emerges from the Nuclear Organizer Regions (NOR).

## .1.2. The Nucleolar Organizer Regions

The NOR is mitosis' counterpart of the nucleolus. Indeed, the nucleolus derives from these specific chromosomal regions. The NORs - holding the rDNA - are located on the small arm of all the 5 acrocentric chromosomes: 13, 14, 15, 21 and 22 (Henderson et al., 1972) (Figure 2). When mitosis commences termination, RNAP1 transcription restarts. This is one of the signals for the NORs, along with some nucleolar proteins such as Fibrillarin, Nucleolin or B23, to form the Pre-Nuclear Bodies (PNBs) (Dundr et al., 2000; Savino et al., 1999; Verheggen et al., 2001). Finally, PNBs fuse together to give rise to the interphasic nucleolus (Dundr et al., 2000; Savino et al., 2001). These studies have lead to the conclusion that the nucleolus is a self-organizing structure (Misteli, 2001).



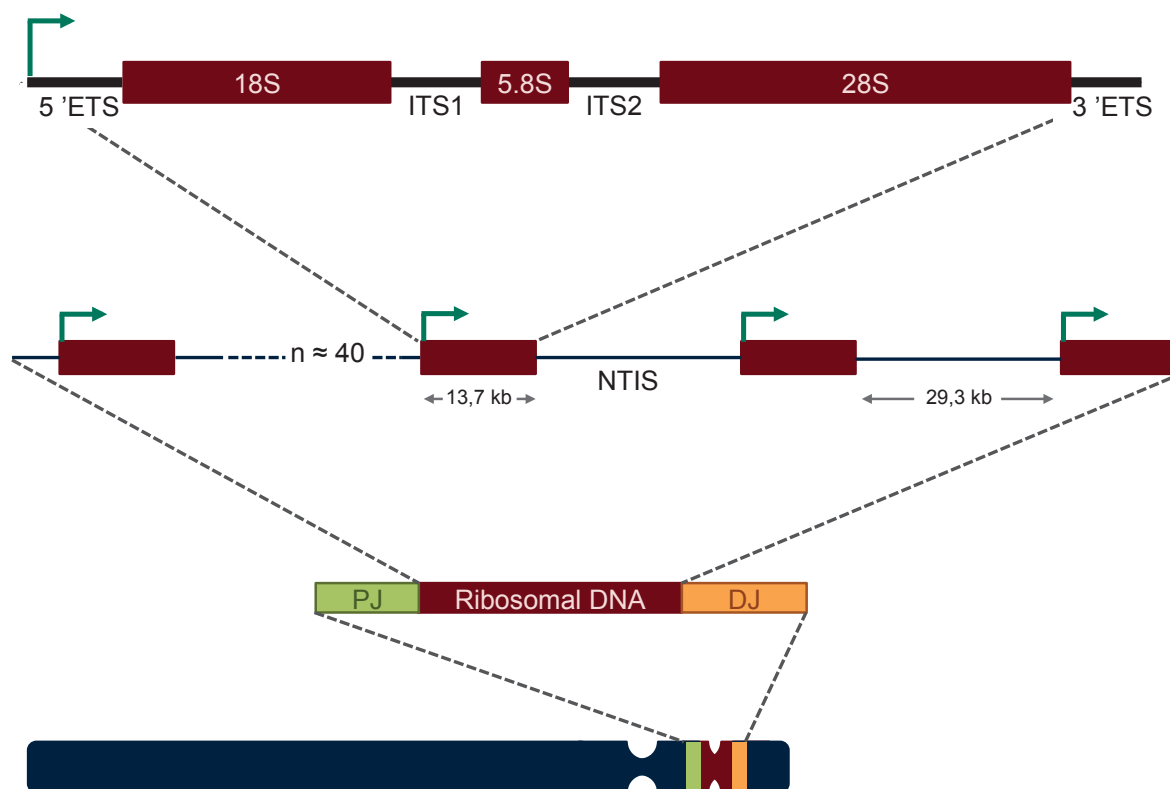
**Figure 2: Nucleolar Organizer Regions location on the chromosome**

*The Nucleolar Organizer Regions (NORs) are located on the short arm of the five acrocentric chromosomes*

## .1.3. Ribosomal DNA

Diploid human cells possess about 400 copies of rDNA (Schmickel, 1973). The rDNA consists of tandemly repeated arrays of a specific transcription unit separated by the Non-Transcribed Intergenic Spacers (NTIS) (Lam and Trinkle-Mulcahy, 2015; Smirnov et al., 2016).

The transcription unit is composed of 3 sequences, coding the ribosomal RNAs: 18S, 5.8S and 28S, along with External/Internal Transcribed Spacers (ETS/ITS) (Figure 3). The transcription units together with the NTIS form the NORs described previously. Proximal and distal regions, flanking the NORs, are conserved across the five acrocentric chromosomes in mammalian cells (Floutsakou et al., 2013). The Distal Junction (DJ), downstream of the rDNA arrays, has been observed at the periphery of the nucleolus. Floutsakou and co-workers (2013) came to the conclusion that DJs were responsible for the rDNA localization in the nucleolus. Additionally, Proximal Junctions (PJs) were described as regions with high recombination rate.



**Figure 3: Transcription unit of the ribosomal DNA.**

*Ribosomal DNA is constituted of head-to-tail tandem repeats of the transcription unit containing the sequences coding for the 18S, 5.8S and 28S and the External and Internal Transcribed Sequences (ETS & ITS1/2). Each of the 13,7kb transcription unit is surrounded by the Non-Transcribed Intergenic Spacer (NTIS; 29,3kb). About 40 ribosomal transcription units are located per acrocentric chromosome. The location of the rDNA into the nucleolus is due to the position of the Distal Junctions (DJ) at the nucleolar periphery. As for the Proximal Junctions (PJ), they present a significant propensity to recombination. Adapted from Floutsakou et al., 2013.*

## **.1.4. Transcription by RNA polymerase I**

In human cells, transcription is performed by the RNA polymerases I, II and III (RNAP1, RNAP2 and RNAP3) (Roeder and Rutter, 1969). These polymerases share five common subunits along with specific ones (Werner and Grohmann, 2011). Each of these polymerases is involved in the transcription of different classes of genes. RNAP2 produces mRNAs and some small nuclear RNAs (snRNAs), whereas RNAP3 gives rise to the 5S rRNA, transfer RNAs (tRNAs) as well as the U6 snRNA (Chandrasekharappa et al., 1983; Turowski and Tollervey, 2016). Unlike RNAP2 and RNAP3 that produce different types of RNAs, RNAP1 is specifically devoted to ribosomal gene transcription into the unique 47S pre-rRNA, which undergoes several cuts leading to the 5.8, 18 and 28S rRNAs (Russell and Zomerdijk, 2006).

In dividing eukaryotic cells, RNAP1 transcription represents 60% of total transcription events, even though 50% of the rDNA is inactive (Conconi et al., 1989; Warner, 1999). Indeed, the transcription initiation step occurs every 1-2 seconds and the elongation rate of RNAP1 is about 95 nucleotides/sec (Dundr, 2002). The large number of RNAP1, about 100-120 per transcription unit, simultaneously loaded on the ribosomal DNA explains this high level of transcription (Dieci and Sentenac, 2003). Moreover, rDNA transcription is cell cycle-dependent. Indeed, transcription does not occur during mitosis, but gradually increases during interphase, with a peak in S and G2 phases (Russell and Zomerdijk, 2005).

### **.1.4.1. Composition of the RNAP1 complex**

The composition of the subunits of the RNA polymerases is highly conserved through evolution (Armaleo, 1987; Fernández-Tornero et al., 2013; Werner and Grohmann, 2011). RNAP1 structure has been extensively studied by crystallography and electron microscopy in yeast (Engel et al., 2013; Kuhn et al., 2007; Tafur et al., 2016). Moreover, structural knowledge about the mammalian RNAP1 enzyme derives initially from biochemical studies on yeast. For this reason, RNAP1 shall refer to the yeast enzyme within this section.

RNAP1 is composed of 14 subunits, for which mammalian homologues have been described, except from the A14 subunit that has not yet been identified (Russell and Zomerdijk, 2006) (Table 1). The core of the enzyme is composed of 10 subunits. 5 of them



(Rpb5; 6; 8; 10 and 12), shared with RNAP2 and RNAP3, form the clamp of RNAP1 together with AC40 and AC19 (Figure 4). Additionally, AC12.2 is part of the core as an RNA cleavage domain. The DNA binding cleft holding the A190 and A135 subunits is also part of the core (Kuhn et al., 2007; Vannini and Cramer, 2012). The stalk of RNAP1 is constituted by the A43 and A14 subunits and is described as a platform for recruitment of the initiation factors (Kuhn et al., 2007). RNAP1 possesses supplemental specific subunits: AC49 and AC34.5 which are involved in the binding/release of Rrn3p (Beckouet et al., 2008). Albert and co-workers (2011) described the heterodimer AC49/34.5 as essential for nucleolar assembly.

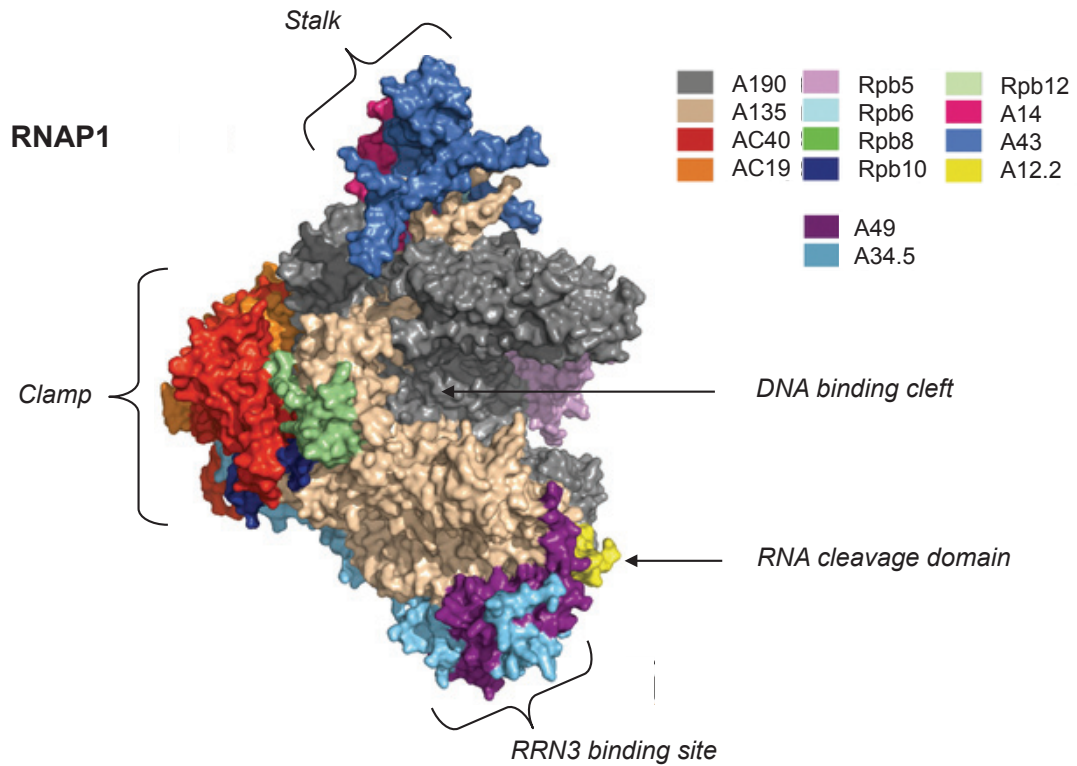
RNA Polymerase I	<i>S. cerevisiae</i>	Human	Unique or shared
Core enzyme subunits	Rpb5	hRPB5	I - II - III
	Rpb6	hRPB6	I - II - III
	Rpb8	hRPB8	I - II - III
	Rpb10	hRPB10	I - II - III
	Rpb12	hRPB12	I - II - III
	A12.2	hRPA12.2	I
	AC19	hRPA19	I - III
	AC40	hRPA40	I - III
	A135	A127	I
	A190	A194	I
Stalk subcomplex	A14	?	I
	A43	TWISTNB	I
Other subcomplex	A34.5	hPAF49	I
	A49	hPAF53	I

**Table 1: Subunits of the RNA Polymerase I in yeast and their homologues in human.**

*“Unique or shared” refers to subunits that are only part of RNAP1 or shared with RNAP2 and/or RNAP3.*

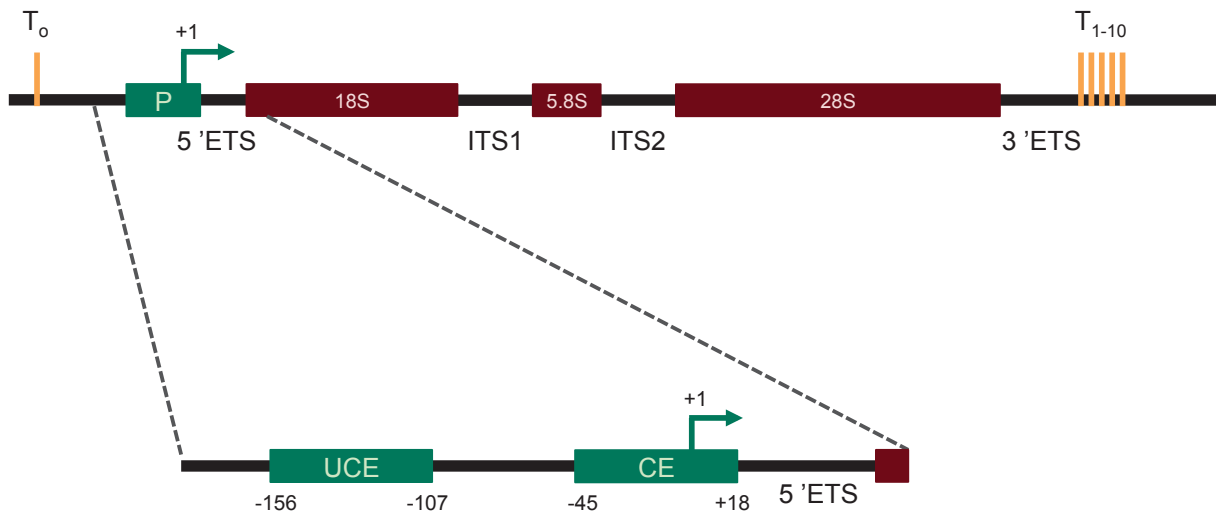
#### **.1.4.2. Pre-initiation complex (PIC) formation and initiation of transcription**

In mammalian cells, the promoter of rDNA consists of a Core Element (CE) – overlapping the transcription start site and needed for accurate initiation of the transcription – together with an Upstream Control Element (UCE) exhibiting a modulatory role for initiation (Goodfellow and Zomerdijk, 2012) (Figure 5).



**Figure 4: Crystal structure of yeast RNAP1.**

In yeast, RNAP1 consists of 14 subunits organized into different structures such as the clamp, the stalk and the DNA binding cleft, constituting the core of the enzyme. The polymerase also presents the RNA cleavage domain and the RRN3 binding site. Adapted from Fernández-Tornero et al., 2013.



**Figure 5: Structure of the promoter and location of the termination sites in the ribosomal DNA.**

The ribosomal promoter consists of the Upstream Control Element (UCE) and the Core Element (CE). The termination sites for ribosomal transcription are located on both parts of the rDNA. Upstream of the ribosomal gene is the termination site T<sub>0</sub> whilst downstream are found up to 10 termination sites (T<sub>1-10</sub>).

The three eukaryotic RNA polymerases use different transcription machineries that all include the recruitment of a TATA-box-Binding Protein (TBP) and several TAFs (TBP-Associated Factors) at different steps of the transcription initiation (Goodrich and Tjian, 1994; Hernandez, 1993; Sharp, 1992). Indeed, because RNA polymerases present a low affinity for promoter sequences, they use different transcription factors such as the Selective Factor1 (SL1) that is a TBP-TAF-containing complex, the Transcription Factor II D (TFIID) which contains the TBP and TFIIIA or TFIIIC that recruit TFIIIB containing the TBP, to initiate transcription for RNAP1, RNAP2 and RNAP3 respectively. (Goodfellow and Zomerdijk, 2012).

Regarding the RNAP1 mechanism, ribosomal transcription starts after the recruitment of RNAP1 and several transcription factors, such as SL1 and the Upstream Binding Factor (UBF), constituting the PIC (Figure 6). UBF and SL1 serve to enhance promoter selectivity. UBF, functioning as a homodimer, binds to the ribosomal DNA both on UCE and CE. A twist on the rDNA, due to the HMG (High-Mobility-Group) boxes of the UBF proteins, allows this double binding (Goodfellow and Zomerdijk, 2012). The binding of UBF is associated with SL1 binding to the rDNA promoter. SL1 is a complex built of the TBP and 4 TAFs (TAF<sub>110</sub>; TAF<sub>63</sub>; TAF<sub>48</sub> and TAF<sub>41</sub>) (Gorski et al., 2007). SL1 recruits RNAP1, in its initiated form (RNAP1 $\beta$ ), on the core element of the promoter. The interaction of SL1 subunits (TAF<sub>110</sub> and TAF<sub>63</sub>) with the RNAP1-specific transcription initiation factor RRN3 and the RNAP1-Associated Factor of 67kDa (PAF67) induces the recruitment of RNAP1. If SL1 and RNAP1 $\beta$  are sufficient for a basal level of ribosomal transcription in vitro, the presence of UBF is required to initiate the transcription in vivo (Russell and Zomerdijk, 2005). Finally, after PIC assembly and RNAP1 recruitment, the ribosomal transcription can start.

#### **.1.4.1. Promoter escape and transcription elongation**

Clearance occurs when the first few ribonucleotides are incorporated, allowing RNAP1 to overcome the inhibitory interactions between the enzyme and the associated factors at the promoter. The promoter escape is concomitant with the release of RRN3, which then is inactivated (Figure 6). Consequently, the RNAP1 $\beta$  initiation-competent

polymerase is converted into its elongation form (RNAP1 $\alpha$ ) (Milkereit and Tschochner, 1998). Although the RNAP1 $\alpha$  can effectively clear the promoter in this condition, it has been shown that an efficient RNAP1 elongation is dependent on the presence of the Transcription Factor II H (TFIIH) (Iben et al., 2002). Indeed, TFIIH is located along the rDNA strand but firstly recruited to the PIC at the promoter even though it has no function in RNAP1 initiation. Moreover, Assfalg and co-workers (2012) have shown that mutations in TFIIH helicase subunits reduce the affinity of this transcription factor for rDNA and impair ribosomal transcription. After RNAP1 $\alpha$  has escaped from the promoter, UBF and SL1 remain on the rDNA promoter in order to recruit the next RNAP1. Hence, they allow the transcription to restart rapidly from the same promoter and so support multiple rounds of transcription (Panov et al., 2001).

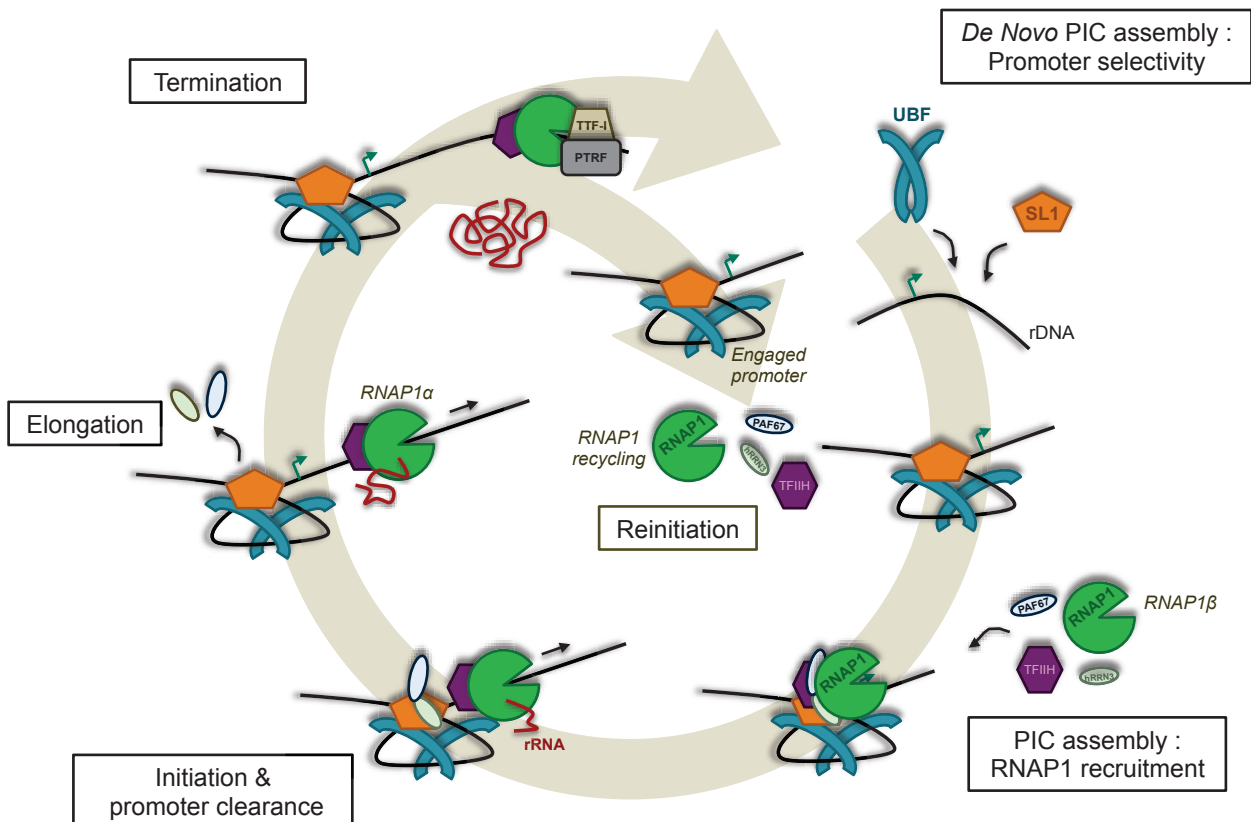


Figure 6: RNA Polymerase I transcription process.

*Adapted from Russell and Zomerdijk, 2005.*

#### **.1.4.2. Termination and re-initiation of transcription**

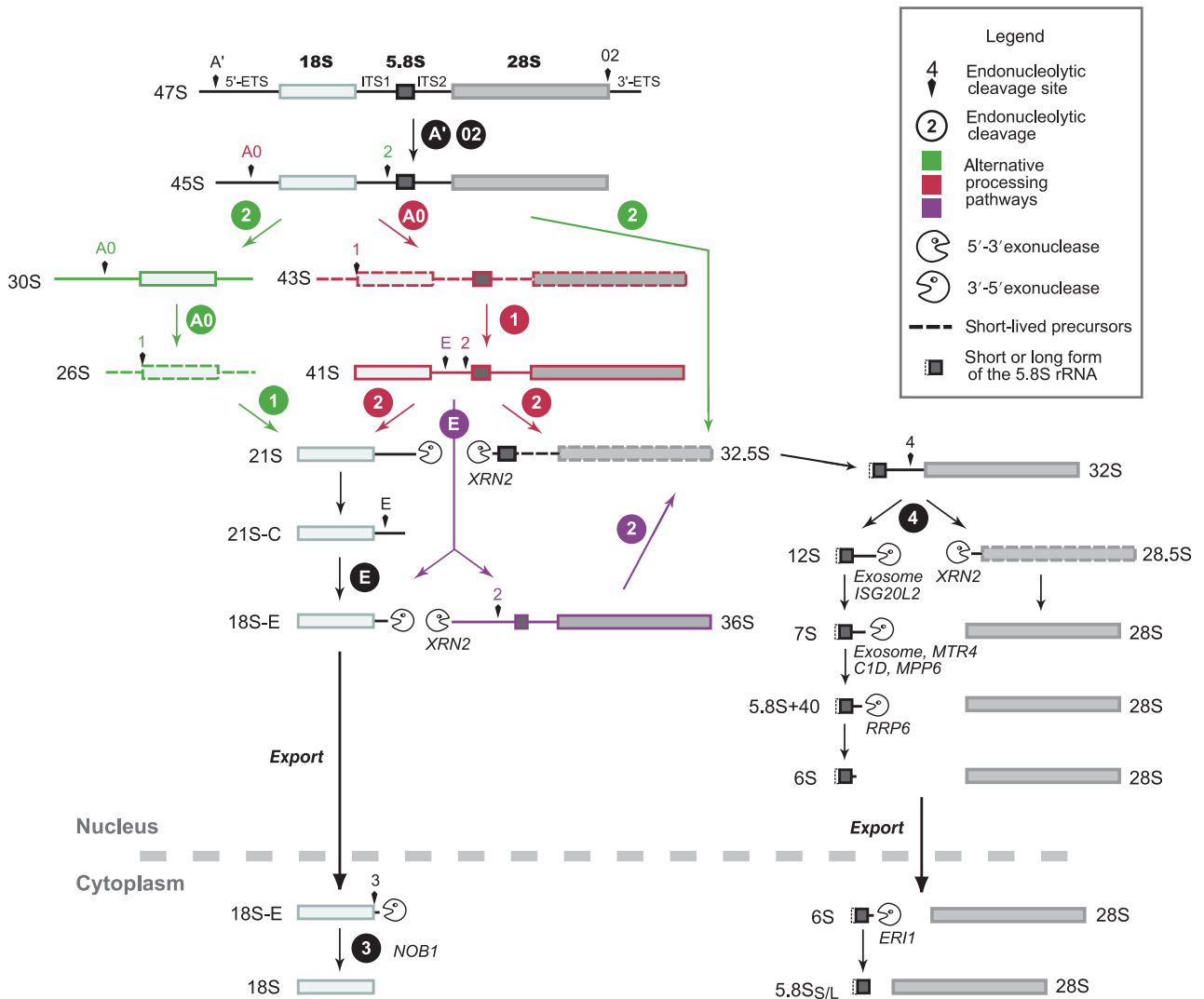
Several transcription termination sites (T) are settled at the 3' end of the transcription unit (Figure 5). The Termination Transcription Factor 1 (TTF-1) binds and twists the 3'-located termination site. Therefore, RNAP1 is forced to pause its activity (Figure 6). TTF-1 cooperates with the "Pol1 and Transcript-Release Factor" (PTRF), inducing transcription termination and the dissociation of the elongating RNAP1 $\alpha$  from the rDNA (Jansa et al., 1998; Richard and Manley, 2009). In this manner, PTRF and TTF-1 facilitate the reinitiation of transcription (Jansa et al., 2001). Furthermore, the components of the released RNAP1 holoenzyme are likely to be recycled to the transcription start site, allowing the PIC to reassemble (Panov et al., 2001).

#### **.1.5. Ribosome factory**

Ribosomes are involved in a mechanism that is essential for cells: the translation of the genetic information, held by the mRNAs, into proteins. The ribosome is a ribonucleoprotein complex composed of 2 subunits, the 40S smaller subunit and the 60S larger subunit, formed of ribosomal RNAs (18S and 5S/5.8S/28S, respectively) and ribosomal proteins (33 and 47, respectively) (de la Cruz et al., 2015). Thus, the formation of the ribosome requires the cooperation of the three RNA polymerases. Indeed, RNAP1 gives rise to the 28S, 18S and 5.8S rRNAs whereas RNAP3 produces the 5S rRNA. Moreover, ribosomal proteins are translated from mRNAs, arisen from RNAP2 transcription.

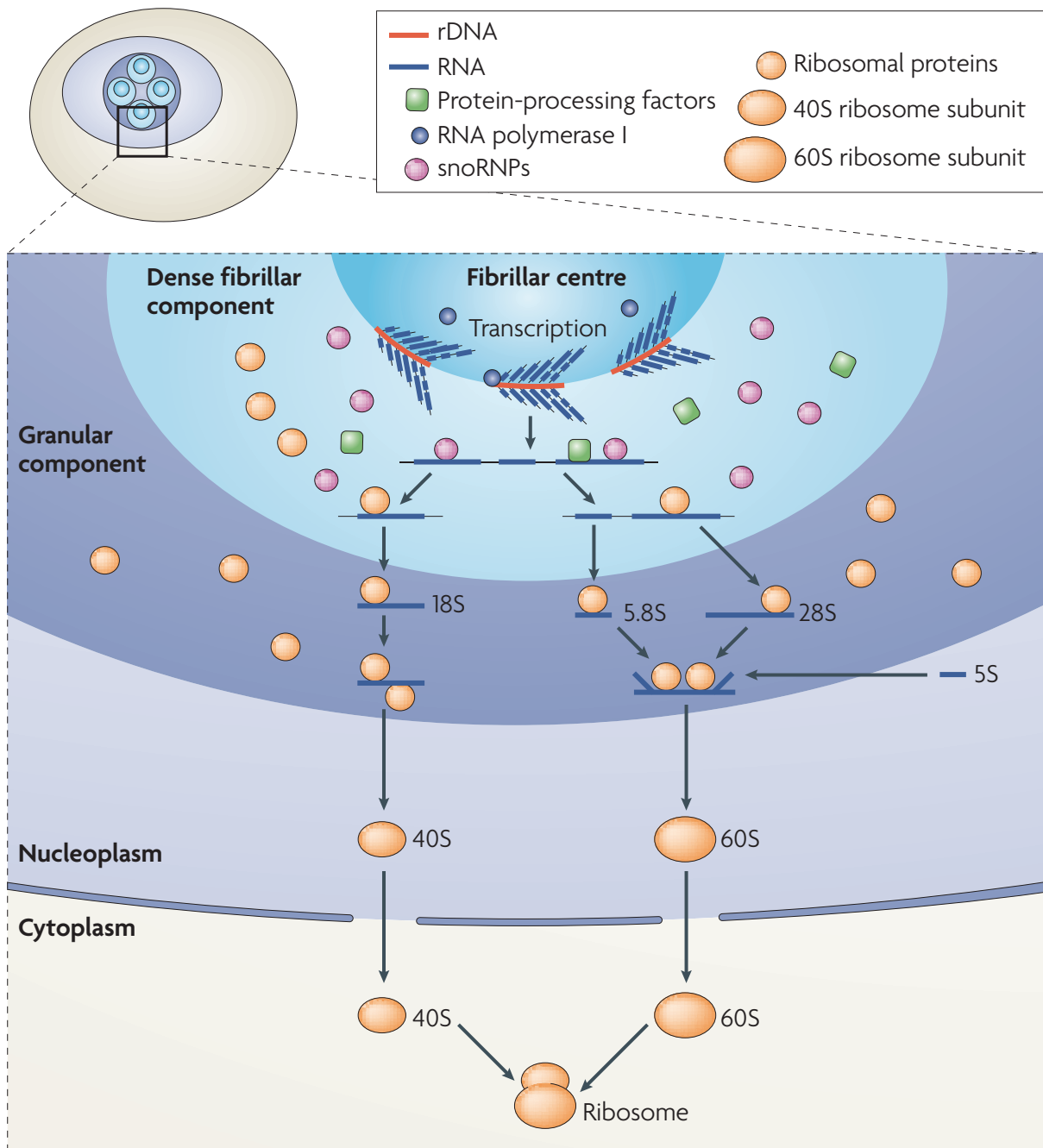
Since most of the steps of ribosome biogenesis take place in the nucleolar compartment, the nucleolus can be considered as a ribosome factory. In effect, ribosome synthesis starts with the transcription of rDNA at the FC/DFC border (see .1.1). In human cells, the first transcript of RNAP1 is the 47S pre-rRNA. Pre-rRNA undergoes extensive processing, that includes several endo/exonucleolytic cuts (Figure 7) and chemical modifications, through the different nucleolar components (DFC/GC) and the nucleoplasm (Henras et al., 2014). In addition, during this maturation process, ribonucleoproteins associate with the pre-rRNAs to form the pre-ribosomal subunits, which are exported

through the nuclear pores to the cytoplasm. Finally, the cytoplasmic pre-ribosomal subunits handle the last step of the maturation process leading to mature ribosome formation, all set up for translation (Figure 8) (Boisvert et al., 2007).



**Figure 7: Pre-Ribosomal RNA processing in human cells.**

*The 47S pre-rRNA is the first ribosomal transcript in human cells. The ribosomal RNA processing consists of several endo/exonucleolytic cuts to give rise to the 18S, 5.8S and 28S rRNA. Alternative maturation pathways are indicated with different colors. Henras et al., 2014.*



**Figure 8: Ribosome biogenesis in human cells.**

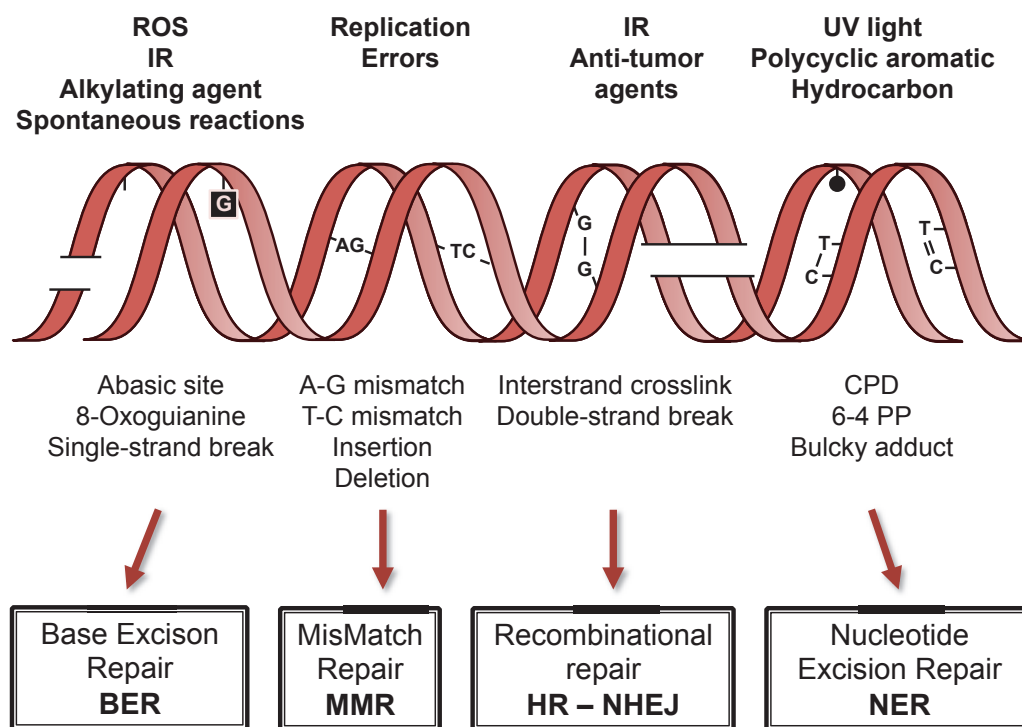
*rDNA transcription is the first step of ribosome biogenesis and occurs at the border between FC and DFC. The first nucleolytic cuts take place in the DFC and the majority of the ribosomal proteins are associated with rRNA in the GC area. Finally, the 40S and 60S ribosomal subunits are assembled in the nucleoplasm and exported to the cytoplasm where they undergo the last processing step in order to form the mature ribosome. Boisvert et al., 2007.*

## .2. The world of DNA repair

### .2.1. DNA damage and repair mechanisms

The genetic information contained in DNA molecules is responsible for the phenotypic characteristics of a human being, such as eye, skin and hair colour, height or sex. Nevertheless, it is also essential at the molecular level for all the cellular mechanisms, including replication, transcription and ribosome biogenesis. Nevertheless, different lesion-inducing agents continuously target DNA. Three causes of DNA damage can be described (Lindhal, 1993): **exogenous/environmental agents** such as air pollutants, cigarette's smoke, chemicals, Ultra-Violet (UV) light or Ionizing Radiation (IR); **endogenous agents** such as Reactive Oxygen Species (ROS) resulting from mitochondrial metabolism; **spontaneous disintegration of chemical bonds**, for instance hydrolysis of nucleotide bonds.

DNA lesions can induce cell death or initiate carcinogenesis. Therefore, it is vital to maintain the genome's integrity to ensure the viability of the entire organism. Fortunately, the cell has developed different mechanisms to repair the lesions induced by these genotoxic agents. Each repair mechanism is specific to a certain type of lesion (Figure 9).



**Figure 9: DNA lesions and associated repair mechanisms in human.**

*Adapted from Hoeijmakers, 2001.*



### .2.1.1. Mismatch repair

The MisMatch Repair (MMR) pathway concerns base-base mismatches and insertion/deletion loops. This repair mechanism occurs throughout the actual DNA replication process or during Homologous Recombination (HR). MMR must happen on the newly synthesized strand to conserve the exactitude of the genetic information (Iyama and Wilson, 2013; Jiricny, 2006; Modrich, 2006). Briefly, in eukaryotes, the major pathway of MMR starts with the recognition of the mismatch by the MutS $\alpha$  (MSH2/MSH6) complex (Figure 10). MutL $\alpha$  (MLH1/PMS2) binds to MutS $\alpha$  and recruits the Proliferating Cell Nuclear Antigen (PCNA) at the lesion. MutL $\alpha$ , activated by PCNA, cuts the strand holding the mismatch. Then, a new and correct DNA strand is synthesized by the DNA polymerase  $\delta$ . The remaining gap is sealed by the DNA ligase 1 (LIG1) (Kunkel and Erie, 2015).

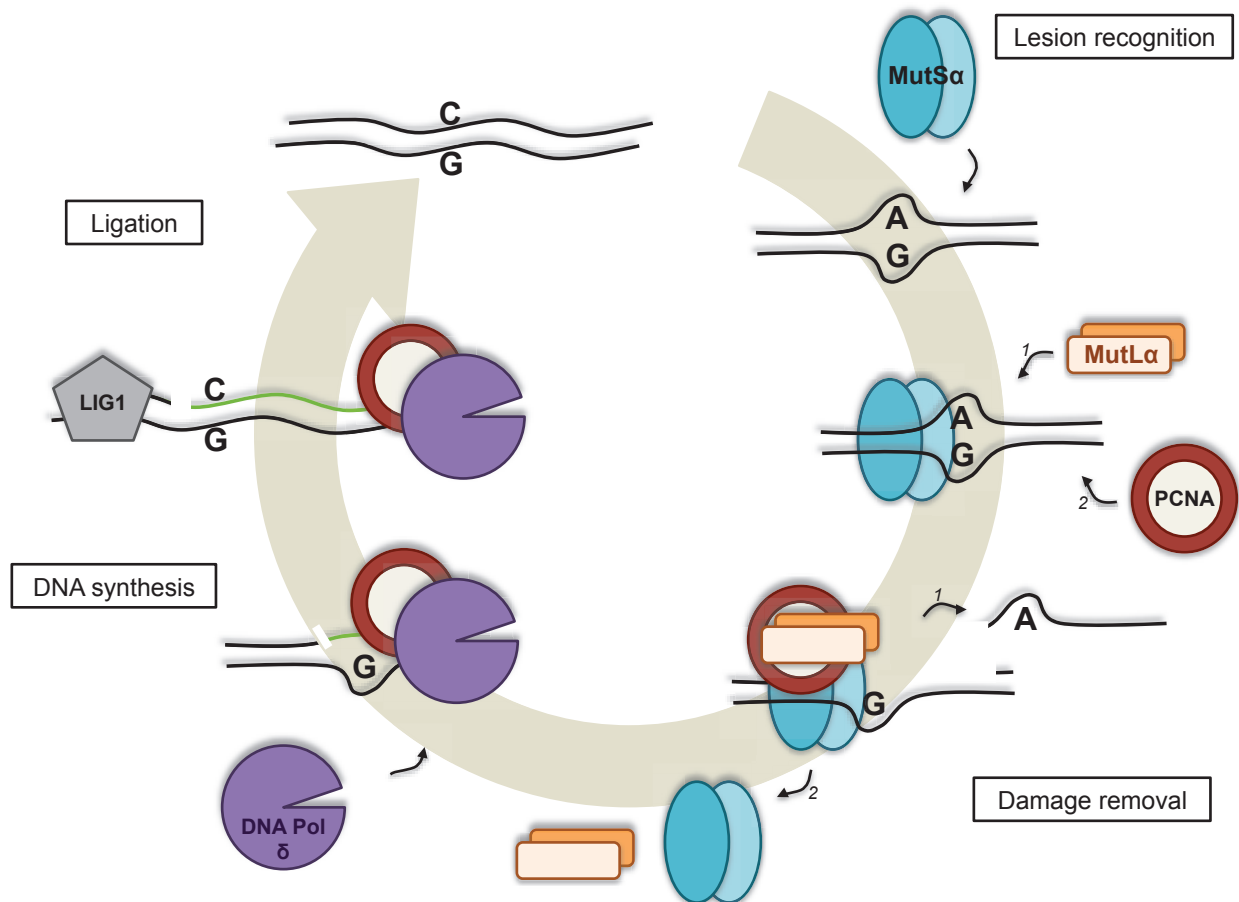


Figure 10: Mismatch repair mechanism.

### .2.1.2. Base excision repair

DNA bases can be modified in different ways, including oxidation by ROS, methylation, alkylation and deamination. These non-bulky lesions lead to DNA and RNA polymerase blockage, activating cell death pathways, but also causing mutagenic issues (Iyama and Wilson, 2013). Once again, in order to maintain the genome's integrity, the cell has developed a specific repair mechanism. The process able to remove these small bases' modifications is the Base Excision Repair (BER) mechanism. The BER mechanism presents different pathways that all include these specific steps (Kim and Wilson, 2012) (Figure 11): *i*) recognition and removal of the damaged base by DNA glycosylases, creating an abasic site in the DNA strand; *ii*) incision of the abasic site by the apurinic/aprimidinic (AP) endonucleases or AP lyase, leading to a single strand break; *iii*) elimination of the sugar residue; *iv*) gap sealing by a DNA polymerase and *v*) ligation of the new DNA strand by LIG1.

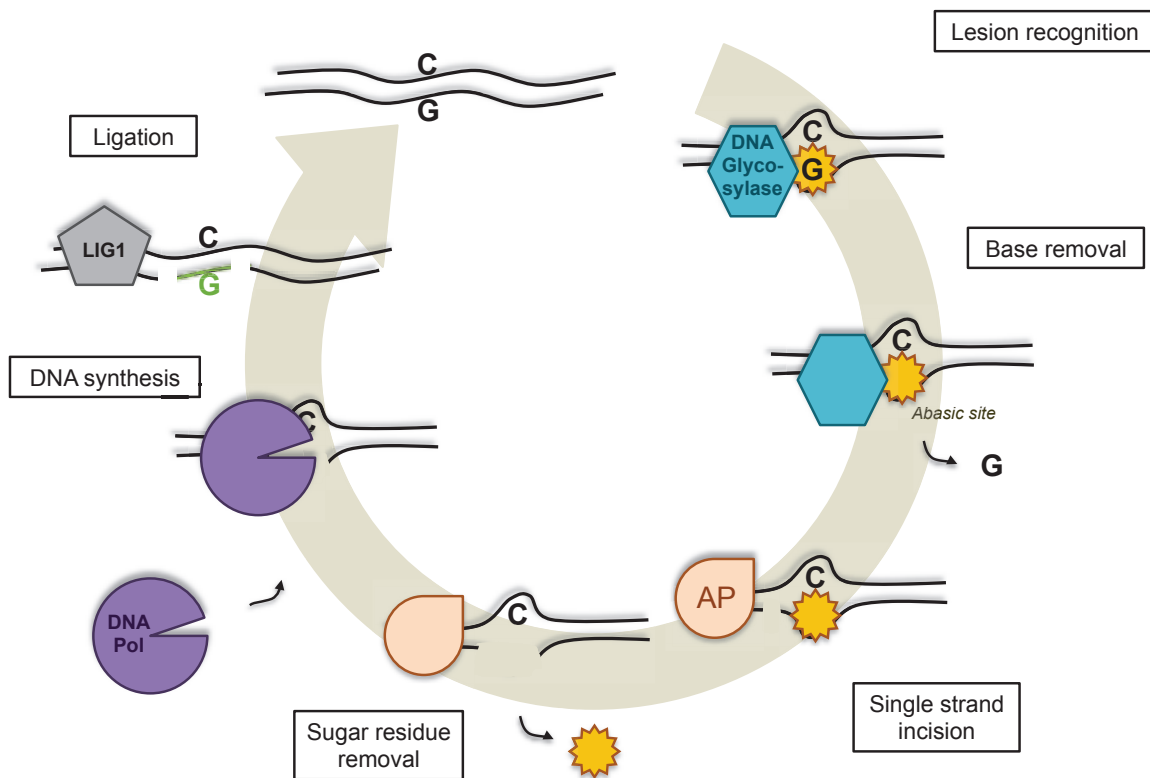


Figure 11: Base excision repair mechanism.

### **.2.1.3. Double-strand break repair**

Double-Strand Breaks (DSBs) are one of the most harmful lesions that happen to DNA. Indeed, DSBs can provoke chromosome rearrangements such as deletion and translocation. If DNA damage is not repaired or not correctly repaired, it will induce mutations, apoptosis or cancer (Bohgaki et al., 2010; Jackson and Bartek, 2009).

DSBs arise from exogenous DNA-damaging agents such as ionizing radiations and anti-cancer treatments. Moreover, endogenous biological events, for instance metabolic production of ROS, replication fork collapse or meiotic recombination, can also induce DSBs (Bohgaki et al., 2010; Jackson, 2002; Lieber, 2010).

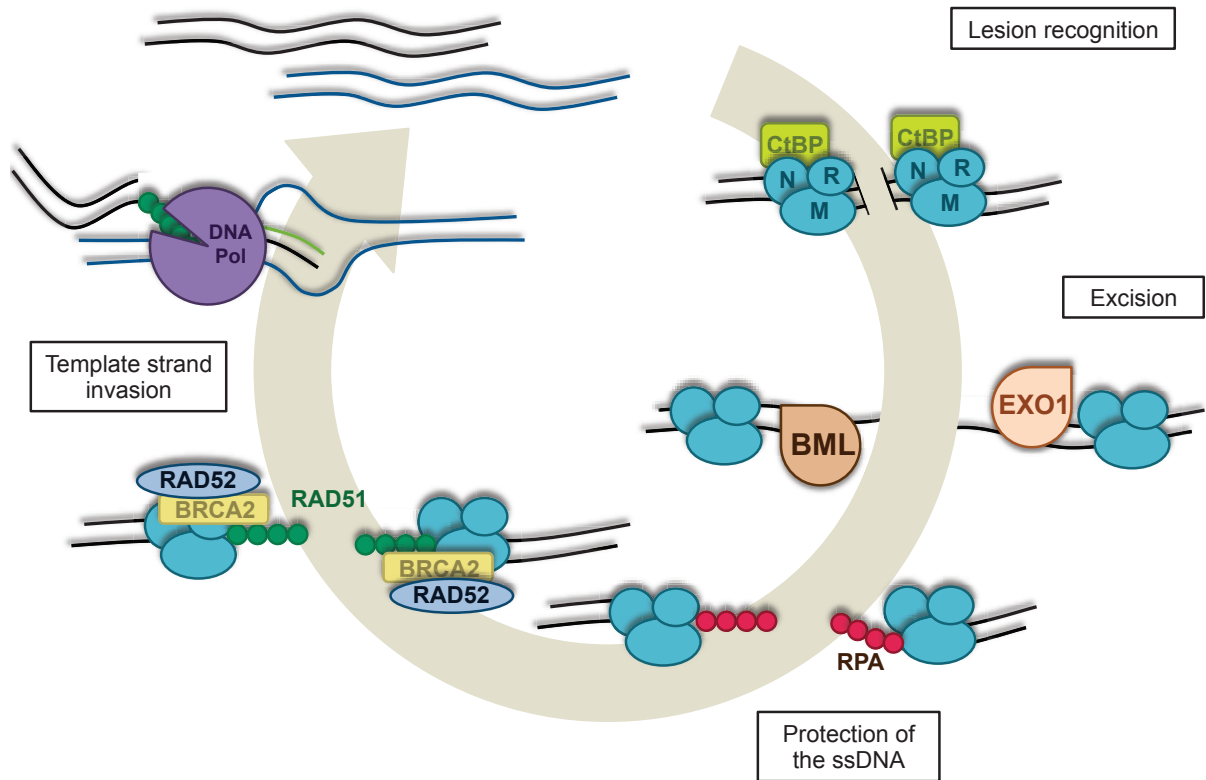
Two pathways are available to repair DSBs: the homologous and the non-homologous repair processes, each of them including two different mechanisms.

#### ***.2.1.3.1. The homologous recombination repair pathway***

The Homologous Recombination (HR) is highly conserved from bacteria to human. HR is a slow mechanism that occurs only during S and G2 phases. Because of its error-free quality and despite its cell cycle-dependent activity, HR represents the first-class repair mechanism for DSBs (Iyama and Wilson, 2013; Mladenov et al., 2016). Indeed, the HR uses the sister chromatid carrying the sequence of homology for the damaged strand. Thus, this repair mechanism is a highly conservative process.

Concisely, the MRN complex (MRE11, RAD50 and NBS1 proteins) recognizes the DSB (Figure 12). Thereupon, the C-terminal Binding Protein (CtBP)-Interacting Protein (CtIP) is recruited to the MRN complex to activate the resectioning of the DNA ends by the exonuclease1 (EXO1). Together with the Bloom syndrome helicase (BLM), EXO1 forms a single-stranded DNA (ssDNA) overhang, which is protected from degradation by the RPA protein. Then, RAD52 and BRCA2 stimulate the replacement of RPA by RAD51. Finally, the RAD51-coated ssDNA can invade the template strand to allow the DNA polymerase to

synthesize the DNA on the damaged strand (Iyama and Wilson, 2013; Mladenov et al., 2016).



**Figure 12: Homologous recombination repair mechanism.**

Another type of homologous recombination, initially described in bacteria and yeast, also repairs DSBs in mammals: the Single-Strand Annealing (SSA) mechanism (Bzymek and Lovett, 2001; Sugawara et al., 2000). This repair pathway occurs in regions with a high concentration of repeated sequences, such as Alu elements and rDNA. In order to repair the lesion, the damaged strand does not use the sister chromatid, but the homologous sequences upstream and downstream of the DNA break. Similarly to HR, the MRN complex, CtIP and EXO1 detect and excise the DNA ends (Figure 13). RPA also protects the overhanging DNA ends, and allows RAD52 to anneal the DNA damage sequence. RAD52 progressively aligns the homologous sequences from both parts of the break. The generated

flapping ssDNA are then cut by the ERCC1/XPF nuclease. In conclusion, because of the deletion of the damaged sequence, SSA is a non-conservative repair mechanism. It is considered as a back-up of HR in case this latter pathway is non-functional (Iyama and Wilson, 2013; Mladenov et al., 2016).

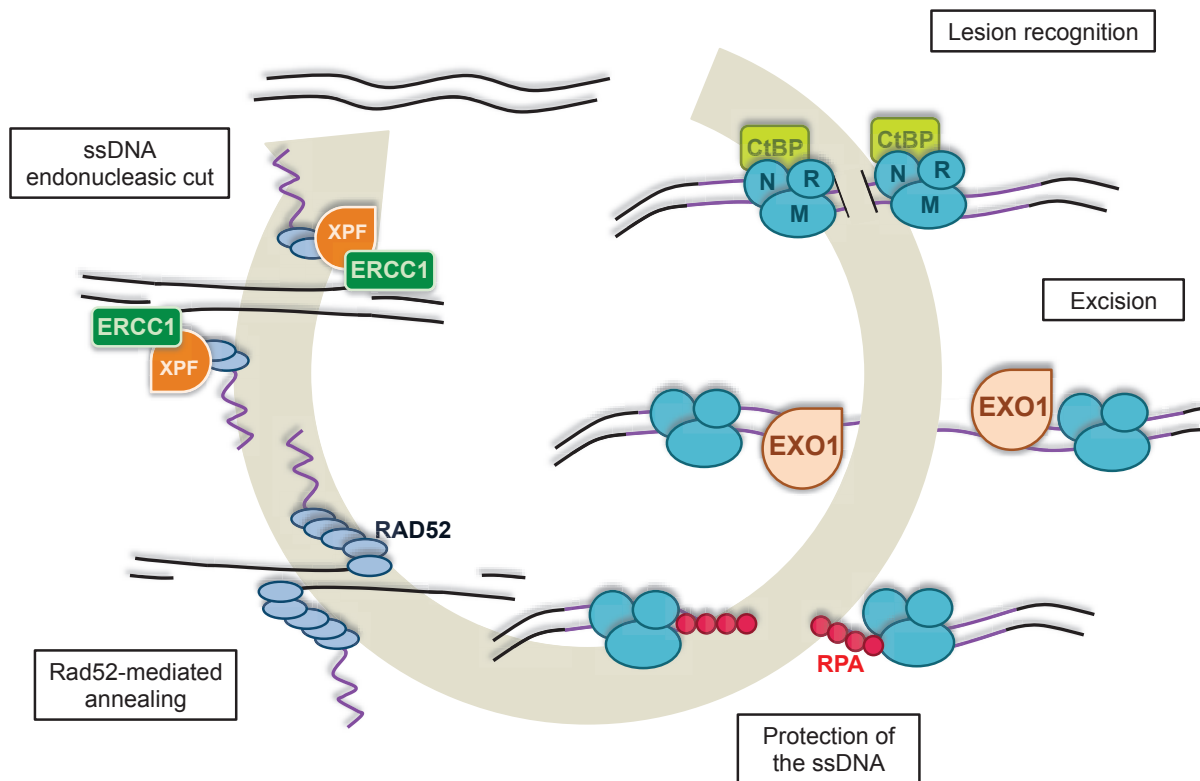
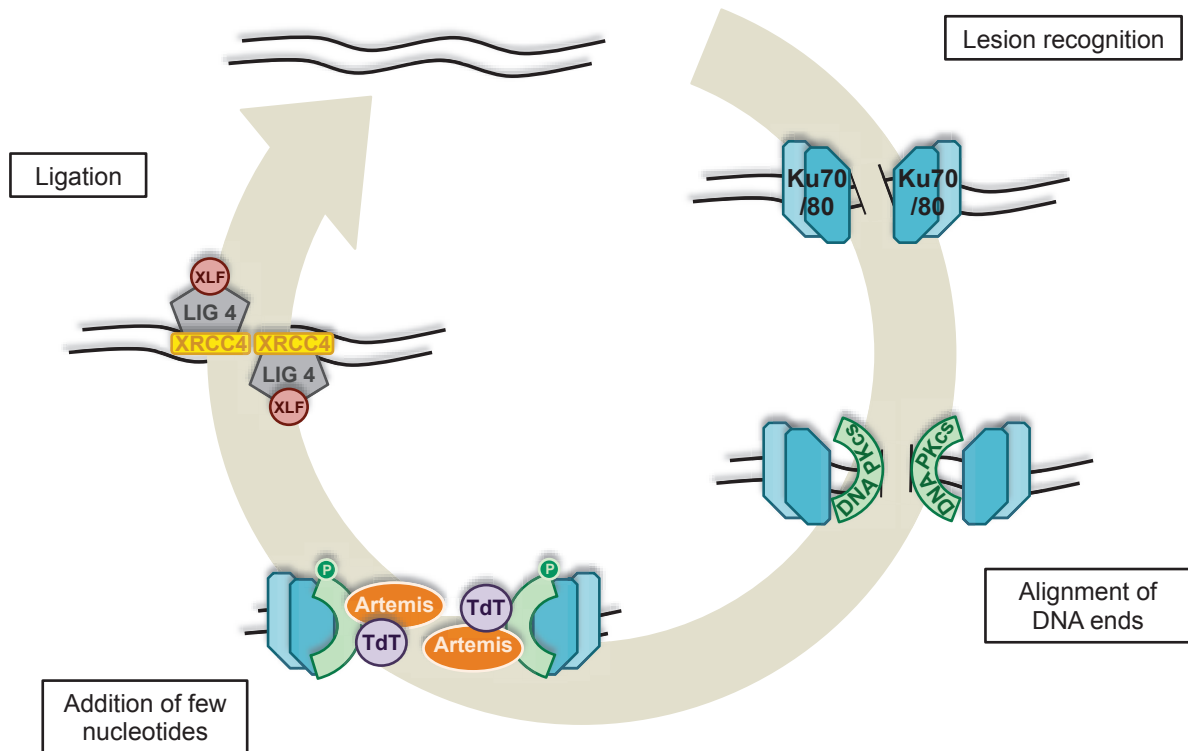


Figure 13: Single-strand annealing repair mechanism.

### .2.1.3.2. The Non-homologous repair pathway

The Non-Homologous End-Joining (NHEJ) process is the major mechanism that repairs DSBs. NHEJ is effective all along the cell cycle but more often during G1 phase when the sister chromatid is absent (Rothkamm et al., 2003). This repair mechanism is rapid but error-prone, due to template strand independence. NHEJ consists of two different pathways: the canonical NHEJ (c-NHEJ) and the alternative NHEJ (a-NHEJ).

In the c-NHEJ pathways, the Ku proteins (Ku 70/80) recognize the DNA ends created by the DSB (Figure 14). The complex of Ku proteins and DNA form a platform to recruit the next repair factor, the DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs) that stabilizes and aligns the DNA ends. Upon DNA-PKcs phosphorylation, several repair factors are recruited to the site of damage, such as the endonuclease Artemis or the Terminal deoxynucleotidyl Transferase (TdT) that adds few nucleotides at the 3' part of the DNA. Finally, the DNA ends are stuck back together with the help of DNA ligase 4 (LIG4) stabilized in a complex with XRCC4 and XLF (Deriano and Roth, 2013; Iyama and Wilson, 2013; Mladenov et al., 2016).



**Figure 14: Canonical Non-homologous end-joining repair mechanism.**

Another repair process, similar to the c-NHEJ, has been described: the a-NHEJ. This alternative mechanism is initiated by the recruitment of the Poly [ADP-Ribose] Polymerase 1 (PARP-1) to the DSB (Figure 15). There is a competition between the Ku heterodimer and PARP-1 to initiate c-NHEJ or a-NHEJ respectively. When PARP-1 binds the DNA damage, the

MRN complex and CtIP (previously described in the HR mechanism, see .2.1.3.1) are recruited to perform the DNA ends resection. The DNA polymerase  $\theta$  synthesizes the new DNA strand. The ends-ligation is performed by the DNA ligases 1 and 3, with or without the activity of XRCC1 (Iyama and Wilson, 2013; Mladenov et al., 2016).

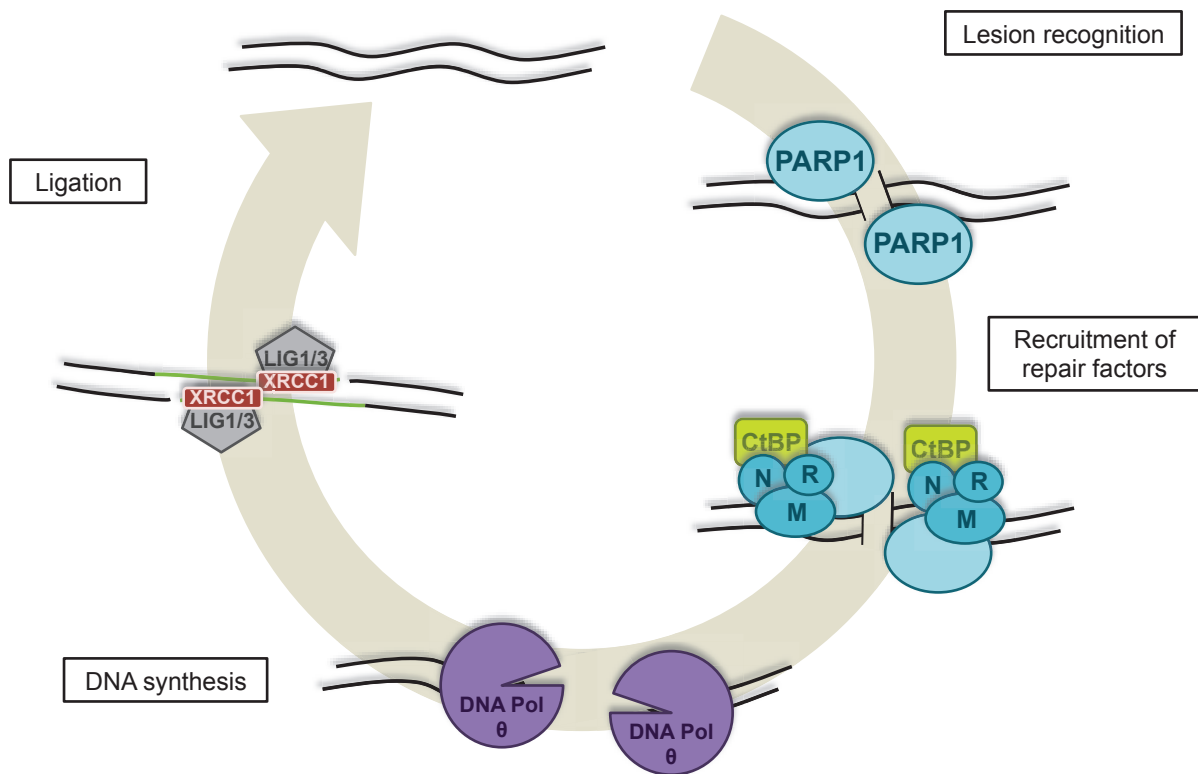


Figure 15: Alternative non-homologous end-joining repair mechanism.

## .2.1.4. Nucleotide excision repair

### .2.1.4.1. Mechanism

The Nucleotide Excision Repair (NER) mechanism is responsible for the removal of a wide range of lesions that induce diverse distortions of the DNA double helix. Indeed, NER has been described to remove UV-induced lesions, such as the Cyclobutane-Pyrimidine Dimers (CPDs) or the 6-4 Pyrimidine-pyrimidone Photoproducts (6-4PPs), but also oxidative damage resulting from metabolic ROS, bulky lesions as well as intrastrand crosslinks (Chalissery et al., 2017; Hoeijmakers, 2009; Menoni et al., 2012).

In humans, NER requires around 30 proteins to achieve a correct DNA repair of the lesions through two different pathways: the Global Genome Nucleotide Excision Repair (GG-NER) and the Transcription-Coupled Nucleotide Excision Repair (TC-NER) (Spivak, 2016). Table 2 shows the main mammalian proteins together with their yeast counterparts. These two subpathways differ from each other only by the DNA damage recognition step: GG-NER detects lesions all over the genome, whereas TC-NER initiates repair mechanism when RNAP2 encounters a lesion on the transcribed strand of active genes. Subsequently, both subpathways fuse into a common path using the same repair factors.

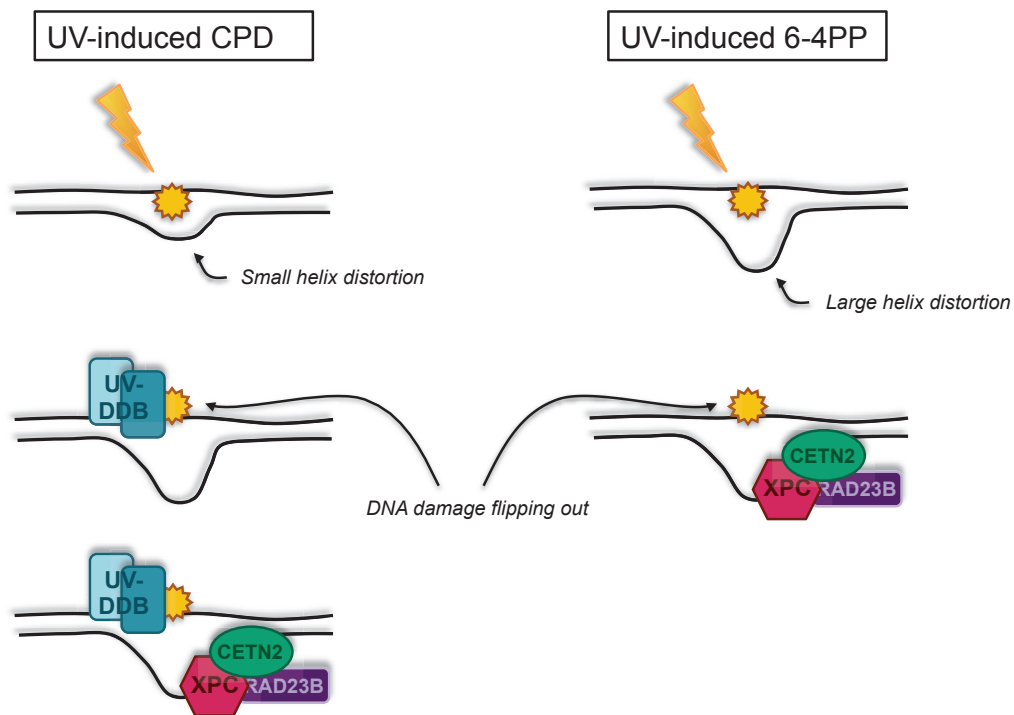
Proteins		NER pathways	
Yeast	Mammals	GG-NER	TC-NER
Rad1	XPF/ERCC4	x	x
Rad2	XPG/ERCC5	x	x
Rad3	XPD/ERCC2	x	x
Rad4	XPC	x	
Rad7	DDB1	x	
Rad10	ERCC1	x	x
Rad14	XPA	x	x
Rad16	DDB2/XPE/p48	x	
Rad23	hRad23B	x	
Rad25	XPB/ERCC3	x	x
Rad26	CSB/ERCC6		x
Rad28	CSA/ERCC8		x
Rad33	CETN2	x	

**Table 2: Mammalian and Yeast NER proteins and their NER pathways**



### .2.1.4.1.1. GG-NER lesion recognition

UV lights produce two different DNA lesions: CPDs and 6-4PPs. These lesions disrupt differentially the DNA molecule, creating a small and a large helix distortion respectively. The GG-NER machinery differentially recognizes 6-4PPs and the twice-abundant CPDs (Marteijn et al., 2014) (Figure 16). Indeed, 6-4PPs modify sufficiently the DNA helix to create a bubble that is recognized, on the non-damaged single strand, by the XPC factor. On the contrary, CPDs do not form a big enough bubble to show a single strand portion of DNA.



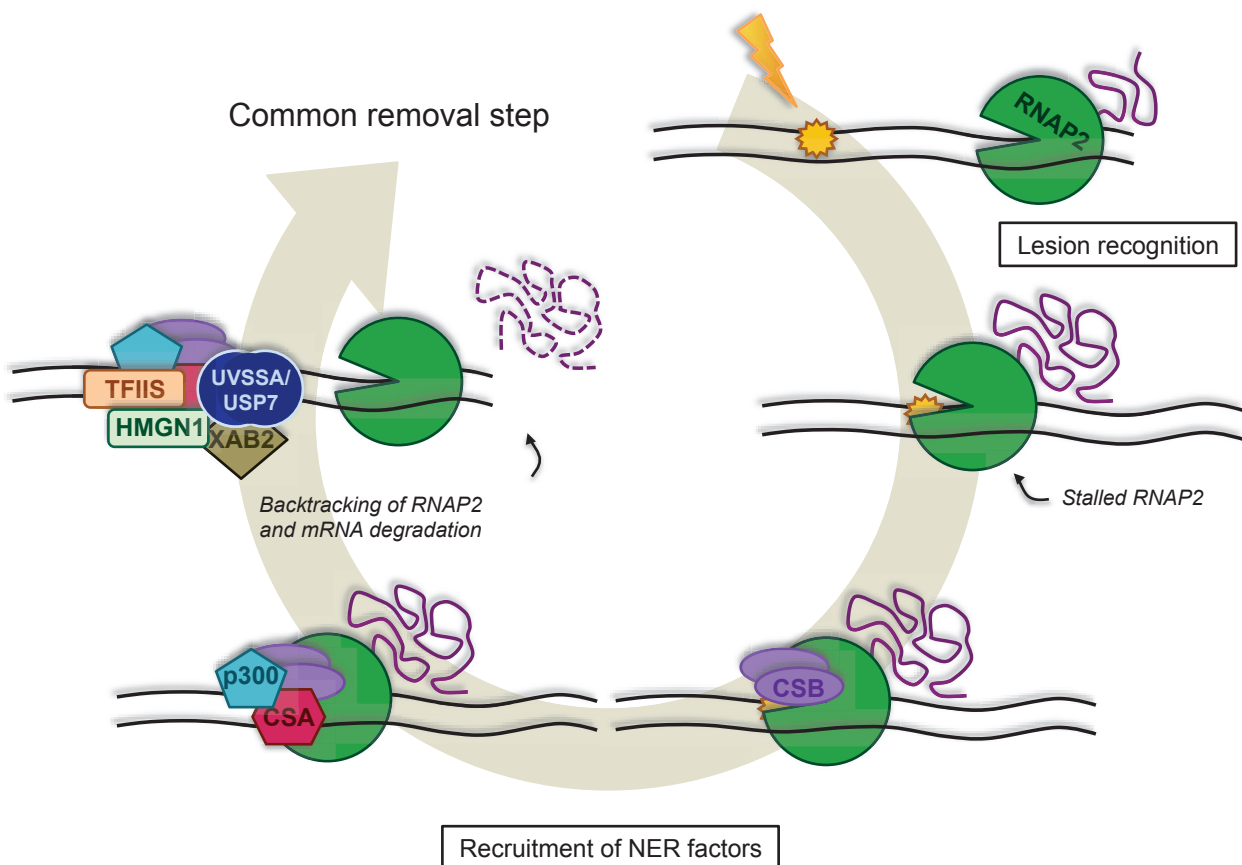
**Figure 16: GG-NER recognition of UV lesions.**

*Adapted from Marteijn et al., 2014.*

Thus, XPC cannot be directly recruited at the lesion site (Reardon and Sancar, 2003). In this case, the UV-DDB (UltraViolet radiation-DNA Damage-Binding protein) complex, which includes DDB1 (also known as XPE-binding factor) and DDB2 (also called XPE) factors, achieves the damage sensor function (Scrima et al., 2008; Tang and Chu, 2002). DDB2 creates a protrusion by taking the CPD into its binding pocket. Thus, a single-stranded DNA is generated, allowing the recruitment of XPC. XPC is stabilized in a complex with hRAD23 and Centrin2 (CETN2) proteins (Nishi et al., 2005; Renaud et al., 2011).

### .2.1.4.1.2. TC-NER lesion recognition

TC-NER machinery is mediated indirectly through RNAP2 transcription activity blockage. Indeed, while transcribing, RNAP2 can encounter DNA damage and recruit NER factors such as Cockayne Syndrome proteins CSA and CSB (Figure 17). Because the lesion is trapped into the area of the stalled RNAP2, several hypotheses have been proposed to decipher the access of the repair protein (Spivak, 2016).



**Figure 17: TC-NER recognition of UV lesions.**

Firstly, a translesional hypothesis has been described for oxidative damages, inducing RNAP2 to bypass the lesion and clear the area for the GG-NER pathway (Charlet-Berguerand et al., 2006). Consequently, the translesional transcription results in mutagenesis (Saxowsky et al., 2008). Secondly, since persistently stalled RNAP2 leads to cell death, degradation of RNAP2 has been proposed as an alternative solution (Anindya et al., 2007; Harreman et al., 2009; He et al., 2016). However, the best explanation of NER factor access to the damage is

the reverse translocation, or backtracking, of RNAP2 (Hanawalt and Spivak, 2008; Lans et al., 2012; . Indeed, the Transcription Factor II S (TFIIS) facilitates the cut of nascent RNA, allowing RNAP2 to reverse translocate from the lesion site in order to let the NER factors access the damage (Tornaletti et al., 1999).

Considering the backtracking hypothesis, the TC-NER progression can be explained as followed. CSB, an elongation factor, works as a homodimer during NER and tightly binds RNAP2 when it is stalled on DNA. The CSB dimer alters the RNAP2/DNA interaction by wrapping the DNA around the CSB protein itself (Beerens et al., 2005). CSA, which is part of the E3 ubiquitin-ligase complex, and the acetyltransferase p300 are recruited by CSB at the site of damage. Then, CSA recruits other NER factors such as UVSSA/USP7 (stabilizing CSB by deubiquitination), the chromatin-remodelling factor HMG1, XAB2 and TFIIS (Nakatsu et al., 2000; Schwertman et al., 2012; Zhang et al., 2012). The wrapping of DNA by CSB and the recruitment of TFIIS together with several chromatin-remodelling factors, promote the RNAP2 backtracking, which allow the following step of the NER process.

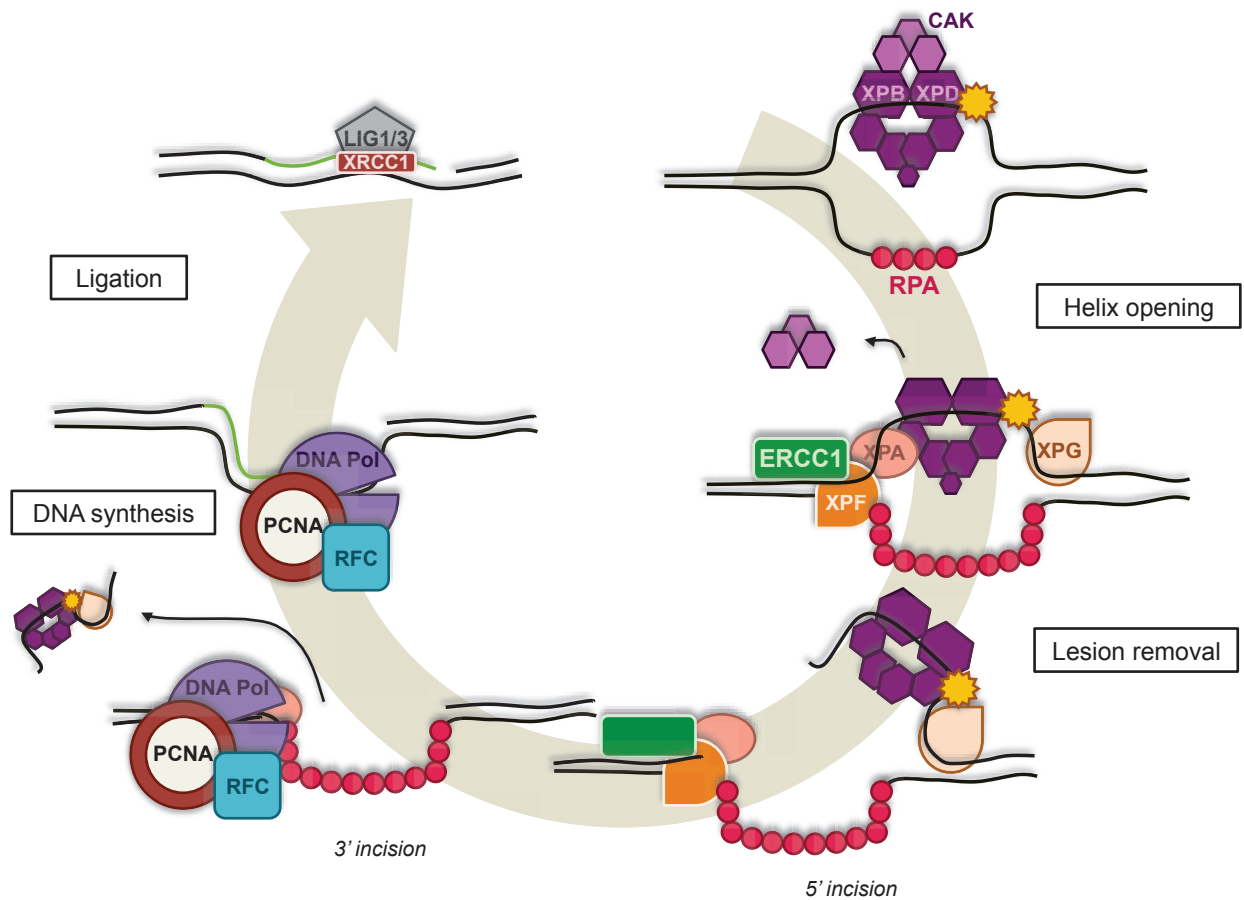
#### *.2.1.4.1.3. Helix opening by TFIIH*

Following the DNA damage detection step, GG-NER and TC-NER join into a unique pathway starting with the recruitment of the transcription/repair factor TFIIH. TFIIH is a 10 subunit-containing complex, including the ATPases/helicases XPB and XPD (Figure 18). TFIIH also contains the CAK (CDK-Activating Kinase) subcomplex, which is only required for transcription initiation. Indeed, in NER context, the XPA protein promotes the CAK dissociation from the TFIIH core complex (Coin et al., 2008). The XPB and XPD subunits are necessary to unwind the DNA helix through their ATPase and ATPase/helicase activities, respectively. The smallest TFIIH subunit, TTDA, is also an essential NER factor, stimulating the XPB and XPD subunits helix opening activity together with the XPG and XPF endonuclease activity (Coin et al., 2006; Giglia-Mari et al., 2006)

#### *.2.1.4.1.4. Lesion removal, DNA synthesis and gap ligation*

After pre-incision complex formation, the Replication Protein A (RPA) and XPA are recruited onto the DNA damage (Figure 18). RPA coats the single strand opposite the lesion

and protects it from degradation. XPA, which is located on the 5' side of the lesion, recruits the endonuclease ERCC1-XPF and interacts with TFIIH, RPA, and later with PCNA.



**Figure 18: NER common subpathway.**

An additional endonuclease, XPG, is recruited on the 3' part of the repair bubble. XPG is not activated until the first incision is performed by the other endonuclease ERCC1/XPF. Few nucleotides can be synthesized before the XPG-dependent second incision has been actually carried out (Fagbemi et al., 2011). A sequence of up to 30 nucleotides, and containing the lesion, is removed after the double incision (Moggs et al., 1996). PCNA recruits the DNA replication machinery including DNA polymerases, Replication Factor C (RFC) and RPA. DNA polymerase  $\epsilon$  is responsible for the DNA repair synthesis in replicative cells, whereas DNA polymerases  $\delta$  and  $\kappa$  function in non-dividing cells (Lehmann, 2011; Ogi et

al., 2010). Similarly, the sealing of the DNA nick is performed by LIG1 and the LIG3/XRCC1 in dividing and non-dividing cells respectively (Schärer, 2013).

#### **.2.1.4.2. Associated syndromes**

Mutations in NER factors are associated with several autosomal recessive diseases exhibiting various symptoms. These diseases have been described in numerous reviews (Brooks, 2013; Cleaver et al., 2009; Kraemer and DiGiovanna, 1993; Lehmann et al., 2011; Spivak and Hanawalt, 2015). The subsequent section shall describe these genetic disorders and their characteristics.

##### **.2.1.4.2.1. Xeroderma Pigmentosum (XP)**

Xeroderma Pigmentosum is a rare and inherited disease characterized mainly by an extreme sensitivity to sunlight. This UV-sensitivity can lead to large sunburns (up to 60% of patients) or to the appearance of lentigines (in the case of 40% of patients), a freckle-like pattern, at an early age.

The diagnosis of XP is principally based on the appearance of these clinical features and can be confirmed by molecular analyses, such as unscheduled DNA synthesis, to detect defective DNA repair (Kraemer and DiGiovanna, 1993).

Moreover, patients with XP syndrome present a higher risk of developing skin cancer compared to unaffected individuals (2000-fold increased risk to declare a melanoma under the age of 20) (Bradford et al., 2011). In addition, several other clinical characteristics are described, such as tongue cancer, keratitis or neuronal degeneration inducing mental disability, hearing loss as well as ataxia (DiGiovanna and Kraemer, 2012; Lai et al., 2013; Lehmann et al., 2011; Viana et al., 2013).

XP disorder derives from mutations in eight distinct XP genes. Seven of them, from XPA to XPG, induce a defect in different steps of the NER process, whereas the eighth (XPV for XP variant) presents a functional repair mechanism but a defective DNA polymerase  $\eta$ . The DNA polymerase  $\eta$  has a DNA translesional synthesis role. Indeed, it is responsible for the replication of unrepaired UV-damaged DNA (Vermeulen et al., 1991).

Other mutations in XPB, XPD, XPF or XPG can induce either TrichoThioDystrophy (TTD) (see .2.1.4.2.3), Cerebro-Occulo-Facial-Skeletal syndrome (see .2.1.4.2.4), XPF-ERCC1 progeroid syndrome (see .2.1.4.2.5) or combined phenotypes such as XP/CS or XP/TTD (Spivak and Hanawalt, 2015).

Although no treatment is available to cure XP disorder, several protective measures can be used to avoid the effect of the sunlight. For instance, special UV filters applied on the windows of homes, schools and even cars. Furthermore, children should rather go out at night but if they do go outside during the daytime, they must wear long clothes, gloves, sunglasses and exposed skin should be covered with sunscreen to prevent sunburn (Lehmann et al., 2011).

#### *.2.1.4.2.2. UV-sensitive syndrome (UV<sup>S</sup>S) and Cockayne Syndrome (CS)*

Patients suffering from the UV<sup>S</sup>S are hypersensitive to UV rays of sunlight as seen for XP and CS patients, but are not prone to develop skin cancer, similar to CS patients (see below). Since UV<sup>S</sup>S is due to a mutation in CSA, CSB or UVSSA (which regulates the turnover of the RNAP2 blocked on a lesion) genes, these patients present defects in the TC-NER mechanism. In addition, UV<sup>S</sup>S is accompanied by the appearance of freckles, skin dryness and sometimes telangiectasia (distension of blood vessels underneath the skin), usually on the nose and the cheeks. Unlike XP and CS, UV<sup>S</sup>S do not show any neurological issues. UV<sup>S</sup>S is a rare autosomal recessive disease but, because of the mild symptoms, could be underdiagnosed (Spivak and Hanawalt, 2015).

Cockayne Syndrome was originally described as a sole DNA repair disease. Indeed, cells from CS patients present a mutation in the NER factor CSA or CSB (Henning et al., 1995; Troelstra et al., 1992), inducing a defective TC-NER pathway, whereas the GG-NER remains fully functional. The UV-light hypersensitivity of the patient suffering from CS is consistent with the NER deficiency. However, CS is a complex disease with a wide set of symptoms different from sunlight sensitivity. For instance, CS patients present gradual hearing loss, growth issues, premature aging and neurological features such as progressive microcephaly and myelin defects (Brooks, 2013; Laugel, 2013).

In a recent review Brooks considers that these features, particularly the neurological ones, cannot be explained by the TC-NER deficiency (Brooks, 2013). Studies on UV<sup>S</sup> have provided the evidence to refute the exclusive TC-NER deficiency explanation. As previously described, patients affected by UV<sup>S</sup> present sun-sensitivity and skin pigmentation abnormalities, similarly to XP and CS patients. Moreover, UV<sup>S</sup> and CS are not cancer-inducing diseases, and both present a defective TC-NER mechanism. However, despite mutations in CSA and CSB genes, UV<sup>S</sup> patients do not have neurological issues, whereas CS patients present severe neurological defects. Therefore, even if a TC-NER deficiency can explain the sun-sensitivity of CS patients, it cannot explain all the other somatic features, including the neurological ones, which are not described in UV<sup>S</sup> (Brooks, 2013).

Brooks (2013) puts forward the transcription deficiency hypothesis. To emphasise this theory, several studies have described the link between CSB, TFIIH and RNAP1 transcription (Bradsher et al., 2002; Iben et al., 2002;). In this way, CS (mutation in CSA; CSB) and combined XP/CS (mutation in XPB; XPD; XPG) symptoms can be explained. Indeed, RNAP1 transcription is responsible for ribosome biogenesis and consequently influences all cellular processes. Hence, impairment of RNAP1 transcription could explain the growth issues together with the neurological defects such as dysmyelination, microcephaly, and so on.

#### .2.1.4.2.3. *TrichoTioDystrophy (TTD)*

Trichotiodystrophy presents similar symptoms to CS: microcephaly, neurological defects, premature aging features, intellectual disability and developmental delay (Cleaver et al., 2009). However, the hallmark that discriminates TTD from CS patients is the brittleness of hair and nails. Indeed, the sulphur-deficiency, due to a reduced level of cysteine in hair proteins, induces the fragility of the hair. This specific characteristic is used for diagnosis via the identification of the tiger-tail-like pattern of the hair under a polarized light (Spivak and Hanawalt, 2015).

There are four mutated genes involved in TTD. Three of them are subunits of the TFIIH transcription/repair complex (XPB; XPD and TTDA) and their mutations are responsible for the photosensitive version of TTD. The fourth gene is TTDN1; its function is still unknown

but its association with the non-photosensitive form of TTD has been described (Cleaver et al., 2009).

#### **.2.1.4.2.4. *Cerebro-Occulo-Facial-Skeletal (COFS) syndrome***

Patients with COFS syndrome exhibit, in addition to similar symptoms of CS patients, reduced birth weight, heart and kidney abnormalities, cataracts and hypotonia. Mutations in CSB, ERCC1, XPD or XPG have been described for this disease (Spivak and Hanawalt, 2015).

#### **.2.1.4.2.5. *XPF-ERCC1 progeroid syndrome (XFEPS)***

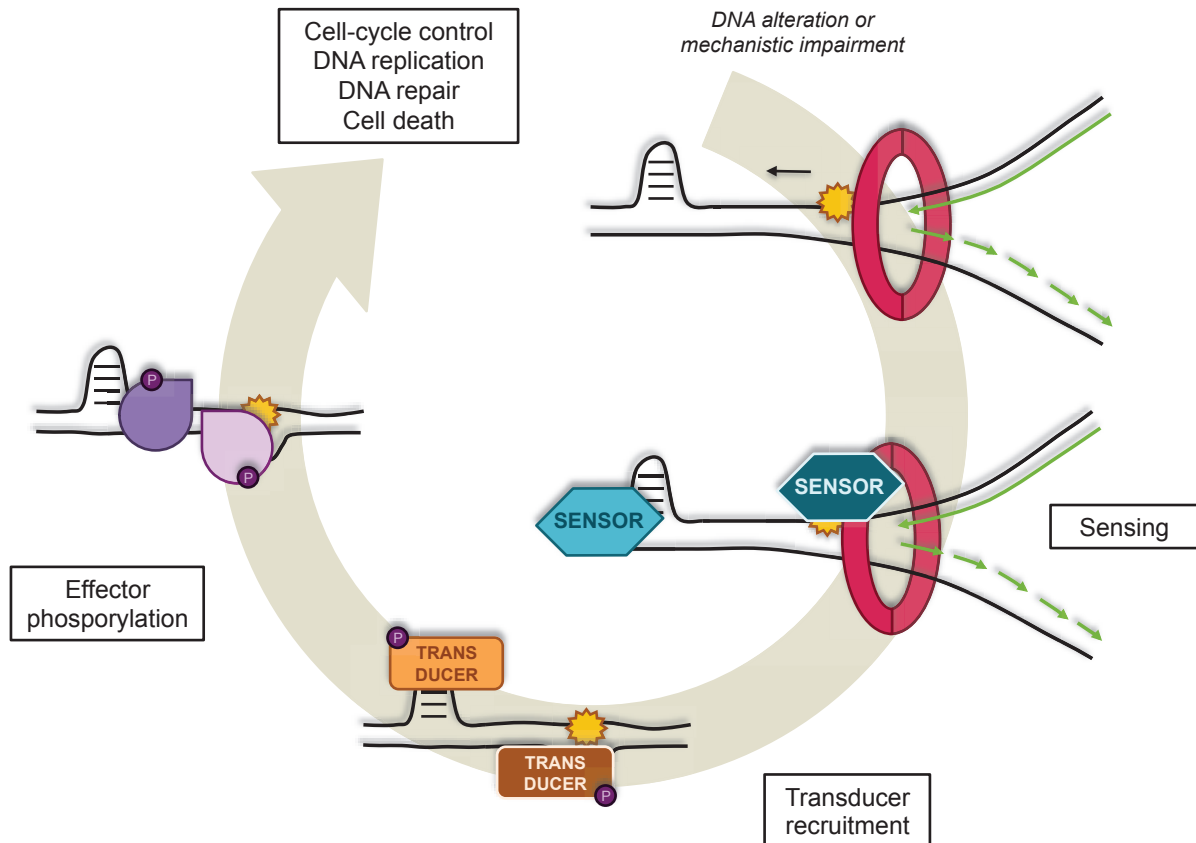
XFEPS patients present mutations in the XPF/ERCC1 endonuclease complex responsible for the first cut of the damaged strand in NER. They display sunlight sensitivity, bird-like faces, dwarfism, microcephaly, premature aging and cachexia (Spivak and Hanawalt, 2015).

### **.2.1.5. The DNA damage response**

The genetic material in cells of a living-organism is continuously confronted with intrinsic and extrinsic stresses, inducing alterations to the DNA double helix molecule as well as impairments of DNA-related mechanisms, such as replication or transcription. In order to resolve these damage and mechanistic issues, the cell has developed a safeguarding response: the DNA Damage Response (DDR). The DDR consists in sensing DNA lesions and impaired mechanisms in order to signal them to the cell, which will trigger specific events to overcome these issues. Indeed, DDR is activated either by proper DNA lesions such as DSBs or by mechanistic issues such as replication fork collapse (Branzei and Foiani, 2010; Cortez, 2015; Larsen and Stucki, 2016). Different sensors detect these types of damage or mechanistic impairments leading to transducer activation. Thus, transducers phosphorylate effectors in order to initiate diverse cellular events, such as cell-cycle arrest, DNA repair or even apoptosis if the issues cannot be settled (Maréchal and Zou, 2013)(Figure 19). Nowadays, it is understood that DDR mainly defines two distinct, but not exclusive, pathways specifically involved with the detection of DSBs and stalled DNA replication forks, i.e. the ATM (Ataxia Telangiectasia Mutated) and ATR (ATM-RAD3-related) pathways respectively. Conversely, according to Hanawalt (2015), a more general term - the Genomic



Stress Response (GSR) - should be used to describe the sensing of all DNA alterations, damaging and non-damaging such as aberrant secondary DNA structures or collision of replication and transcription machinery. The GSR would also include the mechanisms surmounting the DNA aberrations, including the ATM/ATR system (Hanawalt, 2015). Here, the focus is placed on the ATM and ATR transducers in order to decipher their activation processes and their cooperation in the regulation of the DDR.

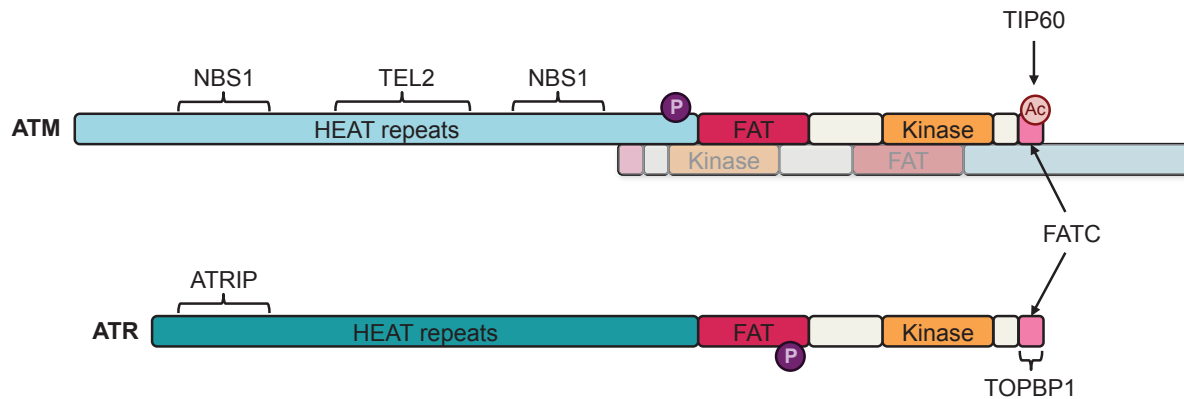


**Figure 19: DNA Damage Response Network**

*The sensing of DNA alterations/mechanical obstruction triggers the DNA Damage Response (DDR). Thus, the sensors recruit the specific transducers, which phospho-activate different effectors required to elicit various events such as cell-cycle arrest, DNA replication, DNA repair and even cell death. Adapted from Maréchal and Zou, 2013.*

Both ATM and ATR proteins present a Phosphoinositide 3-Kinase-related Kinase (PIKKs) activity (Lempiäinen and Halazonetis, 2009; Lovejoy and Cortez, 2009). They also share common domains involved in their activation and cooperation in the DDR network (Figure 20). One of these domains is the  $\alpha$ -helical HEAT (Huntington-Elongation factor 3-protein phosphatase 2A-TOR1) repeats domain that recruits NBS1/TEL2 and the ATRIP (ATR

Interacting Protein) proteins to ATM and ATR respectively. Both ATM and ATR kinases also possess a FRAP-ATM-TRRAP (FAT) domain and a FAT carboxy-terminal (FATC) domain. The FAT domain of ATR is phosphorylated, whereas the FATC domain of ATM is acetylated (Cortez et al., 2001; Falck et al., 2005; Perry and Kleckner, 2003; You et al., 2005).



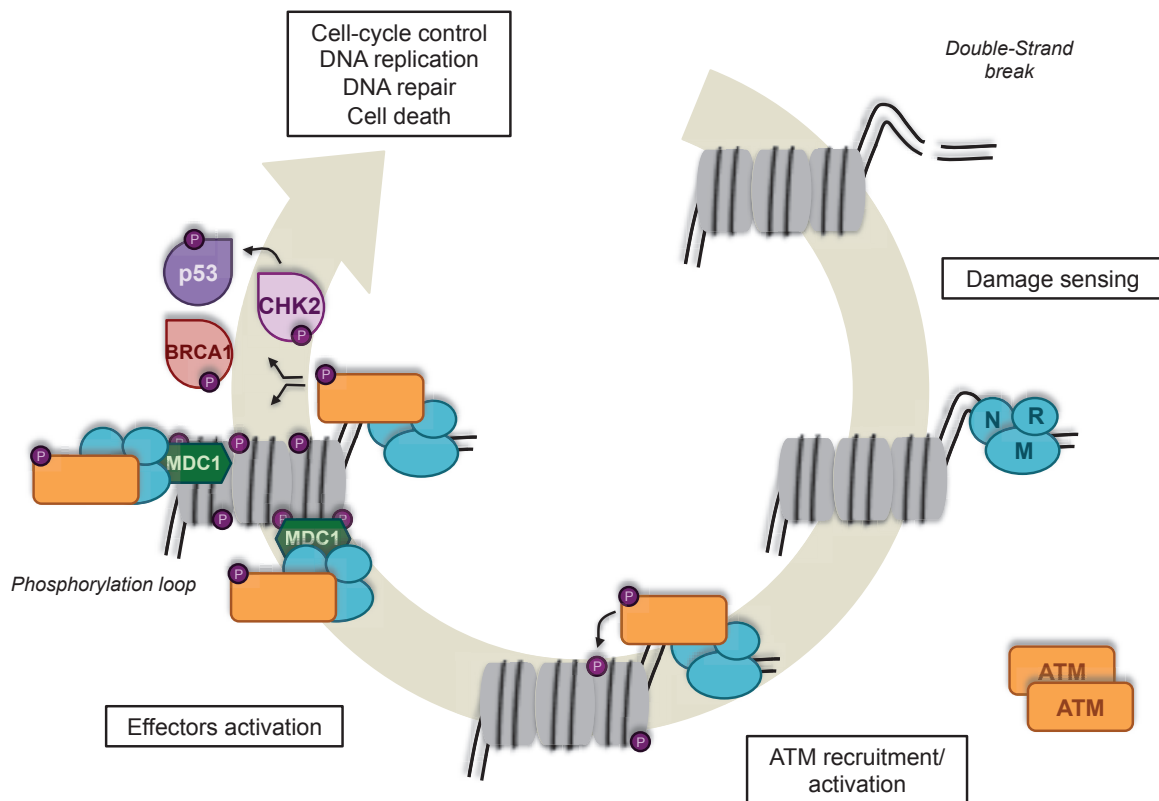
**Figure 20: ATM and ATR proteins structure**

The ATM and ATR proteins are kinases that share common domains such as the HEAT repeats, the FRAP-ATM-TRRAP (FAT) domain and the FAT carboxy-terminal (FATC) domain. The HEAT repeats of ATM and ATR present binding sites for the NBS1/TEL2 and the ATRIP proteins respectively. The FATC domain of ATM is acetylated (K3016) by the TIP60 acetyltransferase to activate ATM, whereas a phosphorylation (Ser1981) occurs next to the FAT domain, into the HEAT repeats. Moreover, in unaltered cells, ATM exists as a dimer through an interaction between its FAT and kinase domains. The binding of the TOPBP1 protein to the FATC domain of ATR facilitates autophosphorylation of the FAT domain (Thr1989). Adapted from Maréchal and Zou, 2013.

### **.2.1.5.1. The ATM pathway**

The ATM protein is the first kinase of a long list of kinases activated through the DDR pathway and located specifically on DSBs. In undamaged human cells, the ATM kinase exists as a dimer that is separated into monomers when recruited to the DNA damage (Bakkenist and Kastan, 2003). The FAT and kinase domains of ATM proteins interact together, thus inhibiting ATM activity (Figure 20). In damaged human cells, this interaction is disrupted when autophosphorylation of ATM (Ser1981) is induced by its recruitment to DNA damage, allowing the activation of the ATM kinase function (Bakkenist and Kastan, 2003). ATM is also activated through its acetylation by the TIP60 protein interacting with the FACT domain of the ATM kinase (Sun et al., 2005).

In brief, the MRN complex (involved in HR, see .2.1.3.1) detects the DSBs and recruits the ATM protein to the damaged site inducing ATM autophosphorylation. ATM activation induces a cascade of phosphorylation of the ATM substrates, such as the histone variant H2AX, CHK2 (Checkpoint kinase 2), p53 and BRCA1 (BReast Cancer 1). Finally, the ATM substrates trigger diverse cellular mechanisms such as DNA repair, cell cycle arrest or apoptosis (Figure 21).



**Figure 21: The ATM pathway**

The ATM pathway is activated through the detection of DSBs by the MRN complex that will recruit the ATM kinase to the damage inducing its autophosphorylation. The phosphorylated ATM ( $\text{P-ATM}$ ) triggers a kinase cascade to activate its substrates such as the histone variant H2AX, p53 and BRCA1. The phosphorylation of H2AX ( $\gamma\text{H2AX}$ ) recruits the MDC1 protein that spreads the phosphorylation of the H2AX through a large domain nearby the DSB (>500kb) by the recruitment of the MRN complex together with  $\text{P-ATM}$ . The effectors impact diverse cellular processes such as cell-cycle progression, DNA replication and DNA repair. Adapted from Maréchal and Zou, 2013.

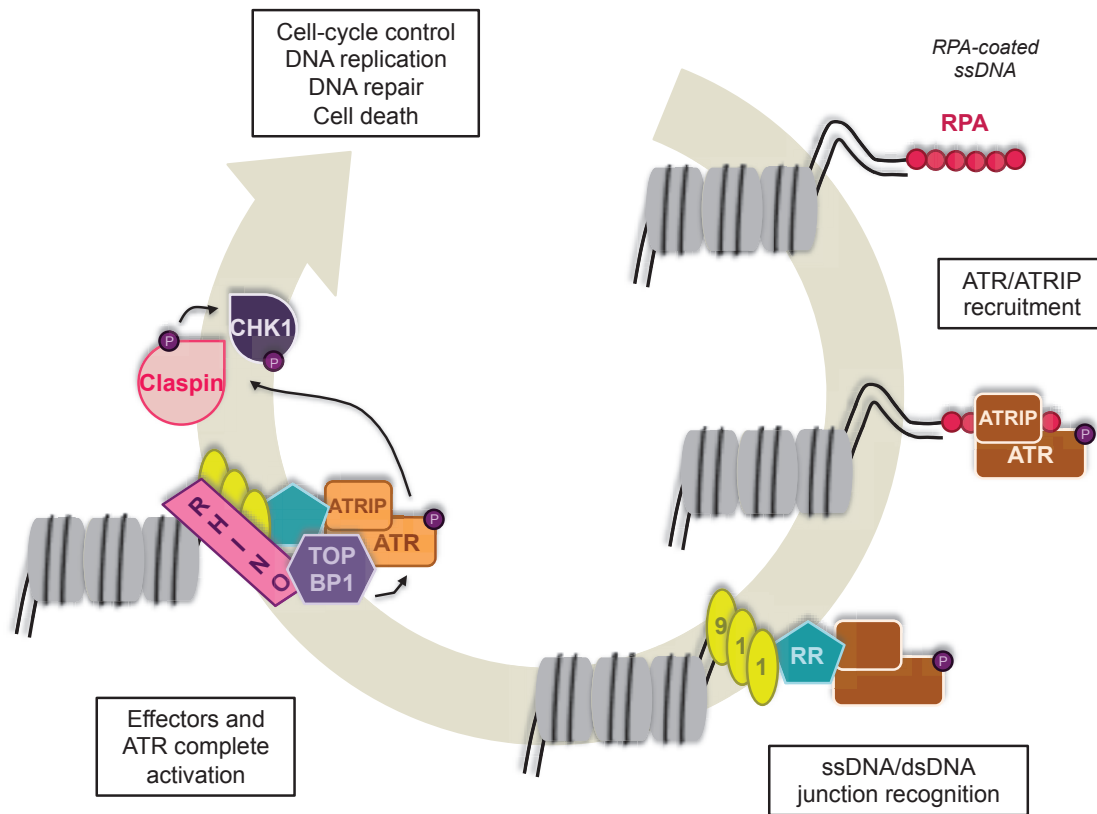
Among the ATM substrates, H2AX is located on the nucleosomes and phosphorylated around the DSBs area. The phosphorylated-H2AX ( $\gamma\text{H2AX}$ ) is recognized by MDC1, allowing a

phosphorylation loop of ATM and H2AX proteins nearby, spreading the phospho-activation over a large chromatin area flanking the DSBs (> 500kb) (Meier et al., 2007; Savic et al., 2009). Phosphorylation of the H2AX proteins induces modifications of the chromatin structure around the DSBs, allowing access to the repair proteins, chromatin-remodelling and checkpoint factors.

### **.2.1.5.2. The ATR pathway**

Whereas DSBs directly activate the ATM pathway, RPA-coated single-stranded DNA (RPA-ssDNA) triggers the ATR pathway in response to replication fork collapse (Branzei and Foiani, 2010; Nam and Cortez, 2011). Therefore, the ATR process is associated with several DNA repair mechanisms such as NER, HR and SSA (see .2.1). The RPA-ssDNA recruits the ATR Interacting Protein (ATRIP), which forms a complex with ATR. Indeed, ATRIP interacts with ATR via its HEAT repeats domain that contains the ATRIP binding site (Figure 20). In addition, activation of the ATR pathway consists of the autophosphorylation of the FAT domain (Thr 1989) facilitating the TOPBP1 binding to the FATC domain in order to stimulate the ATR activation (Figure 22) (Liu et al., 2011). However, the ATR/ATRIP complex is not sufficient to completely induce the ATR cascade. Indeed, single-stranded/double-stranded DNA (ss/dsDNA) junction is a determinant structure to induce the ATR pathway, likewise the RPA-ssDNA (Maréchal and Zou, 2013). The RAD17-RFC2-5 (RR) clamp is recruited to the ss/dsDNA junction allowing the loading of the “9-1-1” complex (RAD9-RAD1-HUS1) on the dsDNA (Lee and Dunphy, 2010). TOPBP1 recruitment to the 9-1-1 complex is facilitated by the RHINO protein and induces complete activation of the ATR/ATRIP complex (Cotta-Ramusino et al., 2011; Delacroix et al., 2007).

After initiation, similarly to ATM, the ATR kinase phosphorylates its substrates such as Claspin and CHK1 (Checkpoint kinase 1). Namely, phosphorylation of Claspin helps to activate CHK1, whose phosphorylation slows down the cell cycle progression to allow the repair process to be completed before cell division (Carr et al., 1995; Kumagai et al., 1998; Liu et al., 2012).



**Figure 22: The ATR pathway**

The ATR pathway consists of the detection of the RPA-coated ssDNA as well as the ssDNA/dsDNA junction by a wide range of proteins such as ATR/ATRIP complex, the RAD17-Rfc2-5 (RR) clamp and the “9-1-1” complex (RAD9-RAD1-HUS1). The ATR/ATRIP complex is fully activated by the TOPBP1 protein, which is recruited to the 9-1-1 complex by the RHINO protein. After ATR/ATRIP is activated, this complex phosphorylates its substrates such as the Claspin protein and the Checkpoint kinase 1 (CHK1). Claspin helps to activate Chk1. Finally the ATR substrates elicit diverse cellular events such as cell-cycle arrest, DNA repair along with apoptosis if damage cannot be repaired. Adapted from Maréchal and Zou, 2013.

### **.2.1.5.3. The ATM/ATR crosstalk**

Even though ATM and ATR pathways are dedicated to different type of DNA aberrations, they can crosstalk. When the initially-ATR-detected DNA fork collapse is unresolved (in ATR mutants for instance), DSBs occur and trigger the ATM pathway (Brown and Baltimore, 2003; Chanoux et al., 2009). Moreover, ATR phosphorylates H2AX, which is known to activate the ATM pathway (Ward and Chen, 2001). Contrarily, DSBs-dependent

ATM pathway will recruit ATR by promoting DNA end resection and RPA-coating of the ssDNA ((Jazayeri et al., 2006; Myers and Cortez, 2006; Shiotani and Zou, 2009). Furthermore, ATM/ATR crosstalk can take place at the substrates level. It has been shown that ATM and ATR phosphorylate the same sites or different sites of the same proteins (Matsuoka et al., 2007)

## **.2.2. DNA repair in yeast**

Yeast is a powerful model organism to study molecular processes such as DNA repair. Indeed, many DNA repair studies have been performed in *S. cerevisiae* and its genome was entirely sequenced in 1996 (Goffeau et al., 1996). Moreover, homologs of *S. cerevisiae* proteins involved in different molecular mechanisms have been found in human cells. Therefore, all mechanisms described in this section shall refer to the budding yeast and present the main differences observed within mechanisms in mammalian cells.

### **.2.2.1. Direct reversal of DNA damage**

Direct reversal of DNA damage consists of a single-step reaction to remove the lesion in the easiest and most accurate way. However, this reaction concerns only few lesions, such as UV damage (CPDs) and methylation of bases ( $O^6$ -methylguanine;  $O^4$ -methylthymine), that are removed by a DNA photolyase and a DNA methyltransferase respectively (Boiteux and Jinks-Robertson, 2013).

### **.2.2.2. Mismatch repair**

Our current understanding of MMR is derived from bacteriological studies, which present three Mut proteins: MutS that binds the mismatch; MutL involved in steps following detection and MutH which cuts the strand to initiate the removal. *S. cerevisiae* presents six MutS (Msh1, Msh2, Msh3, Msh4, Msh5 and Msh6) and four MutL (Mlh1, Mlh2, Mlh3 and Pms1) homologs (Boiteux and Jinks-Robertson, 2013; Reenan and Kolodner, 1992a, 1992b). Msh2 and Msh6 form the MutS $\alpha$  complex specifically involved in base-base mismatch recognition. Msh2, along with Msh3, constitutes the MutS $\beta$  complex that detects the insertion-deletion loop. Another MutS $\gamma$  complex is described and presents a meiotic role.

Yeast possesses several MutL-like complexes: MutL $\alpha$  (Mlh1/Pms1), MutL $\beta$  (Mlh1/Mlh2) and MutL $\gamma$  (Mlh1/Mlh3) (Boiteux and Jinks-Robertson, 2013). MutL $\alpha$  interacts with MutS $\alpha$  and MutS $\beta$ , and MutL $\gamma$  interacts only with MutS $\gamma$  during meiosis. Moreover, PCNA is also part of the process in yeast. However, another protein is specific to the yeast MMR mechanism: the endonuclease Exo1. Exo1, which interacts with Msh2 and Mlh1, presents a structural and enzymatic function (Amin et al., 2001; Tran et al., 2001).

### **.2.2.3. Base excision repair**

BER observed in yeast is similar to the one described in humans. It also consists of a five-step mechanism including abasic site creation, single strand cutting, sugar residue removal, gap refilling and nick sealing by DNA ligase. However, the main difference between yeast and humans lies in the presence of only five N-glycosylases (12 in humans (Kim and Wilson, 2012)) responsible for the abasic site creation. They are also divided into two categories: monofunctional and bifunctional. The monofunctional N-glycosylases (Ung1 and Mag1) cut only the N-glycosylic bond, whereas the bifunctional N-glycosylases/AP-lyases (Ntg1, Ntg2 and Ogg1) cleave the N-glycosylic bond as well as the phosphodiester backbone at the AP site.

### **.2.2.4. Double strand break repair**

As previously mentioned for the other repair mechanisms, an understanding of DSB was gleaned principally from yeast studies. Likewise to human cells, *S. cerevisiae* possesses two different methods to repair DSBs: HR and NHEJ. In contrary to DSB repair in humans, the dominant repair pathway for DSBs occurring in yeast is HR (Shrivastav et al., 2008). HR and NHEJ factors are well conserved across species. Indeed, the MRX (Mre11; Rad50; Xrs2) complex and Sae2 are the yeast counterparts of the human MRN complex and CtIP. During HR, MRN and CtIP are involved in detection and induce DNA ends resection respectively (Critchlow and Jackson, 1998). In addition, Yku70p and Yku80p are the yeast homologs of Ku70 and Ku80 human proteins that initiate NHEJ. However, no homolog was found for DNA-PKcs in yeast NHEJ. Moreover, DSBs are mobile in yeast, while rather immobile in humans (Lemaître and Soutoglou, 2015). They move around the entire nucleus in order to be repaired in specific nuclear structures (DNA repair centres) and persistent DSBs relocate

to the nuclear periphery to be efficiently resolved. However, in humans the DSB mobility is a controversial issue. Indeed differential mobility was observed depending on the location of the DSBs in the genome (Marnef and Legube, 2017). Despite the difference in terms of mobility, DSB repair is compartmentalized and occurs mainly at the nuclear periphery in both human and yeast. Finally, it has been demonstrated that the location regulates the choice of the repair pathway between HR and NHEJ (Lemaître and Soutoglou, 2015).

#### **.2.2.5. Nucleotide excision repair**

NER in yeast has homolog proteins in humans, with few differences in function. For example, Rad4 is required for CPDs removal in actively transcribed genes, whereas XPC (human Rad4 homolog) is implicated in lesion removal in the whole genome, except from the actively transcribed genes. Moreover, Rad28 has no impact in TC-NER, whereas CSA is specifically involved in TC-NER of human cells. Mutation in Rad26 does not induce UV sensitivity, while CSB deficient cells present sunlight sensitivity. Finally, yeast present a Rbp9-dependent TC-NER pathway which is absent in human cells (Boiteux and Jinks-Robertson, 2013).

#### **.2.2.6. DNA lesion bypass**

The last mechanism, referred to as Post-Replication Repair (PRR), is not really a repair pathway. PRR facilitates the bypass of DNA damage through either an error-free or an error-prone subpathway. Both are activated by the ubiquitinylation of PCNA, mediated by the Rad6/Rad8 complex (Huang et al., 2013). The monoubiquitinylation of PCNA triggers the error-prone pathway, whereas further polyubiquitinylation by Ubc13/Mms2/Rad5 complex induces the error-free pathway. However, DNA lesion bypass in human cells does not yet present polyubiquitinylation of PCNA (Kannouche and Lehmann, 2004). Moreover, when the error-prone pathway recruits a translesional polymerase to synthesize DNA across the lesion, the error-free pathway uses the sister chromatid DNA strand as a template for DNA synthesis.



## **.2.3. Ribosomal DNA repair**

### **.2.3.1. Nucleotide excision repair of RNAP1-transcribed genes**

Nucleotide excision repair of ribosomal DNA has been studied in yeast, rodent and humans. These studies have shown contradictory conclusions among the different organisms. Additionally, they have also demonstrated differences with the RNAP2-dependant NER (Balajee et al., 1999; Cohn and Lieberman, 1984; Conconi et al., 2005; Stevnsner et al., 1993; Tremblay et al., 2008; Verhage et al., 1996).

On the one hand, several studies on hamster cells have proven that some forms of UV-induced damage are less efficiently/or not repaired by NER (Balajee et al., 1999; Stevnsner et al., 1993). Indeed, Balajee *et al* have found that the excision repair mechanism for CPDs was deficient, whereas the 6-4PPs lesions were successfully removed (Balajee et al., 1999).

On the other hand, Cohn and collaborators (1984) have demonstrated that UV-induced lesions on rDNA were repaired using the NER mechanism in human cells. Although human rDNA is repaired by NER, this mechanism described for RNAP2-transcribed genes might be different from rDNA-related NER. Effectively, NER on rDNA is slower than NER occurring on RNAP2-transcribed genes (Vos and Wauthier, 1991) and does not involve TC-NER (Christians and Hanawalt, 1993). However, the latter study did not separate the active rDNA from the inactive one. This could explain the TC-NER negative result. Conversely, in yeast rDNA repair the NER mechanism involves GG-NER and TC-NER, and removes CPDs more rapidly in actively transcribed genes (Conconi et al., 2005; Tremblay et al., 2008; Verhage et al., 1996). Despite these encouraging results, Conconi and his coworkers (2005) explained that further investigations have to be carried out to establish whether TC-NER also occurs on ribosomal DNA in human cells.

### **.2.3.2. Double strand break repair in ribosomal DNA**

In the past ten years, several studies have demonstrated the implication of rDNA instability in cancer and aging (Diesch et al., 2014; Ganley and Kobayashi, 2014; Stults et al., 2009). This is why DSBs repair in human rDNA has aroused researchers' interest.

As for the entire genome, DSBs occurring in rDNA are preferentially repaired by NHEJ, but can also trigger HR at any time during the cell cycle (Larsen and Stucki, 2016). Indeed, because of the tandemly repeated structure of the rDNA, DSBs do not need sister chromatids to repair.

Furthermore, several studies have demonstrated that the induction of DSBs induces RNAP1 transcription inhibition, in the area close to DSBs, through the phosphorylation of ATM and the recruitment of MDC1 and NBS1, which are part of the ATM pathway (Kruhlak et al., 2007; Larsen et al., 2014).

A particular event was observed during rDNA damage: the reorganisation of the nucleolus. Nucleolar caps as well as the nucleolar periphery location of rDNA have been described during DSB repair (Franek et al., 2016; Larsen and Stucki, 2016). Conversely, Moore and co-workers (2011) have described nucleolar caps formation after UV-C exposure ( $36\text{J}/\text{m}^2$ ), but not following  $\gamma$ -irradiation. However, RNAP1, UBF and Fibrillarin were observed in these nucleolar caps in both cases (Franek et al., 2016; Moore et al., 2011; Sluis and McStay, 2015). Additionally, nucleolar reorganisation was also observed during RNAP1 transcription blockage (Franek et al., 2016). Indeed, after Actinomycin D treatment blocking RNAP1 activity, RNAP1 was located in nucleolar caps or had formed a necklace structure (Reynolds et al., 1964; Shav-Tal et al., 2005).

Finally, nucleolar caps, together with the rDNA and RNAP1's specific location at the nucleolus' periphery, might be conducive to a more efficient rDNA repair mechanism.

### **.2.3.3. Ribosomopathies**

As described previously (see .1.5), ribosomes consist of rRNAs and ribonucleoproteins that emerge from products of RNAP1, RNAP2 and RNAP3 transcription. Ribosome biogenesis starts with the transcription of rDNA into the 47S pre-rRNA giving rise to the rRNAs, which are parts of the ribosomes.

Any alteration, not only in genes coding for ribonucleoproteins, in proteins involved in regulation of the ribosome biogenesis but also in the rDNA coding for the pre-rRNAs, can

lead to severe ribosomal diseases. Therefore, repairing all mutation occurring in the ribosomal DNA is a priority for the cell. Ribosomopathies define the diseases that present qualitative or quantitative defects in ribosome biogenesis (Nakhoul et al., 2014). One of the first ribosomopathies described was Diamond-Blackfan Anemia (DBA). DBA is a congenital disease characterized by anemia, growth delay, craniofacial malformation and other congenital abnormalities of the heart, eyes and kidneys. It is often diagnosed during the first year of life and presents mutations in a wide range of ribonucleoproteins (Armistead and Triggs-Raine, 2014; Danilova and Gazda, 2015; Nakhoul et al., 2014).

Another ribosomopathy, which has been extensively studied, is the Treacher Collin Syndrome (TCS) associated with craniofacial abnormalities, such as underdevelopment of external ears or absence of cheekbones. This is an autosomal dominant disease which is diagnosed with a prevalence of 1 in 10.000-50.000 births (Yelick and Trainor, 2015). TCS is mainly caused by a mutation in the TCOF1 gene, but could also be induced by mutation in the POLR1C and POLR1D genes. All these genes are involved in rDNA transcription and rRNA processing, reinforcing the identification of TCS as a ribosomopathy (Danilova and Gazda, 2015; Nakhoul et al., 2014).

### **.3. New ribosomal DNA and RNAP1 partners**

#### **.3.1. Actin and Myosins**

##### **.3.1.1. Cytoplasmic functions**

Actin is the major component of the cytoskeleton of the cell and was first described in the cytoplasm. Three isoforms of this protein can be found in different cell types, i.e. the three  $\alpha$ -isoforms present only in muscle cells (cardiac, skeletal and smooth muscle) and  $\beta$ - and  $\gamma$ -isoforms that are found in both muscle and non-muscle cells (Dominguez and Holmes, 2011). Actin is a globular protein that forms microfilaments. Therefore, two different forms of actin are described: the globular/monomeric state called G-actin and the filamentous/polymeric one named F-actin. The actin filaments adapt their architecture according to the cellular structure in which they are located, e.g. parallel bundles in filopodium, anti-parallel organisation in stress fibres, branched and crosslinked network in

cortex or lamellipodium (Blanchoin et al., 2014). Thus, actin presents a large set of functions including among others cellular shape maintenance, cell motility and muscle contraction (Blanchoin et al., 2014; De Lanerolle and Serebryanny, 2011).

In cytoplasm, actin interacts with myosins that are part of the motor protein superfamily. Eighteen classes of myosin have been identified, all involved in different cellular events such as muscle contraction, cell cycle and membrane-cytoskeleton interactions (De Lanerolle and Serebryanny, 2011; Lodish et al., 2000). Each myosin is divided into three different parts: the head, the neck and the tail. The head domain presents the ATPase and the actin-binding activities. The neck linker possesses regulating properties, i.e. calmodulin activity for myosins I and V (Lodish et al., 2000). The tail domain is unique and gives the specificity to each myosin. The myosin head slides through the actin microfilament *via* the ATP hydrolysis.

### **.3.1.2. Nuclear roles**

Actin and myosin were first described in the cytoplasm. However, several studies clearly identified actin and several of the myosins within the nucleus (Almuzzaini et al., 2016; Belin and Mullins, 2013; De Lanerolle and Serebryanny, 2011; Fomproix and Percipalle, 2004; Lanerolle, 2012; Lanerolle et al., 2005).

Actin does not present a nuclear localisation signal and, thus, its nuclear transport requires specific proteins (Iida et al., 1992). Different research groups have identified proteins involved in actin nuclear import and export, e.g. cofilin and the exportin6/profilin complex respectively (Gettemans et al., 2005; Stüven et al., 2003). In the nucleus, actin is mainly monomeric, but 20% of its nuclear pool has the dynamic properties of the polymeric form (McDonald et al., 2006). The monomeric actin interacts with the three RNA polymerases (Grummt, 2006; Hofmann et al., 2004; Hu et al., 2004). Nuclear actin presents a transcription-factor-like function, e.g. it induces a eight-fold increase of RNAP2 transcription activity and binds to RNAP1 in order to increase rDNA transcription by chromatin remodelling (Almuzzaini et al., 2016; Hofmann et al., 2004; Philimonenko et al., 2004). Nuclear actin was also detected in a specific part of the nucleus: the FC of the nucleolus that contains the inactive rDNA (Kyselá et al., 2005).

Nuclear Myosin I (NMI) was the first myosin identified in the nucleus (Pestic-Dragovich et al., 2000). It was later described in a specific component of the nucleolus: the DFC which is the site of active rDNA transcription (Kyselá et al., 2005). Moreover, similar to actin, NMI has been described in correlation with both RNAP1 and RNAP2. However, actin and NMI present slightly different roles in transcription. On the one hand, for example, while actin interacts with RNAP1 directly, RRN3 mediates the indirect interaction between NMI and RNAP1 (Philimonenko et al., 2004). Moreover, Ye and co-workers (2008) have demonstrated that NMI and polymeric actin are required for an efficient rDNA transcription. On the other hand, NMI participates in the formation of the first phosphodiester bond during RNAP2 transcription initiation and stimulates RNAP2 transcription activity although not in the same proportions described for actin (Hofmann et al., 2004, 2006). Furthermore, several research groups have demonstrated that actin and NMI are both responsible for active chromosome locus movements in the nucleus during interphase (Bridger, 2011; Chuang et al., 2006; Mehta et al., 2010). Similarly, NMI is required during DNA repair to promote chromosome territory relocation (Kulashreshtha et al., 2016). The other myosins are implicated in diverse cellular events such as embryonic myoblast differentiation (myosin II), splicing (myosin Va), rDNA transcription (myosin Vb), RNAP2 transcription and DNA repair (myosin VI), cell cycle and proliferation (myosin XVI) and myofibrillar movement (myosin XVIII) (De Lanerolle and Serebryannyy, 2011).

### **.3.2. Fibrillarin**

Fibrillarin (FBL; or Nop1 in yeast) is an S-adenosyl-L-methionine-dependent methyltransferase (Ado-Met-dependent methyltransferase) that can methylate both proteins and RNA (Shubina et al., 2016). FBL is composed of three domains: the GAR domain containing a sequence rich in glycine/arginine residues as well as a Nucleolar Localisation Signal, the RNA-binding domain and the C-terminal domain with an  $\alpha$ -helix structure separated by two spacers (Rodriguez-Corona et al., 2015; Shubina et al., 2016). Together, the RNA-binding and the C-terminal domains form the MethylTransferase (MTase) domain. The highly dynamic FBL is located in the Cajal Bodies and in the nucleolus. In the latter during interphase, FBL is specifically located both at the FC/DFC border and in the DFC. When the cell enters mitosis, the nucleolus disappears and FBL together with nucleolin,

among others proteins, are observed at the chromosome periphery forming the perichromosomal compartment (Rodriguez-Corona et al., 2015).

FBL functions are conserved through evolution. It is implicated in ribosome biogenesis by taking action during pre-rRNA processing, pre-rRNA methylation and ribosome assembly (Tollervey et al., 1993). In yeast, different point mutations on the *NOP1* gene have demonstrated a wide range of effects on rRNA processing, such as decreased synthesis of 18S, 25S and 35S, suggesting diverse targets for Nop1/FBL (Tollervey et al., 1993). FBL induces post-transcriptional methylations or pseudouridylations in rRNA through its interaction with the small nucleolar ribonucleoproteins (snoRNPs) with C/D or H/ACA boxes respectively (Shubina et al., 2016). Moreover, the histone H2A is specifically methylated in glutamine Q104 in human ribosomal DNA (Q105 in yeast) by FBL (Tessarz et al., 2013). This methylation of the histone H2A is specifically recognized by the FACT (Facilitates Chromatin Transcription) protein that induces chromatin remodelling to allow RNAP1 transcription initiation (Rodriguez-Corona et al., 2015). In addition to methylations performed by FBL, the latter can also itself undergo some modifications. Indeed, Protein Arginine MethylTransferases 1 and 5 (PRMT1 and 5) interact with FBL and induce the methylation of its arginine residues; PRMT1 is responsible for the methylation of 45% of the arginine residues (Rodriguez-Corona et al., 2015; Shubina et al., 2016).

Recently, FBL has been presented as an oncogene. Several studies have demonstrated its accumulation in leukaemia and lymphoma cells (Rodriguez-Corona et al., 2015). Moreover, in breast cancer the inhibition of the tumour suppressor p53 upregulates fibrillarin. Subsequently, the high level of FBL modifies the rRNA methylation pattern, the translation fidelity, the amount of ribosomes and lastly contributes to tumorigenesis (Marcel et al., 2013). Interestingly, FBL seems to be involved in DNA repair. Indeed, several studies described either its location at the nucleolar periphery after DNA damage or its interaction with Chk1, a DDR protein involved in the ATR pathway (Franek et al., 2016; Peddibhotla et al., 2011).



# OBJECTIVES





Every day genotoxic agents such as UV lights harm the human body i.e. cells and the entire genome. Fortunately, the cells have developed specific mechanism to remove the lesions. Repair mechanism of UV-induced lesions on RNAP2-transcribed genes has been well described over the past 20 years. Indeed, studies on UV-damage RNAP2 transcribed genes have demonstrated a repair process of UV lesions through NER (see .2.1.4).

Ribosomal transcription is the first and rate-limiting step of the ribosome biogenesis that takes place in the nucleolus. Ribosome synthesis is an energetically costly process involved in translation of all the proteins needed by the cell. Moreover, RNAP1 transcription represents 60% of total cellular transcription in actively transcribing cells (Warner, 1999). Furthermore, alteration in quality and/or quantity of ribosome leads to severe diseases named ribosomopathies (Armistead and Triggs-Raine, 2014; Nakhoul et al., 2014; Yelick and Trainor, 2015).

Therefore, it is of great importance to repair efficiently the rDNA. Several questions came to our mind. How is the rDNA repaired? Is there a RNAP1 transcription-coupled repair process? And last but not least, where is the rDNA repaired? Indeed, rDNA transcription and ribosome biogenesis occur in the nucleolus, which is a specific nuclear and membrane-free structure where repair proteins have not yet been described, with the exception of CSB and TFIIH complex involved in RNAP1 transcription (Assfalg et al., 2012; Bradsher et al., 2002; Iben et al., 2002).



# RESULTS



# PART I



# Mechanistic Insights in Transcription-Coupled Nucleotide Excision Repair of Ribosomal DNA

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Human Ribosomal DNA, Nucleotide Excision Repair, RNAP1 Transcription, UV lesions



## ABSTRACT

Nucleotide excision repair (NER) guarantees genome integrity and proper cellular functions against ultraviolet light induced DNA damage. After UV irradiation, one of the first burdens cells have to cope with is a general transcriptional block caused by the stalling of RNA polymerase II (RNAP2) onto distorting UV lesions. To insure UV lesions repair specifically on transcribed genes, NER is coupled with transcription in an extremely organized pathway known as Transcription-Coupled Repair (TCR). Most of the knowledge about TCR has been gathered from RNAP2 transcription, however, in highly metabolic cells, more than 60% of total cellular transcription results from RNA polymerase I (RNAP1). Despite the importance of RNAP1 transcription, repair of the mammalian transcribed ribosomal DNA (rDNA) has been scarcely studied. Our results show that UV lesions severely block RNAP1 transcription and that the RNAP1 is firmly stalled onto the rDNAs without being degraded. Additionally, our researches describe the displacement of the RNAP1/rDNA complex to the nucleolar periphery after UV irradiation. Our study clearly demonstrated that the full TCR machinery is needed to repair UV-damaged rDNAs and restart RNAP1 transcription.

## INTRODUCTION

DNA integrity is continuously challenged by a large variety of DNA-damaging agents that produce several different DNA lesions. To insure genome stability and to guarantee proper cellular functions, cells have developed different mechanisms to eliminate DNA lesions.

One of the most versatile DNA repair systems involved in genome surveillance is the Nucleotide Excision Repair (NER) pathway. NER removes different structurally unrelated DNA helix-distorting lesions, including the UV-photoproducts cyclobutane pyrimidine dimer (CPD) and pyrimidine 6-4 pyrimidone (6-4PP). During NER, DNA lesions are eliminated via two sub-pathways and the sequential and highly coordinated actions of at least 30 proteins (Wood et al., 2001) that detect the lesion and excise a damage-containing oligonucleotide

then carry out repair synthesis and ligation events to restore the DNA sequence to its original state. One of the NER sub-pathways detects and repairs DNA lesions located on the whole genome, including the non-transcribed strand of active genes, and for this reason is referred to as Global Genome Repair (GGR). In GGR, the protein complex XPC/hHR23B, in concert with the UV-DDB (DDB1 and DDB2/XPE) complex, executes the lesion sensing (Alekseev et al., 2008; Hoogstraten et al., 2008; Min and Pavletich, 2007; Nishi et al., 2009; Scrima et al., 2008; Sugasawa, 2016). After the initial lesion recognition, the damaged DNA segment is opened over a stretch of ~30 nucleotides by the XPD helicase activity of the NER/basal transcription factor TFIIH (Coin et al., 2007). XPA together with RPA (Replication Protein A) organize the repair machinery around the lesion. The two structure-specific endonucleases XPG (O'Donovan et al., 1994; Staresincic et al., 2009; Zotter et al., 2006) and ERCC1-XPF complex (Houtsmuller et al., 1999; Sijbers et al., 1996; Staresincic et al., 2009) (responsible for the 3' and 5' incisions, respectively) excise a stretch of 27-29 nucleotides containing the lesion and finally the resulting single strand gap is filled in by normal DNA replication proteins (Shivji et al., 1995) and sealed by DNA ligases (Barnes et al., 1992; Moser et al., 2007).

The second sub-pathway is directly coupled to transcription elongation and detects and repairs DNA lesions located on the transcribed strand of active genes. This sub-pathway is referred to as Transcription-Coupled Repair (TCR). This dedicated NER sub-pathway specifically and efficiently removes lesions that block RNA Polymerase II (RNAP2) elongation, effectively avoiding accumulation of the very cytotoxic lesion-stalled transcription machinery and allowing quick resumption of transcription (Hanawalt, 1994). The CS factors (CSA and CSB) play an essential role during the initiation and proper progression of TCR by the lesion-stalled RNAP2 (Donahue et al., 1994). After the lesion recognition step TCR follows a similar cascade of events as for GGR (see above).

Several mutations in diverse NER associated genes have been reported in patients suffering from different genetic disorders such as Xeroderma Pigmentosum (XP), the Cockayne Syndrome (CS), the Trichothiodystrophy (TTD) or a combination of these diseases (XPCS). Although these syndromes present a wide range of symptoms, they show a common

feature: UV light sensitivity (DiGiovanna and Kraemer, 2012; Karikkineth et al., 2017; Stefanini et al., 2010).

Damaging agents harm randomly the whole genome and block many cellular functions. As mRNA production is impaired due to the stalling of the RNAP2, transcription of ribosomal genes (rDNA) should also be blocked when RNA Polymerase I (RNAP1) stalls on a helix-distorting DNA lesion. DNA lesions on ribosomal genes should be hence repaired to restore the RNAP1 cellular activity.

RNAP1 transcription is the first and rate-limiting step of a very complex cellular function known as ribosome biogenesis. Energetically, ribosome biogenesis is the most costly activity in actively growing or high-metabolism cells (e.g. neurons) and more than 60% of the total cellular transcription results from RNA Pol I activity (Grummt, 2003). This specific transcription has the uniqueness of being specifically localized in a dedicated sub-nuclear compartment: the nucleolus. RNAP1 transcription is solely dedicated to the transcription of ribosomal genes. rDNAs are located in the nucleolus and are grouped into several copies organized in tandem repeats, some of which are actively transcribed and others are silent. This redundancy is important to guarantee that at low damage levels one rDNA gene can be temporarily silenced without affecting the overall RNAP1 transcription rate. Nevertheless, when DNA repair is defective or overloaded, many rDNAs copies could be affected, disturbing the whole RNAP1 transcription process and later on modifying the ribosome content of cells, directly interfering with cellular protein production.

Therefore, it is of fundamental importance for the cell to maintain a functional RNAP1 transcription by repairing DNA lesions on the rDNAs active copies.

While the mechanism of RNAP1 transcription has been elucidated to a large extent (Russell and Zomerdijk, 2005), the repair of bulky lesions on rDNA genes has been very poorly investigated.

In yeast, a remarkable work has been done to try to disclose how rDNA is repaired by the NER system after UV irradiation (Conconi et al., 2002; Tremblay et al., 2014). Indeed, in

*Saccharomyces cerevisiae*, active rDNAs are repaired by a CSB-independent TCR-like process (Verhage et al., 1996) that implies the dissociation of the RNAP1 (Tremblay et al., 2014) to allow the action of a still undefined repair process, of which the mechanistic details remain obscure.

However, the largest gap of knowledge remains the repair process of ribosomal genes in mammalian cells. For many years, the idea was that after UV-irradiation mammalian rDNAs were simply not repaired (Balajee et al., 1999; Christians and Hanawalt, 1993, 1994) but these studies did not distinguish between active and inactive rDNA genes and some of them used murine cells to detect CPDs removal. Nevertheless, murine cells are nowadays known to be generally defective in CPDs repair (Vreeswijk et al., 1994). Moreover, these studies put forward the idea that in mammalian cells rDNAs, being located within the nucleolus, are not accessible to repair proteins (Christians and Hanawalt, 1993). However, it is common knowledge nowadays that some repair proteins are part of the nucleolus (Bradsher et al., 2002; Iben et al., 2002) and that mutations in repair proteins can affect ribosome biogenesis (Nonnekens et al., 2013).

These new evidences prompted us to investigate a possible role of these repair proteins in the removal of helix-distorting lesions on rDNAs in human cells. We made use of a novel approach, by measuring specifically RNAP1 transcription reduction and recovery after UV exposure, just as generally is used for the measurement of TC-NER on RNAP2 transcribed regions.

Our results show that transcription of the rRNA genes is blocked shortly after irradiation and recovers over time. We show here, for the first time that, in mammalian cells, the TC-NER machinery repairs UV lesions in the rDNAs and that this process is CSB and CSA dependent, but XPC independent. We could also show that RNAP1 is not released from the rDNAs sequences and not degraded. Finally, we could show that the repair reaction takes place at the periphery of the nucleolus, where all the repair proteins can access the damaged rDNAs. Importantly, we demonstrated that UV lesions present on the rDNAs specifically trigger the displacement of the rDNAs at the periphery of the nucleolus.

## **MATERIALS & METHODS**

### **RPA43-GFP fusion protein production and expression in transformed human fibroblasts.**

The full-length RNAP1 subunit (RPA43) was cloned in-frame into the pEGFP-N1 vector (Clontech). The construct was sequenced prior to transfection. Transfection in MRC5, CSA, CSB and XPC SV40-transformed human fibroblasts was performed using Fugene transfection reagent (Roche) Stably expressing cells were isolated after selection with G418 (Gibco) and single cell sorting using FACS (FACScalibur, Beckton Dickinson).

### **Cell culture and treatments**

The cells used in this study were: (i) wild type SV40-immortalized human fibroblasts (MRC5); (ii) GGR deficient SV40-immortalized human fibroblast: XPC (XP4PA); (iii) TCR deficient SV40-immortalized human fibroblast: CSA (CS3BE); CSB (CS1AN); (iv) NER (GGR & TCR) deficient SV40-immortalized human fibroblast: XPA (XP12RO); XPB (TTD6VI); XPB (XPCS2BA); XPD (XP6BE); XPD (XPCS2); TTDA (TTD1BR); (v) MRC5, CSB and XPC stably expressing RPA43-GFP (RNAP1 subunit; G418 selected 0.2 mg/ml). Wild type Embryonic Stem (ES) cells were derived from the XPB-YFP mouse model (Giglia-Mari et al., 2009). HT-1080 cells stably expressing an adapted Lac Operator/Lac Repressor (LacO/LacR) system (selected using BlasticidinS and Hygromycin, 5 $\mu$ g/ml and 100 $\mu$ g/ml respectively), were used to detect the rDNA as previously described (Chubb et al., 2002; Robinett et al., 1996).

Human fibroblasts were cultured in a 1:1 mixture of Ham's F10 and DMEM (Lonza) supplemented with 1% antibiotics (penicillin and streptomycin; Lonza) and 10% foetal bovine serum (Gibco). Embryonic Stem (ES) cells were cultured in a 1:1 mixture of DMEM (Lonza) and BRL conditioned medium, supplemented with antibiotics (penicillin and streptomycin; Lonza), 10% foetal bovine serum (Gibco), non-essential amino acids (Gibco),  $\beta$ -Mercaptoethanol (Sigma) and ESGRO Leukemia Inhibitory Factor (LIF, Merk-Millipore). HT-1080 cells with LacO/GFP-LacR were cultured in DMEM (Lonza), supplemented with 1% antibiotics (penicillin and streptomycin; Lonza) and 10% foetal bovine serum (Gibco). All

human cells were incubated at 37°C with 20% O<sub>2</sub> and 5% CO<sub>2</sub>. ES cells were incubated at 37°C with 3% O<sub>2</sub> and 5% CO<sub>2</sub>.

DNA damage was inflicted by UV-C light (254nm, 6 Watt lamp). Cells were globally irradiated with different doses of UV-C (1J/m<sup>2</sup>, 2J/m<sup>2</sup>, 4J/m<sup>2</sup>, 5J/m<sup>2</sup>, 6J/m<sup>2</sup>, 8J/m<sup>2</sup>, 10J/m<sup>2</sup>, 12J/m<sup>2</sup> and 16J/m<sup>2</sup>). Experiments were performed at different time points after UV exposure (0.5h, 1h, 3h, 16h, 24h, 36h and 40h post UV). Mock-irradiated cells (untreated) were used as control.

## **RNA FISH**

Cells were grown on 12 mm coverslips, washed with warm PBS and fixed with 4% paraformaldehyde for 15min at 37°C. Coverslips were washed twice with PBS. Cells were permeabilized by washing with PBS 0.4% Triton X-100 for 7min at 4°C. Cells were washed rapidly with PBS before incubating them with pre-hybridization buffer (2x SSPE and 15% formamide) (20x SSPE, [pH 8.0]: 3M NaCl, 157mM NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O and 25mM EDTA) for at least 30min. 1.5µl of probe (10ng/ml) was diluted in 30µl of hybridization mix (2x SSPE, 15% formamide, 10% dextran sulphate, 0.5 mg/ml tRNA) and heated to 90°C for 1 min. Hybridization of the probe was conducted overnight at 37°C in a humid environment. Subsequently, cells were washed twice for 20min with pre-hybridization buffer, then once for 20min with 1x SSPE and finally mounted with Vectashield (Vector Laboratories) and kept at -20°C. The probe sequence (5' to 3') is: Cy5-AGACGAGAACGCCTGACACGCACGGCAC. At least three biological replicates were obtained and at least 30 cells were imaged for each condition of each cell line.

## **Northern blot**

Extractions of total RNAs from cells were performed using TRI Reagent (Sigma-Aldrich). Briefly, sub-confluent growing cells were trypsinized and washed in PBS. The pellet was suspended in 1mL TRI Reagent and processed as recommended by the manufacturer. An additional step of phenol/chloroform extraction was performed on the RNA-containing soluble fraction before isopropanol precipitation.

4µg of total RNAs was separated on a 1.2% agarose gel as described in “Molecular Cloning”, Sambrook and Russell, CSHL Press (“Separation of RNA According to Size: Electrophoresis of Glyoxylated RNA through Agarose Gels”). RNAs were transferred to Amersham Hybond N+ membranes (GE Healthcare), which were hybridized with <sup>32</sup>P-labeled oligonucleotides using Rapid-hyb buffer (GE Healthcare). The membranes were exposed to Phosphor Screens developed in Phospho Imager apparatus and quantified with ImageQuant software. The oligonucleotides sequences used as probes were:

45S: AGACGAGAACGCCTGACACGCACGGCAC

18S: ATGTGGTAGCCGTTTCTCAG.

### **Chromatin Immunoprecipitation (ChIP) on ES nuclear extract**

ChIP was performed as described previously (Nonnekens et al., 2013). Briefly, ES cells were grown under optimal growth conditions, UV irradiated with 16 J/m<sup>2</sup> UV-C and after 1 hour cells were fixed and harvested by scraping in PBS. Cell lysis was performed with lysis-buffer and nuclei washed with wash-buffer. Afterwards, nuclei were suspended in IP-buffer and sonicated in a Bioruptor UDC-200 (set up high for 30min, with cycles of 30s on/1 min off; Diagenode) to yield DNA fragments with an average size of 300bp. Samples were centrifuged at 14000g for 5min to remove insoluble material and measured with a nanodrop at 260nm. Optimal amounts of ES extracts to maximize ChIP ratio were incubated in 150µl total volume with antibody (RPA194 C-1, sc-48385 Santa Cruz) (ChIP) or no antibody (Mock), overnight. Immunoprecipitation (IP) was performed for 1 hour with 40µl of washed magnetic Bio-Adembeads Protein G (Ademtech). After IP, the beads were washed and DNA and proteins eluted with elution buffer. DNA from ChIP, Mock and input preparations were decrosslinked and purified by phenol-chloroform extraction. Samples were amplified by real-time PCR (qPCR) using the Power SYBR Green PVR master mix (Applied Biosystems) on a 7300 real-time PCR system (Applied Biosystems). ChIP data were normalized to the input (to take copy number into account) and subtracted with the background (Mock). Biological replicates were generated for each experiment. Primer sequences for qPCR can be found in (Nonnekens et al., 2013).

## Chromatin extracts

MRC5 were grown in 14.5cm plate. Cells were irradiated as described above and washed once with PBS. In vivo crosslinking was performed as described (Orlando et al., 1997; Parekh and Maniatis, 1999) with few modifications. All procedures were carried out at 4°C unless otherwise stated. Briefly, control or irradiated cells were cross-linked with 12ml of 1% formaldehyde (in PBS) prepared from an 11% stock (0.05 M HEPES [pH 7.8], 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 11% formaldehyde) for 16 min. Cross-linking was neutralized with 12ml of glycine solution (PBS, glycine 0.125M, [pH 6.8]) for 7 min, followed by 2 washes with cold PBS. The cells were collected by scraping in cold PBS (PBS, 1mM EDTA) and spinned down 10 min at 1200-1500 rpm 4°C.

All buffers used for cell extraction contained, among others, 1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, and a mixture of proteinase and phosphatase inhibitors (EDTA-free protease inhibitor tablets; Roche). Just before use, Napy-sodium pyrophosphate (0.33M stock) was added.

Cell pellet was washed twice with cold PBS. The cell pellet was suspended in Chro-lysis buffer (1 ml per  $12.5 \times 10^6$  cells; 50mM Hepes-KOH [pH 7.8], 0.14 M NaCl, 1mM EDTA [pH 8.0], 0.5mM EGTA [pH 8.0], 0.5% NP-40, 0.25% Triton, 10% glycerol) and rotated for 10 min. The suspension was spinned down (1200-1400 rpm, 10 min, 4°C). Cell pellet was washed with Wash buffer (1 ml per  $12.5 \times 10^6$  cells; 0.01M Tris-HCl [pH 8.0], 0.2 M NaCl, 1mM EDTA [pH 8.0], 0.5mM EGTA [pH 8.0]) rotated for 10 min, spinned down (1200-1400 rpm, 10 min, 4°C), suspended in RIPA buffer (1 ml RIPA buffer per  $25\text{-}35 \times 10^6$  cells; 0.01M Tris-HCl [pH 8.0], 0.14 M NaCl, 1mM EDTA [pH 8.0], 0.5mM EGTA [pH 8.0], 1% Triton, 0.1% Na-Deoxycholate, 0.1% SDS) and incubated for 30 min.

The nuclear suspension was sonicated on ice-cooled water using a Bioruptor UDC-200 for 45 min (power setting High; 30sec ON; 1min OFF; Diagenode) to yield DNA fragments with an average size of 300bp. After the sonication, samples were spinned down (10.000 rpm, 10min, 4°C) and the supernatant that contained the crosslinked chromatin was aliquoted, freezed with liquid nitrogen and stored at -80°C.



## Western blot

Protein concentration was determined by using the Bradford method. Samples were diluted with 2X Laemmli buffer, heated at 95°C (1x30 min, spin down, 1x 25 min, spin down) and loaded on a SDS-PAGE gel. Proteins were separated on 8% and 14% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane (PVDF, 0.45µm Millipore). The membrane was blocked in 5% Milk PBS 0.1% Tween (PBS-T) and incubated for 1.5h with the following primary antibodies in milk PBS-T. The loading was controlled with the anti-Histon3 antibody. Subsequently, membrane was washed with PBS-T (3x 10min) and incubated with the secondary antibody in milk PBS-T. After the same washing procedure, protein bands were visualised via chemiluminescence (ECL Enhanced Chemo Luminescence; Pierce ECL Western Blotting Substrate) using the ChemiDoc MP system (BioRad).

## Western blot antibodies

- Primary antibodies:

Mouse anti-RPA194(C1) (sc-48385) 1/500; mouse anti-UBF(F-9) (sc13125) 1/500; rabbit anti-TWISTNB (ab99305) 1/2000; rabbit anti-hPAF49 (GTX102175) 1/250; mouse anti-POLR1E (sc398270) 1/500; rabbit anti-Pol II CTD repeat YSPTSPS Phospho Ser2 (ab5095) 1/250; rabbit anti-Histone H3 (ab1791) 1/10000.

- Secondary antibodies:

Goat anti-mouse HRP conjugate (170-6516; Biorad) 1/5000, Goat anti-rabbit HRP conjugate (170-6515; Biorad) 1/5000.

## Immunofluorescence assay

Cells were grown on 24mm coverslips, washed with warm PBS and fixed with 2% paraformaldehyde for 15 min at 37°C. Cells were permeabilized with PBS 0.1% Triton X-100 (3x short + 2x 10 min). Blocking of non-specific signal was performed with PBS<sup>+</sup> (PBS; 0.5%BSA; 0.15% glycine) for at least 30 min. Then, coverslips were incubated with 100µl of primary antibody mix (Mouse anti-RPA194; 1/500 in PBS<sup>+</sup>; sc-48385) for 2h at room

temperature in a moist chamber, washed with PBS (3x short; 2x 10min), quickly washed with PBS<sup>+</sup> before incubating with 100µl of secondary antibody mix (Goat anti-mouse Alexa Fluor<sup>®</sup> 488 and Goat anti-mouse Alexa Fluor<sup>®</sup> 594; 1/400 in PBS<sup>+</sup>; A-11001 and A-11005 Invitrogen respectively) for 1h at room temperature in a moist chamber. After the same washing procedure, coverslips were finally mounted using Vectashield with DAPI (Vector Laboratories) and kept at -20°C. At least three biological replicates were performed and at least 15 cells were imaged for each condition of each cell line.

### **Fluorescent Recovery After Photo-bleaching (FRAP)**

FRAP experiments were performed on a Zeiss LSM 710 NLO confocal laser scanning microscope (Zeiss), using a 40x/1.3 oil objective, under a controlled environment (37°C, 5% CO<sub>2</sub>). Briefly, RNAP1-GFP foci were selected in the nucleolus and photo-bleached at 100% laser intensity of the 488 line of a 25mW Argon laser. Then, recovery of fluorescence was monitored at 1% laser intensity, every 30sec directly after the bleach and up to 6 min after bleaching. All FRAP data were normalized to the average pre-bleached fluorescence after background removal. The loss of fluorescence induced by the measure every 30sec was also taken in account. Every plotted FRAP curve is an average of at least 30 measured cells.

### **Fluorescent imaging and analysis**

Imaging has been performed on a Zeiss LSM 780 NLO confocal laser-scanning microscope (Zeiss), using a 60x/1.4 oil objective. Images were analysed with ImageJ software. For all images of this study, nuclei and nucleoli were delimited with dashed and dotted line respectively, using DAPI staining or transmitted light.

### **Statistical analysis**

Error bars represent the Standard Error of the Mean (SEM) of the biological replicates. Student's t-test was used to assess whether the mean values of the replicates were statistically significant, assuming equal variance. A P-value of 0.05 or less was considered as significant. (\* p<0.05 ; \*\* p<0.01 ; \*\*\* p<0.001).

## RESULTS

### **RNAP1 retention on the rDNAs after UV irradiation**

Ribosomal DNA transcription represents 60% of the total cellular transcription and is the first and rate-limiting step of ribosome biogenesis (Grummt, 2003; Henras et al., 2014). Despite the fact that the mechanism of RNAP1 transcription has been thoroughly studied, little is known about the repair mechanism of UV-induced lesions in rDNA and the implication of the RNAP1 in this process. Recent studies in yeast have described the dissociation of the RNAP1 from the ribosomal DNA after UV irradiation. In order to investigate whether the mammalian RNAP1 behaves in the same manner as yeast RNAP1 in response to UV-induced damage, we performed Chromatin Immunoprecipitation coupled with quantitative PCR (ChIP-qPCR) assays against RNAP1, in absence of UV lesions and 1h after UV irradiation. ChIP-qPCR experiments were performed using different couples of primers (14 couples); their location is indicated in figure 1A. The set of 14 primers covered completely the ribosomal DNA transcription unit (including the promoter) and the IntraGenic Spacer (IGS). Our results described the binding of the RPA194 subunit of RNAP1 along the rDNA gene in both untreated and irradiated cells. In untreated cells the binding profile did not differ from the previously published results (Nonnekens et al., 2013). Interestingly, RPA194 binding profile on rDNA was drastically modified 1h after the UV exposure, showing that, more of this subunit bound the ribosomal DNA in response to UV irradiation (Figure. 1B). This result was in contrast to what have been described for yeast RNAP1 (Tremblay et al., 2014) and for mammal RNAP2 (Bregman et al., 1996; Ratner et al., 1998) after UV irradiation. To verify whether the whole RNAP1 complex is more bound to chromatin after UV-irradiation, we prepared chromatin extracts at different time after UV exposure and we carried out western blots against several RNAP1 subunits (RPA194, PAF49, TWISTNB and POLR1E), the Upstream Binding Factor (UBF) and RNAP2 (Figure 1C).

Quantification of the western blots clearly showed that RNAP1 subunits accumulated on chromatin 3h after UV irradiation then partially detached from the chromatin upon repair 40h after UV irradiation. Interestingly, at this time point the binding of RNAP1 was still higher than the one measured in untreated cells, suggesting that it would take more

time for RNAP1 to restore its binding dynamics after UV damage. In our conditions and as expected, RNAP2 unbound the chromatin after UV exposure (Figure 1D). Differently from the RNAP1 binding behaviour, the binding of the RNAP1 associated transcription factor UBF on the chromatin did not change upon UV irradiation.

Together, these data described a surprising and specific outcome for the RNAP1 after UV-induced damage: more RNAP1 bound to rDNA when it encountered a UV lesion.

### **RNAP1 transcription arrest after UV irradiation**

We showed that UV irradiation induced a modification of RNAP1 binding on rDNA and we wondered whether this modification could affect RNAP1 transcriptional activity. In order to measure the transcriptional activity of RNAP1, we performed RNA FISH using a probe that specifically recognized the first transcript of the RNAP1: the 47S pre-rRNA (Figure 2A). The designed probe, including a cyanin5 fluorochrome, was conceived to recognize the 5' part of the 47S pre-rRNA, upstream from the first cleavage site, rapidly processed during rRNA processing. For this reason RNA FISH performed with this specific probe is a reliable indicator of the ribosomal transcription level and speed (Cui and Tseng, 2004). In order to quantify transcriptional activity after UV damage induction, cells were UV-irradiated at different doses (5, 10 or 16 J/m<sup>2</sup>) and fixation was performed at different time after UV exposure (1h, 3h or 16h post UV).

Our results showed that UV irradiation induced a decrease of the 47S pre-rRNA level, indicating that RNAP1 transcription was rapidly hindered (Figure 2B) and reached a minimum level between 1 and 3 hours depending on the UV dose. Namely, a higher UV dose (16 J/m<sup>2</sup>) blocked the transcriptional activity faster than a lower UV dose (5 J/m<sup>2</sup>) (Figure 2C).

Our results also demonstrated that, no matter the dose used, 3-4 hours post-irradiation RNAP1 transcription progressively restarted. To determine when the RNAP1 transcription levels were completely restored, we measured the 47S pre-rRNA level up to 48h after UV irradiation. Equal quantities of total RNA were analysed by northern blot with a probe targeting the same area of the 47S pre-rRNA as described in the RNA FISH

experiment (Figure 2D). Quantification of the northern blots revealed a total resumption of the ribosomal transcription between 36 and 48h after UV exposure.

### **TC-NER dependent UV lesions repair of rDNA**

We showed that RNAP1 transcription is fully restored 36 to 48 h after UV irradiation, meaning that UV lesions, hindering RNAP1 activity, have been removed. Because in mammalian cells NER pathway repairs UV damage, we decided to further investigate the implication of NER factors into UV lesions removal on rDNA and RNAP1 transcription restart.

Therefore, we studied RNAP1 transcription activity after UV irradiation in NER deficient cell lines and to distinguish between TC-NER and GG-NER we specifically used cell lines that are deficient for these sub-pathways. Hence, we performed RNA FISH and northern blot experiments on wild type (WT), TC-NER (CSA & CSB) and GG-NER (XPC) deficient cells at different time after UV irradiation (Figure 3A-B).

Both Northern Blot assays (Figure 3A) and RNA FISH labelling (Figure 3B) showed that 47S pre-rRNA level decreased 3h after the irradiation in all cell types. However, while in WT cells and GG-NER deficient cells (XPC) RNAP1 transcription slowly resumed over time, in TC-NER deficient cells (CSA and CSB) no resumption of RNAP1 activity could be detected (Figure 3C). Our results showed that RNAP1 transcription inhibition was due the presence of UV lesions on the rDNAs and that resumption of transcriptional activity was due to the specific repair of UV lesions on the actively transcribed strand of rDNAs. Moreover, these results indubitably involved TC-NER factors CSA and CSB, but not the GG-NER factor XPC in repair of the actively transcribed rDNAs.

### **rDNAs repair involves the complete TC-NER machinery**

We showed that the specific TC-NER factors CSA and CSB were required for rDNA UV lesion repair and we wondered whether other known NER factors could be involved in this repair mechanism. We, therefore, conducted RNA FISH assays in different NER mutant cell lines (XPA, XPB<sup>XPCS</sup>, XPB<sup>TTD</sup>, XPD<sup>XPCS</sup>, XPD<sup>XP</sup>, TTDA) (Figure 4A). As seen previously for the WT, GG-NER (XPC) and TC-NER (CSA & CSB), the NER deficient cells (XPA, XPB, XPD and TTDA)

showed a decrease of the 47S pre-rRNA production 3h after UV exposure. Similarly to CSA and CSB cells (TC-NER deficient), but unlike the WT and XPC cells (GG-NER deficient), no resumption of 47S pre-rRNA production was observed in these cells lines (Figure 4B). After UV irradiation, RNAP1 transcription did not recover in these NER mutants evidently implying that the whole TC-NER machinery was required for the ribosomal genes repair of UV-induced lesions.

### **Displacement of RNAP1 and rDNA during TC-NER**

We showed that NER-factors were implicated in rDNA repair of UV lesions. Nevertheless, some basic NER-factors were excluded from the nucleolus (XPC, XPA, ERCC1-XPF) and it remains unrevealed if and how these repair proteins reach the rDNA within the nucleolus to repair it.

In order to determine the location of the rDNA during repair we used a LacO-LacR-GFP reporter system to visualise the rDNA by microscopy in cells. Several LacO genes were inserted downstream from the ribosomal transcription unit. These cells (HT80, (Rasheed et al., 1974) were subsequently transfected with a plasmid carrying the LacR-GFP reporting system. The LacR-GFP, when expressed, targeted the LacO repeats downstream from the ribosomal genes allowing one to visualise the rDNA (Figure 5A). To detect both the rDNA and the RNAP1, we performed an immunofluorescence (IF) assay against the biggest subunit of RNAP1 (RPA194) in these cells. We observed that in the untreated cells, the ribosomal DNA (green) and the RNAP1 (red) were located within the nucleolus. However, 3h after the irradiation the rDNA, together with the RNAP1, relocated to the periphery of the nucleoli and after the repair reaction is completed, both rDNA and RNAP1 returned within the nucleolus (Figure 5B). The fact that not only rDNA was displaced but also RNAP1 confirms our results from the CHIP-qPCR experiments (Figure 1B) showing that after UV damage, RNAP1 did not dissociate from the rDNA.

In order to investigate whether this relocation is due to the DNA repair reaction, in the first place we performed a UV-dose assay in wild type cells untreated (NT) or 3h after a UV-irradiation at different UV doses (1; 2; 4; 6; 8; 10; 12 and 16J/m<sup>2</sup>). Our results showed

that RNAP1 was displaced to the nucleolar periphery in a UV-dose dependent manner (Figure S1). Namely, UV doses from 1 to 10J/m<sup>2</sup> did not affect RNAP1 position within the nucleolus. However, at 12J/m<sup>2</sup>, RNAP1 gathered in few foci within the nucleolus but closer to the nucleolar periphery, whereas at 16J/m<sup>2</sup> the RNAP1 foci were clearly located at the nucleolar periphery. Because of these results, we decided to use 16J/m<sup>2</sup> as the standard UV-dose. At this dose in WT cells, in which NER is functional, RNAP1 was displaced at the nucleolar periphery 3h after UV irradiation and returned within the nucleolus at later time points (36h) after UV exposure, when repair was achieved (Figure 5C). In CSA and CSB cells, which are TC-NER deficient, at 3h post-UV a displacement of RNAP1 at the nucleolar periphery was observed as in WT cells. However, no return of the RNAP1 within the nucleolus was described at later time points (Figure 5C). Furthermore, NER deficient cells (XPA, XPB, XPD and TTDA) showed a similar RNAP1 behaviour after UV damage induction (Figure S2).

### **UV-lesions on rDNA induce retention of RNAP1 at the nucleolar periphery**

To investigate whether the return of RNAP1 within the nucleoli depends on transcription blockage (Figure 4), we performed an IF assay in a GG-NER deficient cell line (XPC). In XPC cells, UV-lesions located on non-transcribed strands of DNA were not repaired. However, in these cells, UV-lesions located on active rDNA genes were repaired and RNAP1 transcription restarted (Figures 4, 6A, 6B). This situation was different from CSB cells, in which UV-lesions located in active rDNA were not repaired and their presence inhibited transcription (Figures 4, 6A, 6B). Within these GG-NER (XPC) and TC-NER deficient cells (CSB), we performed IF against RPA194. As previously described, RNAP1 relocated to the nucleolar periphery 3h after irradiation in all cell types (Figure 6C). As previously shown (Figures 5C and 6C), in WT cells RNAP1 returned within the nucleolus at 36h post-UV and transcription restarted (Figures 6A-B). In contrast, in CSB cells RNAP1 remained at the nucleolar periphery (Figure 6C) concomitantly with a transcription blockage (Figures 6A-B). Surprisingly RNAP1 remained at the nucleolar periphery in XPC cells despite the ribosomal transcription resumption (Figures 6A-B), (Figure 6C). We were able to verify these results in living cells by expressing a GFP-tagged version of the subunit RPA43 in WT, XPC and CSB

cells (Figure 6D). Namely, in XPC cells RPA43-GFP did not return in the nucleolus after DNA damage induction and repair of the transcribed rDNA.

We further investigated RNAP1 behaviour by performing Fluorescent Recovery After Photobleaching (FRAP) on RNAP1-GFP foci in wild type, CSB and XPC cells at different times after UV irradiation (3h, 16h and 40h). Our FRAP data (Figures 6E-G) showed a recovery of fluorescence of the bleached foci, in about 6 minutes, indicating an almost complete turnover of rDNA-bound RNAP1 in that time interval. In WT cells, our FRAP data showed that 3h after UV exposure the turnover of RNAP1-GFP was incomplete compared to untreated cells (Figure 6E). 40h after UV, although a small change in the FRAP profile was seen in the first part of the curve, RNAP1-GFP turnover remained incomplete compared to non-irradiated WT cells. This partial recovery of fluorescence indicated that RNAP1 is more bound within the nucleolar foci, 3h and 40h after UV exposure, consistent with the western blot observations in Figure 1C. In CSB cells, UV irradiation did not significantly impact RNAP1-GFP fluorescence recovery (Figure 6F) i.e. the bound fraction of RNAP1 was not modified by UV in these TC-NER deficient cells. The same FRAP on foci procedure performed on GG-NER deficient XPC cells, showed that 3h after UV, the turnover of RNAP1-GFP was also incomplete (Figure 6G). The effect observed 40h after UV was even stronger, indicating that RNAP1 appeared progressively more bound.

## DISCUSSION

Nucleotide Excision Repair has been extensively studied in correlation with RNAP2-transcribed genes (Chitale and Richly, 2017; Coin et al., 2008; Hanawalt and Spivak, 2008; Marteiijn et al., 2014; Spivak, 2015). However, the repair mechanism of UV-damaged rDNA has been poorly investigated in mammals and the few studies carried out have shown contradictory results ((Christians and Hanawalt, 1994; Cohn and Lieberman, 1984; Conconi et al., 2002; Tremblay et al., 2014). Our research has focused on ribosomal gene repair after UV irradiation in human cells.



In this study, we have reported a decrease of RNAP1 transcription shortly after UV irradiation, followed by a slow recovery of rDNA transcription between 36h and 48h after UV exposure (Figure 2). The resumption of RNAP1 transcription is slower than the RNAP2 transcription recovery, which occurs within 16-24h (Bohr et al., 1985). It is our opinion that the slow resumption of RNAP1 transcription could be due to the structure in which the transcription takes place: the nucleolus.

Although some studies have identified some NER factors interacting with RNAP1 in the nucleolus (Assfalg et al., 2012; Bradsher et al., 2002; Iben et al., 2002; Koch et al., 2014), most of the repair proteins have not yet been found to localise in this nuclear section. For this reason, it is plausible that the transcription machinery needs to be relocated at the periphery of the nucleolus in order to be repaired, as in other cellular processes such as replication (Dimitrova, 2011) and chromatin remodelling (Franek et al., 2016).

Indeed, rDNA and/or RNAP1 were located at the nucleolar periphery during rDNA replication and DSB repair of ribosomal genes (Dimitrova, 2011; Franek et al., 2016; Larsen and Stucki, 2016). In order to confirm this hypothesis, we have investigated the RNAP1 location during repair of UV lesions. Our data have demonstrated that RNAP1 shifted to the nucleolar periphery after UV exposure and returned into the nucleolus, when repair was completed 36h after the irradiation in wild type cells (Figure 5C). Moreover, we have reported that RNAP1 bound strongly to the rDNA after UV irradiation (Figure 1). Consistent with these findings, we have observed that the rDNA is also displaced to the nucleolar periphery after UV irradiation and returned into the nucleoli after repair (Figure 5B). Our results support the hypothesis that the rDNA/RNAP1 complex is transferred to the nucleolar periphery in order to access specific repair factors.

More than three decades ago, Cohn and Lieberman (1984) have shown that rDNA was repaired through NER in human. Conversely, later on, several studies have established that rDNA was not repaired through the TC-NER mechanism in mammalian cells (Balajee et al., 1999; Christians and Hanawalt, 1993; Vreeswijk et al., 1994). However, these studies have worked on total rDNA, making no difference between active and inactive regions of the rDNA. Indeed, 50% of the rDNA is silent (Conconi et al., 1989). In order to circumvent

this bias, different studies performed in yeast have separated the inactive rDNA from the active one and have described the implication of TC-NER in repair of UV lesions on active rDNA (Charton et al., 2015; Conconi et al., 2002), even though the NER factor CSB did not seem to be involved (Verhage et al., 1996).

Despite the absence of TC-NER of rDNA in mammals but in view of the fact that many similarities have been previously described between yeast and human processes, implication of TC-NER factors have been investigated during repair of UV-damaged rDNA in human cells. In this study we have reported that a complete TC-NER mechanism was required to repair the UV lesions present on the active rDNA (Figures 3-4). During TC-NER of active rDNA, RNAP1 bound tightly to its substrate, as demonstrated by the FRAP experiments conducted in WT and XPC cells (GG-NER deficient but TC-NER proficient). Indeed, in WT and XPC cells, the turnover between the RNAP1 bound to rDNA and its free pool in the nucleolus was slower after UV irradiation, whereas no difference was observed in CSB cells (TC-NER deficient) before and after UV exposure (Figure 6E). This result was concomitantly confirmed by ChIPqPCR experiments (Figure 1B) and western blot results, showing an accumulation of RNAP1 on chromatin after UV exposure in WT cells (Figures 1C-D). Interestingly, these results are different from those found in previous studies in mammals showing that TC-NER is not involved in rDNAs repair (Balajee et al., 1999; Christians and Hanawalt, 1993; Vreeswijk et al., 1994). Moreover, differently from yeast (Tremblay et al., 2014), mammalian RNAP1 does not dissociate from its substrate during rDNA repair. Furthermore, in contrast with what has been shown for RNAP2 i.e. backtracking or unbinding of the DNA followed by its degradation during repair (Cheung and Cramer, 2011; Marteiijn et al., 2014; Wilson et al., 2013), RNAP1 is not degraded during DNA repair but accumulates on rDNA and relocates at the periphery of the nucleolus as described in this study.

Intriguingly, RNAP1 remained strongly bound to rDNA up to 40h after UV irradiation even though rDNA repair was completed and transcription has restarted (Figures 1C-D, 2E & 6E). This result indicated that RNAP1 requires a certain time to recover its binding dynamic after UV irradiation. On the contrary, UBF, which is an RNAP1 transcription partner, did not

change its binding profile after UV irradiation, meaning that UBF was not more recruited to neither excluded from rDNA during repair. This result can be explained by the fact that UBF is structurally needed to give the rDNA a conformation suitable for transcription, but it does not participate enzymatically to the transcription reaction *per se*. Indeed, in mammals, in addition to its implication in RNAP1 initiation (Jantzen et al., 1990), UBF induces the formation of the enhancosome by modifying structurally the rDNA in order to allow the RNAP1 elongation through the ribosomal gene (Stefanovsky and Moss, 2008).

Interestingly, in this study we have described the relocation of RNAP1/rDNA complex at the nucleolar periphery after UV irradiation (Figures 5A&C). Several research groups have shown the formation of nucleolar caps at the periphery of the nucleolus, containing the rDNA, after Actinomycin D treatment and DSBs induction inhibiting RNAP1 transcription (Franek et al., 2016; Reynolds et al., 1964; Sluis and McStay, 2015). Another study has clearly demonstrated that DSBs inhibited RNAP1 transcription through the ATM pathway, and has indicated the relocation of RNAP1 at the nucleolar periphery (Kruhlak et al., 2007). However, Moore and co-workers (2011) have shown opposite conclusions i.e. nucleolar caps were formed after UV irradiation ( $36\text{J/m}^2$ ), but not following ionizing radiation (IR). These contradictory results could be explained by the way DSBs have been induced. Indeed, Moore and co-workers used IR, whereas in the other studies micro-irradiation, IR, the CRISPR/Cas9 technique or the transfection of a plasmid holding the sequence of the endonuclease I-PpoI were used (Franek et al., 2016; Kruhlak et al., 2007; Sluis and McStay, 2015). Despite these differences, the converging idea in the field was that the stall of transcription induced the displacement of RNAP1 at the nucleolar periphery. Our study challenged this accepted view.

We have investigated the relocation of RNAP1 and rDNA at the nucleolar periphery during DNA repair in various NER-deficient cell lines. We have shown that while in WT cells, 36h after UV irradiation, ribosomal transcription restarted and RNAP1 returned into the nucleolus (Figures 6A-D), in TC-NER deficient cells (CSB, CSA) transcription was permanently inhibited and the RNAP1 remained at the periphery of the nucleolus. Remarkably, in XPC cells that are TC-NER proficient but GG-NER deficient, even though RNAP1 transcription

restarted, the RNAP1 protein remained at the nucleolar periphery. In fact, in these cells, while the UV lesions on the active rDNA were repaired (proficient TC-NER), the UV lesions on the inactive genome in proximity of rDNA genes (active or inactive) were not repaired and were still present on the DNA. These results support two main ideas. Firstly, RNAP1 position at the nucleolar periphery is not always associated with transcription inhibition and transcription recovery is not enough to allow the RNAP1 to return into the nucleolus. Secondly and most importantly, unrepaired UV lesions on silent area nearby active rDNA or in inactive rDNA are sufficient to maintain RNAP1 to the nucleolar periphery. The presence of unrepaired UV-lesions could be a signal for the transport of RNAP1/rDNA and/or the signal for its retention at the nucleolar periphery. But also, the unrepaired UV-lesions could induce specific chromatin remodelling around the lesion that would imply a relocation of the rDNA for the recruitment of repair proteins nearby the lesion.

Ribosomal DNA repair of UV-induced lesions has been poorly studied in human cells and the few studies were performed more than 20 years ago. However, rDNA transcription represents a crucial step of the ribosome biogenesis, an essential process for cells that has been involved in several diseases called ribosomopathies. Thus, we decided to focus on rDNA repair in human cells. We demonstrated, for the first time in human, that rDNA repair after UV irradiation involves the complete TC-NER mechanism. We also determined that the relocation of RNAP1/rDNA at the nucleolar periphery during repair and return of RNAP1/rDNA within the nucleolus is strictly dependent on the repair of all UV-lesions (in transcribed and untranscribed DNA) rather than subordinate to transcription resumption.

Many questions remain to be elucidated. In the next years, it will be important to investigate the mechanistic aspects of this displacement. Further studies should be set up in order to identify the partners involved in the relocation of RNAP1/rDNA, detecting chromatin modifications during this displacement, as well as decipher its impact on RNAP1 transcription at long term and on ribosome biogenesis.

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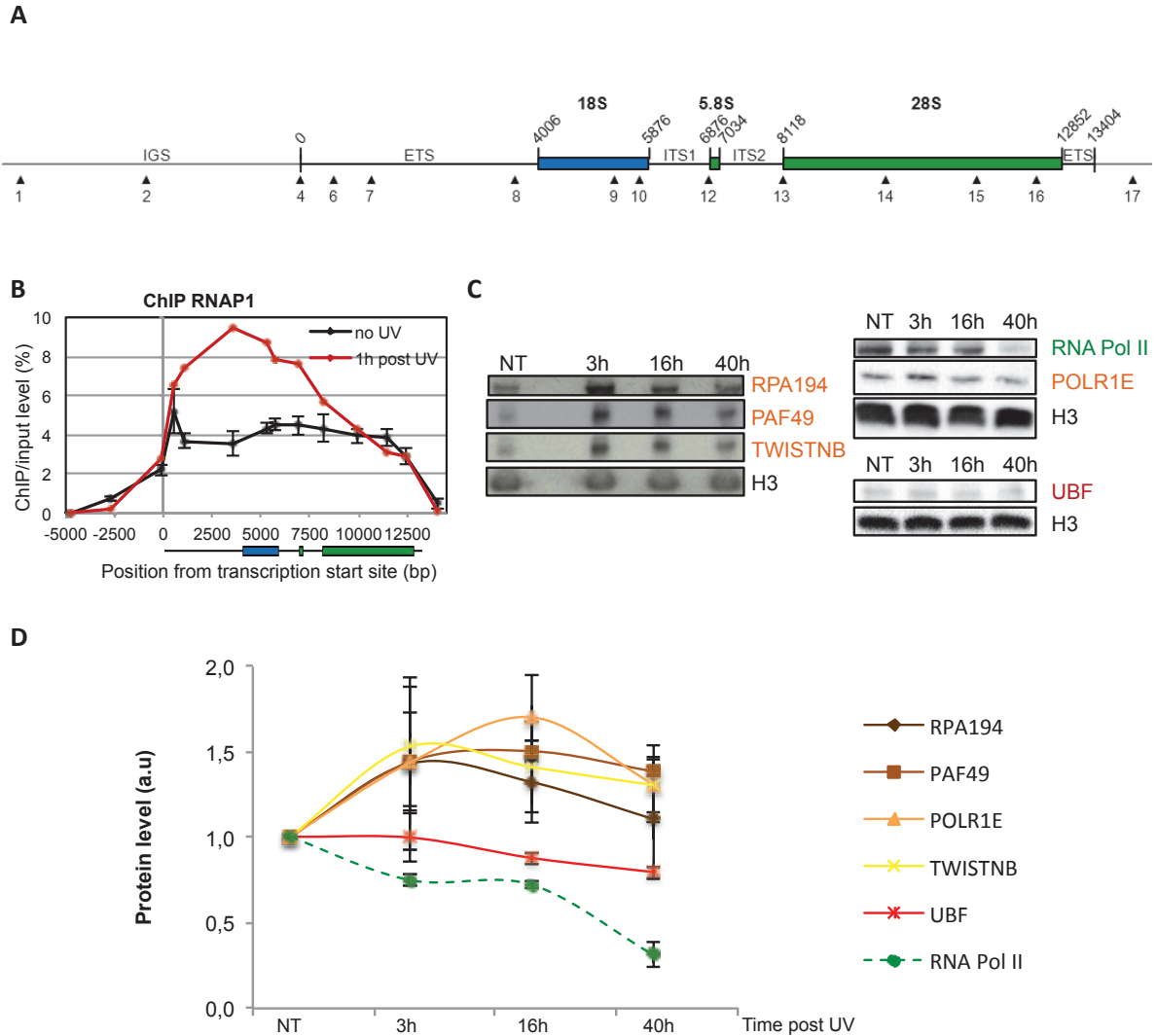
## **AUTHOR CONTRIBUTIONS**

GGM, POM and LD designed the experiments. LD, EC, JN, LMD, CC and SZ performed the experiments and analysed the data. POM assisted with the microscopy imaging and analysis. GGM and LD wrote the paper.

## **CONFLICTS OF INTEREST**

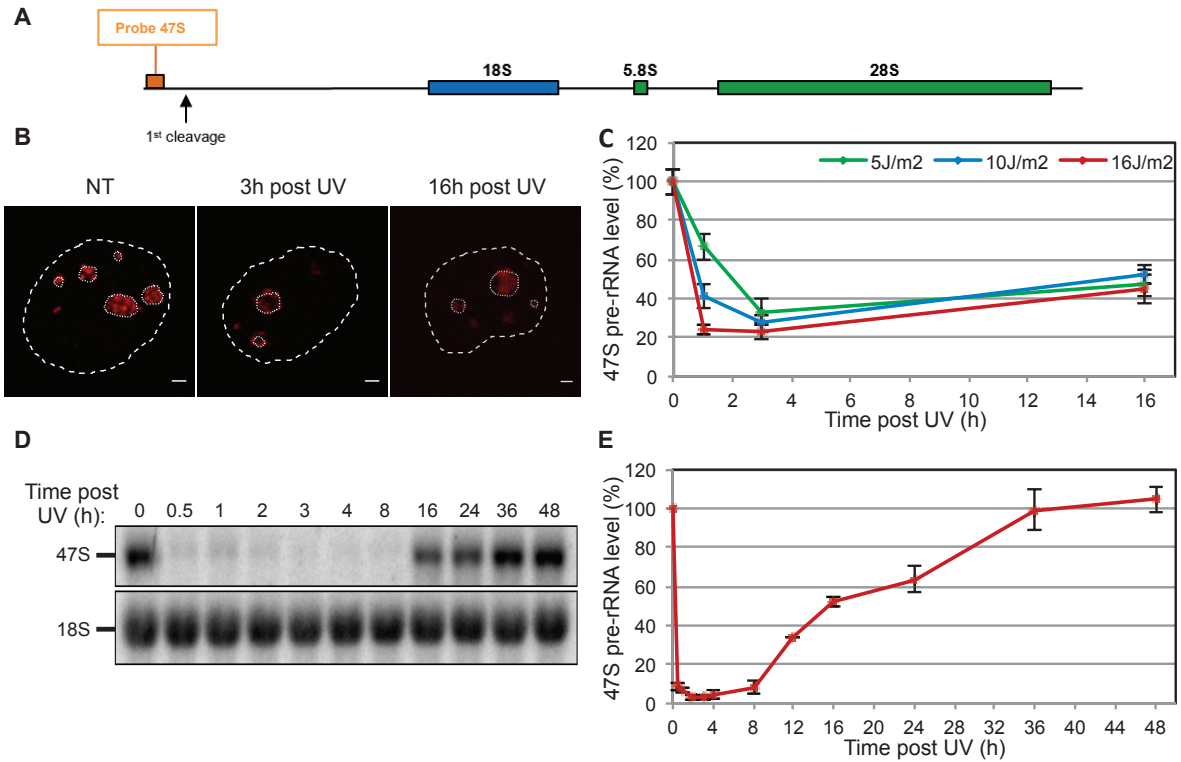
The authors disclose no potential conflict of interest.

## **FIGURES**



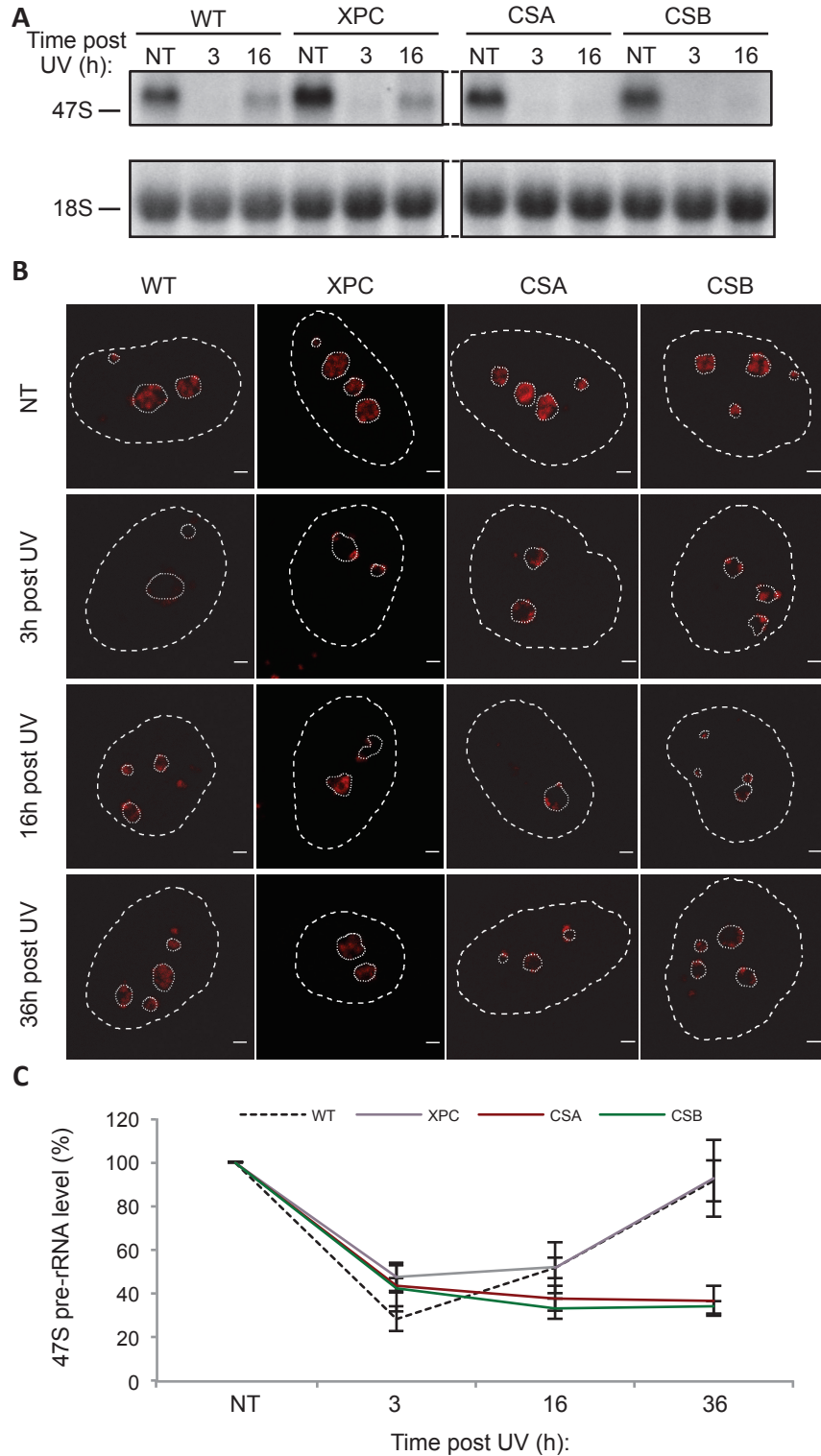
**Figure 1. After UV irradiation, RNAP1 is retained on the rDNAs without being degraded**

**A)** Scheme of the murine ribosomal DNA unit. Positions of the primers used for the ChIP qPCR assay are notified with arrowheads and numbers. IGS: intergenic spacer ; ETS : External Transcribed Spacer ; ITS : Internal Transcribed Spacer. **B)** ChIP-qPCR results showing the binding profil of RNAP1 on ribosomal DNA in murine embryonic stem cells, not treated or after UV-C exposure (1h, 16J/m<sup>2</sup>). The y-axis depicts the ChIP/Input ratio minus background (Mock/Input ratio) and error bars represent the SEM. **C)** Western blot of RNAP1 subunits (blue) on chromatin extracts from WT human fibroblast at different times after UV exposure. The proteins H3 and RNA Pol II were respectively used as loading and positive control. **D)** Quantification of western blots of RNAP1 subunits (from brown to yellow), UBF (red) and RNA Pol II (PhosphoSer2; green) on chromatin extracts from WT cells. Error bar show the SEM of three biological replicates.



**Figure 2. UV-irradiation blocks RNAP1 transcription**

**A)** Schematic representation of rDNA unit and localisation of 47S pre-rRNA probe. **B)** Confocal images of RNA FISH experiment showing 47S pre-rRNA level in wild-type cell. Cells were irradiated or not (NT) with 16J/m<sup>2</sup> of UV-C and fixation was performed 3h or 36h after UV exposure. Scale bar corresponds to 2 $\mu$ m. **C)** Quantification of 47S pre-rRNA level after different doses of UV-C (5 ; 10 and 16 J/m<sup>2</sup>). **D)** Northern blot showing overtime the 47S pre-rRNA level after UV-C irradiation. The amount of 18S rRNA is used as control. **E)** Northern blots quantification of 47S pre-rRNA level overtime after UV-C exposure. Error bars show the SEM of 3 independent experiments.

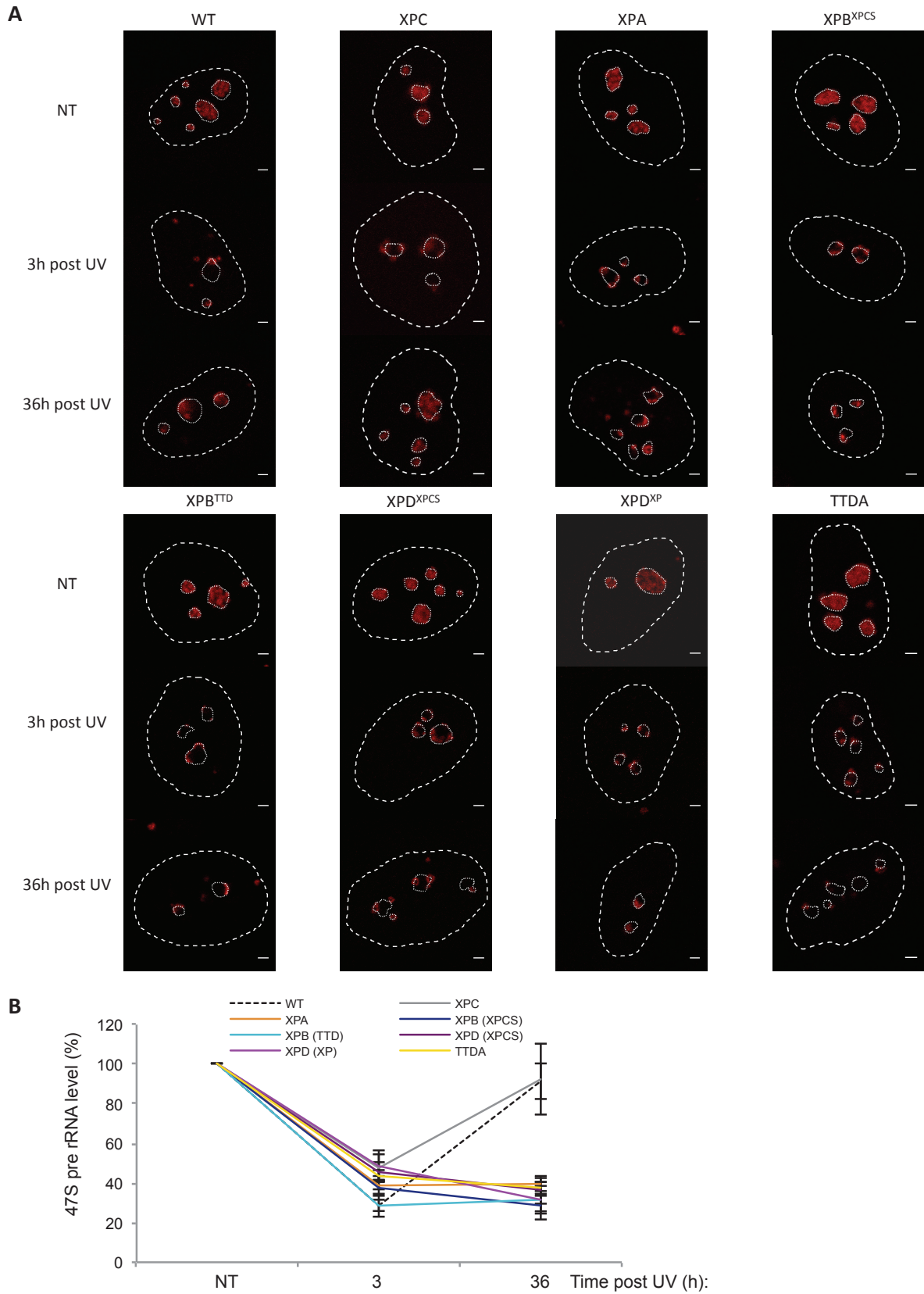


**Figure 3. Repair of the transcribed rDNA is performed by TC-NER**

**A)** Northern blot using a probe targeting the 47S pre-rRNA in WT, XPC, CSA and CSB cells was performed at different time after UV-C irradiation. 18S was used as control. **B)** Confocal images of the 47S pre-rRNA level. RNA FISH was performed using a 47S-Cy5 probe on WT, XPC (GG-NER deficient), CSA and CSB (TC-NER deficient) cells. Cells were irradiated or not (NT) at 16 J/m<sup>2</sup> and fixed 3h, 16h or 36h after the UV-C exposure. Nuclei and nucleoli are delimited by dashed and dotted lines



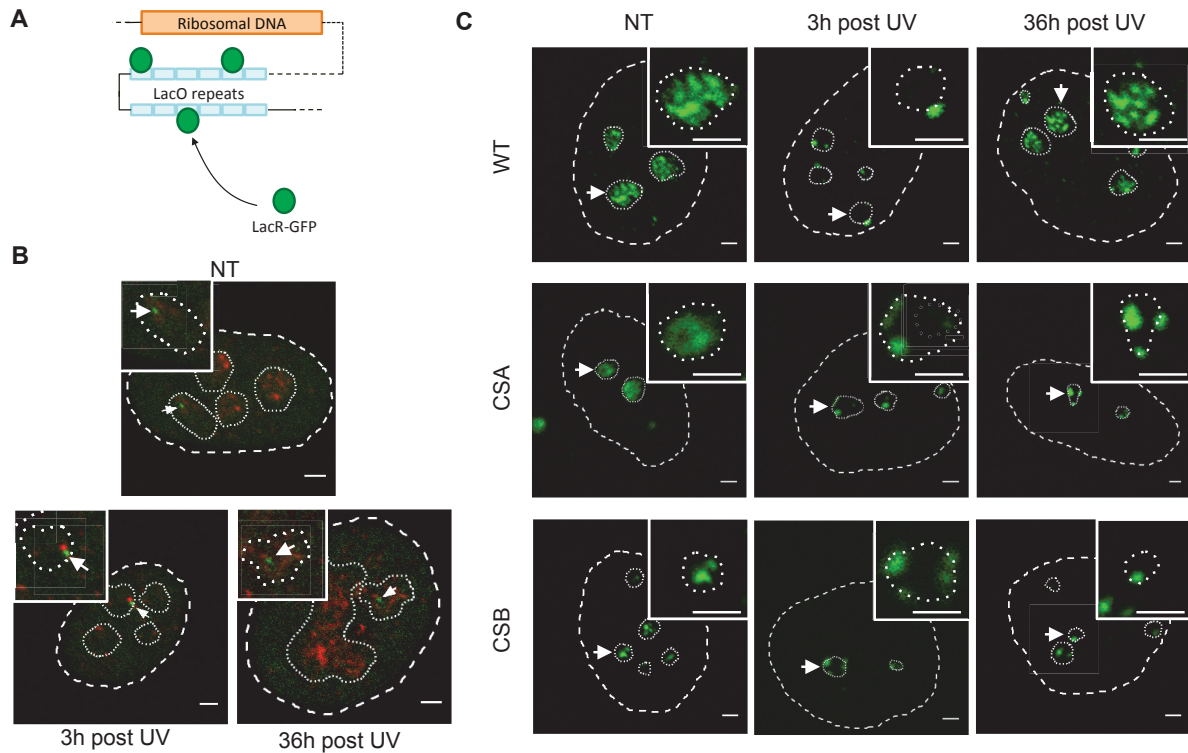
respectively. Scale bar corresponds to 2  $\mu\text{m}$ . **C)** Quantification of RNA FISH assay showing the 47S pre-rRNA level in WT, XPC, CSA and CSB cells. Error bars represent the SEM of 3 independent experiments.



**Figure 4. The complete TC-NER machinery is involved in the repair of rDNAs**

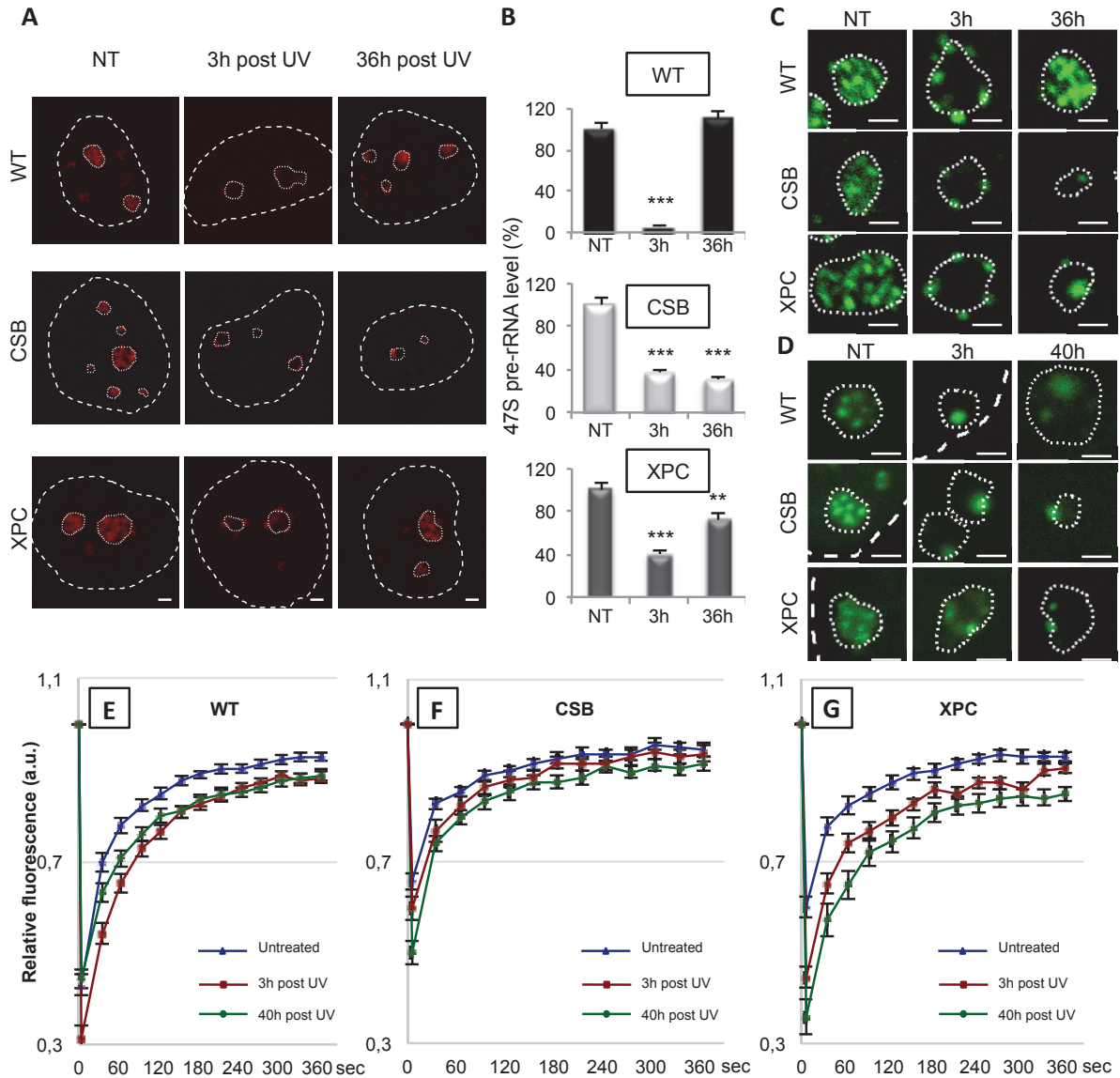
**A)** Confocal images of RNA FISH experiment performed on WT, XPC (GG-NER deficient), XPA, XPB<sup>XPCS</sup>, XPB<sup>TTD</sup>, XPD<sup>XPCS</sup>, XPD<sup>XP</sup> and TTDA (GG and TC-NER deficient) cells and showing the 47S pre-rRNA level.

Cells were irradiated or not (NT) at  $16 \text{ J/m}^2$  and fixed 3h or 36h after the UV exposure. Nuclei and nucleoli are delimited by dashed and dotted lines respectively. Scale bar corresponds to  $2 \mu\text{m}$ . **B)** Quantification of RNA FISH assay representing the 47S pre-rRNA level in WT, XPC, XPA,  $\text{XPB}^{\text{XPCS}}$ ,  $\text{XPB}^{\text{TTD}}$ ,  $\text{XPD}^{\text{XPCS}}$ ,  $\text{XPD}^{\text{XP}}$  and TTDA cells. Error bars represent the SEM of 3 independent experiments.



**Figure 5. Displacement of RNAP1 and rDNA during TC-NER**

**A)** Schematic representation of the LacO/LacR system used to visualize the ribosomal DNA. Several Lac Operon genes were integrated downstream of the rDNA of the cells, which were also transfected with a plasmid carrying the Lac Repressor gene tagged with the GFP coding sequence. **B)** Confocal images of immunofluorescence stained against RNAP1 (red) in LacR-GFP (green) expressing cells. Cells were exposed or not (NT) to  $16 \text{ J/m}^2$  of UV-C and fixation was performed 3h or 36h after UV exposure. Nuclei and nucleoli are indicated by dashed and dotted lines. White arrows indicate the LacO array (GFP green dot). Insets zoom into the nucleoli of interest. Scale bar corresponds to  $2 \mu\text{m}$ . **C)** Confocal images of immunofluorescence staining against RNAP1 (green) performed on WT, CSA and CSB cells. Cells were exposed or not (NT) to  $16 \text{ J/m}^2$  of UV-C and fixation was performed 3h or 36h after UV exposure. Nuclei and nucleoli are indicated by dashed and dotted lines respectively. Insets zoom into the nucleoli indicated with arrows. Scale bars correspond to  $2 \mu\text{m}$ .



**Figure 6. Unrepaired UV-lesions keep the RNAP1 at the periphery of the nucleolus despite the restart of transcription**

**A)** Confocal images of RNA FISH experiment showing the 47S pre-rRNA level in WT, CSB and XPC cells. Fixation was performed 3h or 36h after UV-C exposure. Untreated cells (NT) were used as control. Nuclei and nucleoli are delimited by dashed and dotted lines respectively. Scale bar corresponds to 2 $\mu$ m. **B)** Quantification overtime of the 47S pre-rRNA level in WT, CSB and XPC cells. Error bars represent the SEM of 3 independent experiments. \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . **C-D)** Localization, overtime after UV-C exposure, of endogenous (C) and GFP-tagged RNAP1 (D) in the nucleolus of living WT, CBS and XPC cells. **E-F-G)** FRAP curves of RNA Pol I-GFP protein stably expressed in WT, CSB and XPC cells respectively, untreated (blue) or treated with UV-C (16 J/m<sup>2</sup>), 3h before (red) or 40h before (green) photobleaching.

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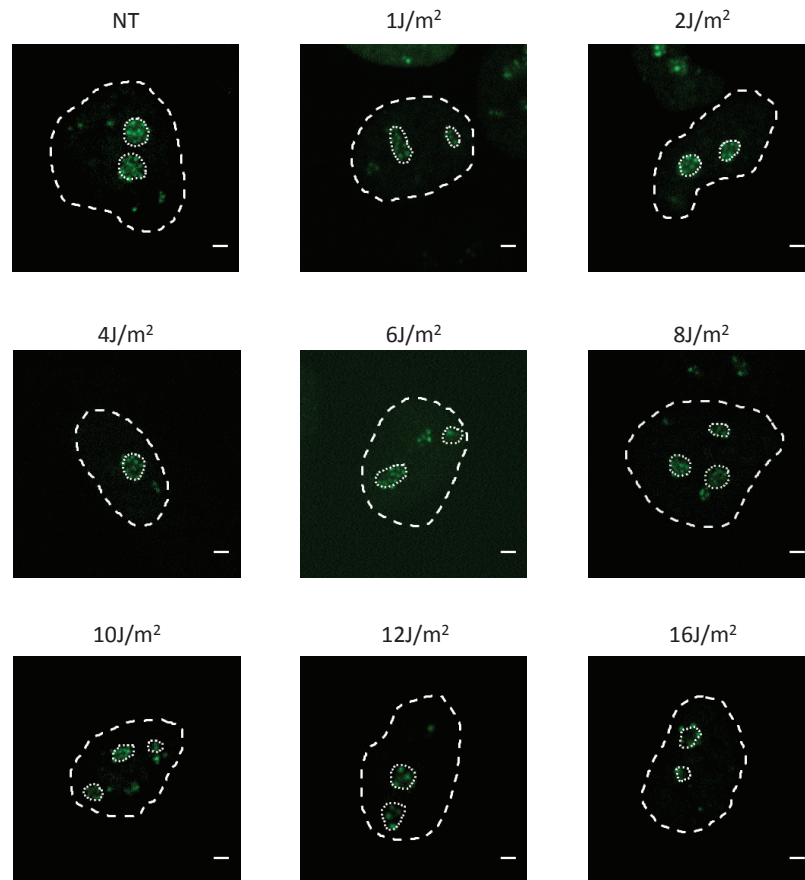
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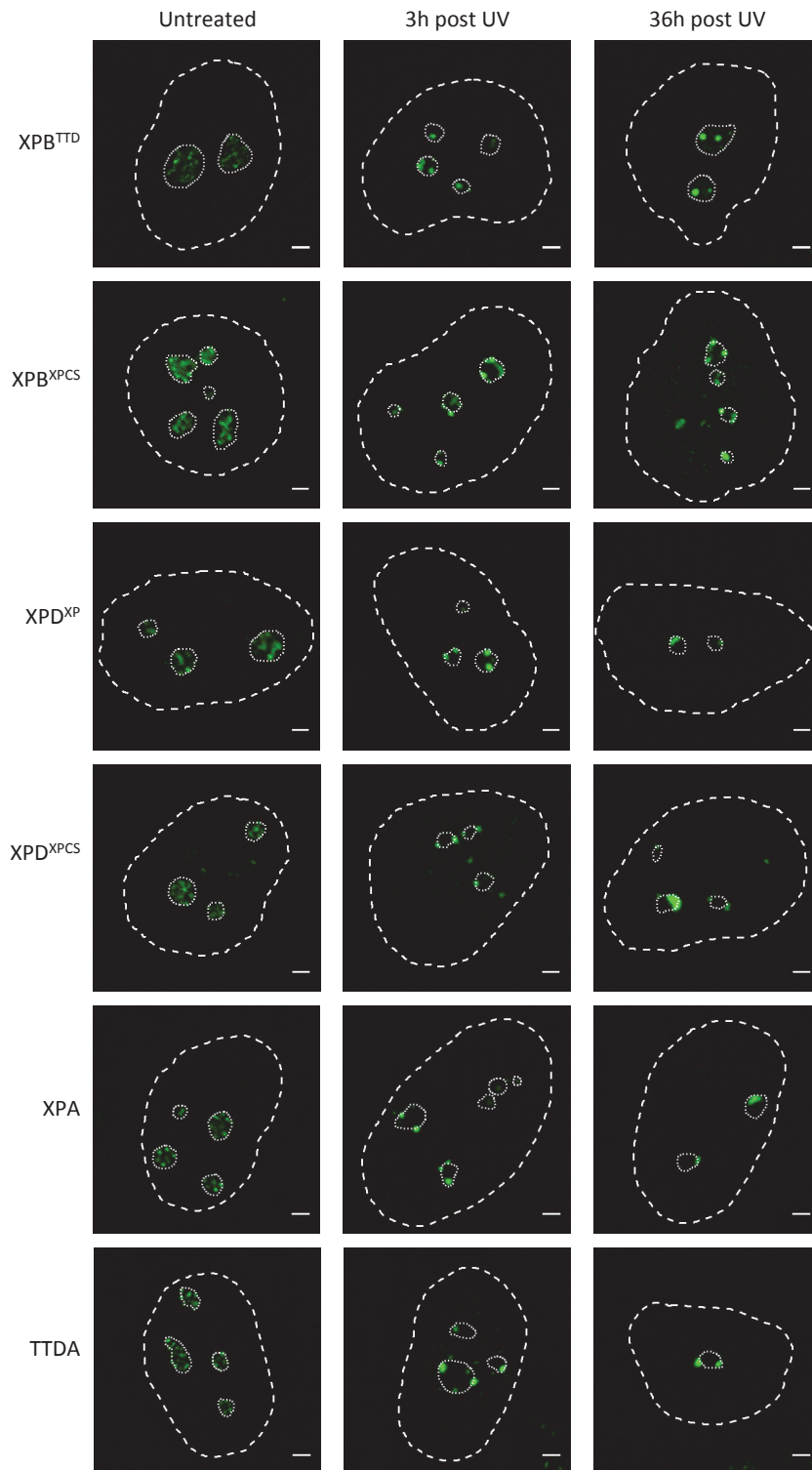
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## SUPPLEMENTAL DATA



**Figure S1. RNAP1 relocation at the periphery of the nucleolus is UV-C dose dependant**

*Confocal images of human fibroblasts stably expressing RNAP1-GFP. Cells were exposed or not (NT) to different doses of UV-C (1; 2; 4; 6; 8; 10; 12 and 16J/m<sup>2</sup>) and fixation was performed 3h after irradiation. Nuclei and nucleoli are indicated by dashed and dotted lines respectively. Scale bar 2 $\mu$ m.*



**Figure S2. RNAP1 location in NER deficient cells upon UV**

Confocal images of immunofluorescence staining against RNAP1 (green) performed on NER deficient cells. Cells were exposed (or not) to  $16\text{J/m}^2$  of UV-C and fixation was performed 3h or 36h after UV exposure. Nuclei and nucleoli are indicated by dashed and dotted lines respectively. Scale bar  $2\mu\text{m}$ .



# PART II





# **Nuclear Beta-Actin and Nuclear Myosin I are required for RNAP1 Return into the Nucleolus After Repair of UV-Damage Ribosomal DNA**

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## **Keywords:**

Beta-Actin; Nuclear Myosin I; RNAP1 relocation, rDNA Transcription, UV lesions, Nucleolus,  
Nucleotide Excision Repair

## ABSTRACT

The nucleolus is a specific nuclear structure in which the ribosomal genes are transcribed by the RNA polymerase I (RNAP1). Many nuclear proteins are excluded from the nucleolus and because of this some nucleolar processes, such as replication and DNA repair, cannot occur inside this structure. In order to be replicated and repaired rDNA needs to be displaced at the nucleolar periphery. To the best of our knowledge no molecular mechanism has been proposed for rDNA displacement. During DNA repair of UV lesions, the complex RNAP1/rDNA is displaced at the periphery of the nucleolus and returns within the nucleolus after DNA repair completion. Our results demonstrate that both nuclear  $\beta$ -actin and nuclear myosin I are implicated in RNAP1/rDNA movements after UV irradiation and specifically they control the step of re-entering the nucleolus when DNA repair is successfully completed.

## INTRODUCTION

In order to enable certain molecular mechanisms, the nucleolus and more generally the nucleus need to exhibit a certain dynamic. For example, during mitosis, the replicated genome is contained into condensed chromosomes that are initially spread into the whole nucleoplasm (Gurley et al., 1978). Then, microtubules drive these chromosomes to the equatorial plane where sister chromatids are separated and migrate to opposite sides of the mother cell which will undergo cytokinesis to give rise to two genetically identical daughter cells (Glotzer, 2001).

Aside from the mitotic vision of the nuclear dynamic, several studies have investigated nuclear motion during interphase. Even though chromosome movements were thought to be specific from mitosis, they have also been observed during interphase in different organisms such as yeast, *Drosophila* and mammals (Csink and Henikoff, 1998; Gunawardena and Rykowski, 2000; Heun et al., 2001; Walter et al., 2003; Wiesmeijer et al., 2008). In yeast and *Drosophila*, chromosome motion has been directly linked to the cell

cycle. Indeed, their mobility is more important during G1 phase than S phase (Csink and Henikoff, 1998; Gunawardena and Rykowski, 2000; Heun et al., 2001). However, in mammalian cells, chromosome displacements show a different cell-cycle dependence, e.g. only the early G1 phase present a higher mobility of the chromosome compared to the mid/late G1 phase and S phase (Walter et al., 2003; Wiesmeijer et al., 2008). Furthermore, different research groups have demonstrated that some parts of the genome regularly change their location according to their replicational or transcriptional status (Chuang et al., 2006; Croft et al., 1999; Ferreira et al., 1997; Finlan et al., 2008; Osborne et al., 2004; Taddei and Gasser, 2012). Indeed, early and late replicated origins are located within the nucleus and at the nuclear periphery, respectively (Chuang et al., 2006; Croft et al., 1999; Ferreira et al., 1997). Inactive genes are found at the nuclear periphery (Finlan et al., 2008) whereas active ones are located within transcription factories (Osborne et al., 2004) or nearby the nuclear pores (Taddei and Gasser, 2012).

Interestingly, nuclear  $\beta$ -actin (ACT $\beta$ ) and Nuclear Myosin I (NMI), two proteins belonging to different families of proteins firstly identified in the cytoplasm and involved in several cellular events such as cell migration, muscle contraction or organelle movement (De Lanerolle and Serebryanny, 2011; Pollard and Cooper, 2009), have been implicated in nuclear dynamics as well. Several studies have demonstrated that ACT $\beta$  and NMI are required for active chromosome locus movements in the nucleus during interphase (Bridger, 2011; Chuang et al., 2006; Mehta et al., 2010). Similarly, NMI is required during DNA repair to promote chromosome territories relocation (Kulashreshtha et al., 2016). Intriguingly, ACT $\beta$  and NMI are also involved in RNA Polymerase I (RNAP1) transcription (Fomproix and Percipalle, 2004; Philimonenko et al., 2004; Sarshad et al., 2013; Ye et al., 2008).

The nucleolus, a particular region of the nucleus, is considered as the ribosome factory. Indeed, this nuclear structure encloses ribosomal genes (rDNA) transcribed, by the RNAP1, into a pre-ribosomal RNA (pre-rRNA) that undergoes several processing steps in order to form, with different ribonucleoproteins, the ribosomal subunits (Henras et al., 2014). The large (60S) and the small (40S) ribosomal subunits are exported to the cytoplasm

in order to assemble into the mature ribosome (Boisvert et al., 2007). Moreover, the nucleolus is composed of different components, the Fibrillar Centre (FC) encompassed by the Dense Fibrillar Component (DFC) and the Granular Component (GC) (Sirri et al., 2008), in which all these molecular processes take place, e.g. rDNA transcription at the FC/DFC limit, early and late rRNA processing in the DFC and GC respectively (Boisvert et al., 2007; Grob and McStay, 2014).

Although some studies have identified some NER factors interacting with RNAP1 in the nucleolus (Assfalg et al., 2012; Bradsher et al., 2002; Iben et al., 2002; Koch et al., 2014), most of the repair proteins have not yet been found to localise in this nuclear structure. Because of this and due to the particular structure and function of the nucleolus, some nucleolar processes, such as replication and DNA repair (Daniel et al, manuscript submitted; Dimitrova, 2011; Franek et al., 2016; Larsen and Stucki, 2016; Sluis and McStay, 2015) cannot take place within this cellular structure. For this reason, it is necessary for the rDNA to be displaced outside the nucleolus to be replicated (Dimitrova, 2011) and repaired (Franek et al., 2016; Larsen and Stucki, 2016; Sluis and McStay, 2015).

One of the repair mechanisms available for cells is the Nucleotide Excision Repair (NER) process that corrects UV-lesions. NER consists of two different pathways: the Global-Genome Nucleotide Excision Repair (GG-NER) and the Transcription-Coupled Nucleotide Excision Repair (TC-NER). GG-NER detects lesions on the whole genome while TC-NER detects lesions in the actively transcribed genes (Spivak, 2015). During GG-NER, detection of the lesion happens through the recruitment of the XPC protein (together with its partners RAD23B and CETN2) at the site of damage, whereas TC-NER is triggered by RNA Polymerase II (RNAP2) stalling at UV lesions inducing the recruitment of the CSA and CSB proteins (Marteijn et al., 2014).

During rDNA repair, e.g. Double Strand Break (DSB) repair and more recently NER (Daniel et al, manuscript submitted), as well as rDNA replication (Dimitrova, 2011), RNAP1 and rDNA have been described to be displaced at the nucleolar periphery (Dimitrova, 2011; Franek et al., 2016; Larsen and Stucki, 2016; Moore et al., 2011; Sluis and McStay, 2015; Daniel et al, submitted). Although the displacement (two distinct steps: eviction and re-

entry phases) of the RNAP1/rDNA complex at the nucleolar periphery has been described during replication and repair, no studies have been conducted up to now, to unravel the molecular mechanism underlying this phenomenon. This ascertainment together with all the studies revealing nuclear functions for both nuclear actin and myosin I (see above), lead us to investigate the role of NMI and ACT $\beta$  during RNPA1/rDNA displacement to the nucleolar periphery after UV exposure.

Our results show that ACT $\beta$  and NMI are required for the second step of the RNAP1/rDNA displacement, e.g. its re-entry into the nucleolus, after DNA repair completion. Our study identifies new components of the machinery responsible for the RNAP1 relocation during UV induced lesion repair and more generally responsible for DNA movements within the nucleus.

## **MATERIALS & METHODS**

### **Cell culture and treatments**

The cells used in this study were: wild type SV40-immortalized human fibroblasts (MRC5), GGR deficient SV40-immortalized human fibroblast: XPC (XP4PA) and TCR deficient SV40-immortalized human fibroblast CSB (CS1AN). Human fibroblasts were cultured in a 1:1 mixture of Ham's F10 and DMEM (Lonza) supplemented with 1% antibiotics (penicillin and streptomycin; Lonza) and 10% foetal bovine serum (Gibco) and incubated at 37°C with 20% O<sub>2</sub> and 5% CO<sub>2</sub>.

HT-1080 cells (Rasheed et al., 1974) stably expressing an adapted Lac Operator/Lac Repressor (LacO/LacR) system (selected using BlasticidinS and Hygromycin, 5 $\mu$ g/ml and 100 $\mu$ g/ml respectively), were used to detect the rDNA as previously described (Chubb et al., 2002; Robinett et al., 1996). These cells were cultured in DMEM (Lonza), supplemented with 1% antibiotics (penicillin and streptomycin; Lonza) and 10% foetal bovine serum (Gibco), and at 37°C with 3% O<sub>2</sub> and 5% CO<sub>2</sub>.

DNA damage was inflicted by UV-C light (254nm, 6 Watt lamp). Cells were globally irradiated with a  $16\text{J}/\text{m}^2$  dose of UV-C or locally irradiated with a  $100\text{J}/\text{m}^2$  dose of UV-C through a filter ( $5\mu\text{m}$  of diameter). Experiments were performed at different time points after UV exposure (3h, 16h and 40h post UV). Mock-irradiated cells (untreated) were used as control.

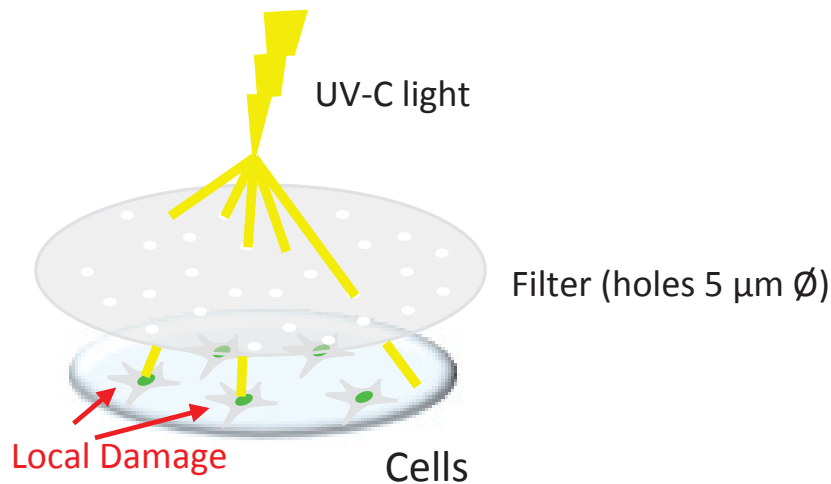


Diagram of local UV-irradiation through a filter

A calcium free medium was used in some experiments. The composition of this medium is the following: 1:1 mixture of Ham's F10 and Calcium-free DMEM (21 068-028; Gibco) supplemented with 1% antibiotics (penicillin and streptomycin; Lonza) and 10% chelated foetal bovine serum. Chelex chelating ion exchange resin (BioRad) was used to obtain the chelated foetal bovine serum

Cisplatin (Cis-Platinum (II) Diamine Dichloride; P4394; Sigma) was used at  $20\mu\text{g}/\text{ml}$  to induce lesions that specifically trigger the NER mechanism. Cells were incubated with Cisplatin for 3h or 6h, and allowed to recover up to 16h after the drug removal.

Cells were incubated with Cordycepin ( $50\mu\text{g}/\text{ml}$ ) for 1h before fixation performed as described in the Immunofluorescence (IF) paragraph of this Materials & Methods section.

### **Transfection of small interfering RNAs (siRNAs) and UV-irradiation**

On day 0, 100 000 cells were plated in a 6-wells plate. The first and second transfections were performed on day 1 and day 2, using Lipofectamine® RNAiMAX reagent (Invitrogen; 13778150). The transfections were carried out as recommended by the manufacturer. Then, on day 3 (40h time point), on day 4 (16h time point) or on day 5 (3h time point), cells were UV-irradiated as mentioned above. Finally, cells were fixed on day 5 according to protocols described in the RNA FISH and IF paragraphs of this Materials & Methods section. SiRNA efficiency was confirmed by western blot. The characteristics of the siRNAs are described in Table 1.

siRNA	Concentration	Full name or sequence	Reference
siMock	10nM	SMARTpool: ON-TARGETplus Non-targeting siRNA #2	Dharmacon D-001810-02-05
siACT $\beta$	10nM	SMARTpool: ON-TARGETplus ACTB siRNA	Dharmacon L-003451-00-0005
siCETN 2	5nM	AAGCUCUUUGAUGAUGAUGAA / UUCAUCAUCAUCAAGAGCUU	Sigma
siNMI	10nM	AACCCGUCCAGUAUUUCAACA	Sigma
siXPF	10nM	Human ERCC4	Thermoscientific M-01994600

Table 1: small-interfering RNA characteristics

## Whole cell extracts

Cells were collected using trypsin and spinned down 10 min at 1400 rpm. Firstly, cell pellet was washed with PBS and spund down 10 min at 1400 rpm. Secondly, cell pellet was incubated with Lysis buffer (ProteoJET™ Mammalian Cell Lysis Reagent; #K0301; Fermentas) complemented with the Protease Inhibitor Cocktail (Sigma Aldrich; 05056489001), for 10 min at room temperature on a shaker (500rpm). Finally, samples were spund down at 16000g for 15 min and supernatant was freezed at -80°C.

## Western blot

Protein concentration was determined by using the Bradford method. Samples were diluted with 2X Laemmli buffer, heated at 95°C (1x30 min, spin down, 1x 25 min, spin down) and loaded on a SDS-PAGE gel. Proteins were separated on 8% and 14% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane (PVDF, 0.45 $\mu$ m Millipore). The membrane was blocked in 5% milk PBS 0.1% Tween (PBS-T) and incubated for 1.5h with the



following primary antibodies in milk PBS-T. The loading was controlled with the anti-Histone3 antibody. Subsequently, membrane was washed with PBS-T (3x 10min) and incubated with the secondary antibody in milk PBS-T. After the same washing procedure, protein bands were visualised via chemiluminescence (ECL Enhanced Chemo Luminescence; Pierce ECL Western Blotting Substrate) using the ChemiDoc MP system (BioRad).

## Western blot antibodies

- Primary antibodies:

Mouse anti-RPA194(C1) antibody (sc-48385; Santa Cruz) 1/500; Rabbit anti-Nuclear Myosin I antibody (M3567; Sigma) 1/1000; Mouse anti- $\alpha$ -Tubulin antibody (T5168; Sigma) 1/50000; Mouse anti- $\beta$ -Actin antibody (A5316; Sigma) 1/10000; Mouse anti-XPF Ab-1 (219) (MS-1381-T1; Neomarkers) 1/500; Rabbit anti-Centrin2 antibody (ABE480; Millipore) 1/500.

- Secondary antibodies:

Goat anti-mouse IgG HRP conjugate (170-6516; Biorad) 1/5000, Goat anti-rabbit IgG HRP conjugate (170-6515; Biorad) 1/5000.

## RNA FISH

Cells were grown on 12 mm coverslips, washed with warm (37°C) PBS and fixed with 4% paraformaldehyde for 15min at 37°C. Coverslips were washed twice with PBS. Cells were permeabilized by washing with PBS 0.4% Triton X-100 for 7min at 4°C. Cells were washed rapidly with PBS before incubating them with pre-hybridization buffer (2x SSPE and 15% formamide) (20x SSPE, [pH 8.0]: 3M NaCl, 157mM NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O and 25mM EDTA) for at least 30min. 1.5 $\mu$ l of probe (10ng/ml) was diluted in 30 $\mu$ l of hybridization mix (2x SSPE, 15% formamide, 10% dextran sulphate, 0.5 mg/ml tRNA) and heated to 90°C for 1 min. Hybridization of the probe was conducted overnight at 37°C in a humidified environment. Subsequently, cells were washed twice for 20min with pre-hybridization buffer, then once for 20min with 1x SSPE and finally mounted with Vectashield (Vector Laboratories) and kept at -20°C. The probe sequence (5' to 3') is: Cy5-AGACGAGAACGCCTGACACGCACGGCAC. At

least three biological replicates were obtained and at least 30 cells were imaged for each condition of each cell line.

### **Immunofluorescence assay**

Cells were grown on 24mm coverslips, washed with warm (37°C) PBS and fixed with 2% paraformaldehyde for 15 min at 37°C. Cells were permeabilized with PBS 0.1% Triton X-100 (3x short + 2x 10 min). Blocking of non-specific signal was performed with PBS<sup>+</sup> (PBS; 0.5%BSA; 0.15% glycine) for at least 30 min. Then, coverslips were incubated with 100µl of primary antibody mix (Mouse anti-RPA194; 1/500 in PBS<sup>+</sup>; sc-48385) for 2h at room temperature in a moist chamber, washed with PBS (3x short; 2x 10min), quickly washed with PBS<sup>+</sup> before incubating with 100µl of secondary antibody mix (Goat anti-mouse Alexa Fluor® 488 and Goat anti-mouse Alexa Fluor® 594; 1/400 in PBS<sup>+</sup>; A-11001 and A-11005 Invitrogen respectively) for 1h at room temperature in a moist chamber. After the same washing procedure, coverslips were finally mounted using Vectashield with DAPI (Vector Laboratories) and kept at -20°C. At least three biological replicates were performed and at least 15 cells were imaged for each condition of each cell line.

### **Unscheduled DNA Synthesis (UDS)**

MRC5 cells were grown on 18mm coverslips. SiRNA transfections were performed 48h and 24h before UDS assay. Transfected cells were locally UV-irradiated (100 J/m<sup>2</sup>) through a 5-µm-pore polycarbonate membrane filter (Moné et al., 2001) and UDS experiment was performed using the Click-iT EdU imaging kit (Thermo Fisher) according to the manufacturer's recommendations.

### **Fluorescent imaging and analysis**

Imaging has been performed on a Zeiss LSM 780 NLO confocal laser-scanning microscope (Zeiss), using a 60x/1.4 oil objective. Images were analysed with ImageJ software. For all images of this study, nuclei and nucleoli were delimited with dashed and dotted line respectively, using DAPI staining or transmitted light.

### **Statistical analysis**

Error bars represent the Standard Error of the Mean (SEM) of the biological replicates. Student's t-test was used to assess whether the mean values of the replicates were statistically significant, assuming equal variance. A P-value of 0.05 or less was considered as significant. (\*  $p < 0.05$  ; \*\*  $p < 0.01$  ; \*\*\*  $p < 0.001$ ).

## RESULTS

### UV-independent relocation of RNAP1 to the nucleolar periphery

We previously demonstrated that RNAP1/rDNA was relocated at the nucleolar periphery after UV irradiation (Daniel et al, manuscript submitted). In order to verify whether the relocation of RNAP1/rDNA was either induced by UV or more generally lesion-dependent, we treated the cells with another genotoxic agent. For this purpose, we treated cells with cisplatin. Cisplatin induces bulky lesions (Eastman, 1987; Kelland, 1993; Pinto and Lippard, 1985; Siddik, 2003) and similarly to UV-lesions induces the NER DNA repair system (Enoiu et al., 2012; Reed, 1998; Rosell et al., 2003). An advantage of the cisplatin treatment is that its effect is reversible when removed from the medium.

In order to mimic UV-lesions optimal conditions for RNAP1 transcription inhibition, we tested different cisplatin incubation time (3h and 6h) and monitored the RNAP1 transcription level through RNA FISH targeting the 47S pre-rRNA. We demonstrated that already at 3h of treatment with cisplatin RNAP1 transcription was significantly decreased (Figure 1A). However, 6h of cisplatin treatment showed a more important reduction of RNAP1 transcription that could be compared with levels observed 3h after UV irradiation (Daniel et al, manuscript submitted). Because of this result, hereafter, we used a cisplatin incubation time of 6 hours.

We performed RNA FISH and immunofluorescence (IF) assay after cisplatin treatment on wild type, GG-NER (XPC) and TC-NER (CSB) deficient cell lines to monitor the RNAP1 transcription level and the position of RNAP1 within the nucleolus, respectively. In wild type cells treated for 6h with cisplatin, we observed a decrease of the 47S pre-rRNA level and the

relocation of RNAP1 at the nucleolar periphery (Figures 1B-C). The RNAP1 transcription restarted progressively 16h after drug removal, whereas the RNAP1 remained at the periphery of the nucleolus. In the XPC (GG-NER deficient) cisplatin-treated cells, the situation was similar to the one observed in the wild type cells, except that the transcription resumption appeared slower. In CSB cells (TC-NER deficient), RNAP1 transcription decreased after a 6h-treatment with cisplatin, and RNAP1 relocated to the nucleolar periphery. However, 16h after the drug removal RNAP1 remained at the periphery and its transcription level was still low, as observed for UV-lesions (Daniel et al, manuscript submitted). These results confirmed that RNAP1 relocation at the nucleolar periphery is dependent on the presence of lesions rather than being a UV-dependent process.

### **Transcription-dependent relocation of RNAP1 and rDNA**

We demonstrated that the presence of UV-induced (Daniel et al, manuscript submitted) or cisplatin-induced (see above) lesions on rDNA triggered the relocation of RNAP1 at the nucleolar periphery. However, UV irradiation as well as cisplatin incubation induced DNA damage but also inhibited transcription. Therefore, we decided to investigate whether RNAP1 and rDNA relocation were affected only by transcription inhibition, rather than lesions and lesion-induced transcription inhibition. In order to investigate this point, we used cordycepin, an analogue of adenosine, which incorporates into DNA and inhibits transcription by causing elongation termination (Dundr, 2002; Zeevi et al., 1982).

In order to determine the location of the rDNA during transcription inhibition we used a LacO/LacR-GFP reporter system to visualise the rDNA by microscopy in cells. Several LacO genes were inserted downstream from the ribosomal transcription unit. These cells HT80 were subsequently transfected with a plasmid carrying the LacR-GFP reporting system. The LacR-GFP, when expressed, targets the LacO repeats downstream from the ribosomal genes allowing the visualisation of the rDNA (Figure 2A). To detect both the rDNA and the RNAP1, we performed IF against the biggest subunit of RNAP1 (RPA194) in these cells (Figure 2B). We observed that in the untreated cells, the ribosomal DNA (green) and the RNAP1 (red) were located within the nucleolus. However, 1h after cordycepin incubation, RNAP1 transcription is fully inhibited (Figure 2C) and the rDNA, together with the RNAP1,

relocated to the periphery of the nucleoli. Therefore, transcription inhibition alone seemed to be sufficient to induce the RNAP1 and rDNA relocation at the nucleolar periphery.

### **Calcium-independent and centrin2-dependent RNAP1 relocation**

We demonstrated that the RNAP1/rDNA relocation (eviction phase) was either caused by transcription blockage (see above) or the presence of lesions (Daniel *et al*, manuscript submitted). We have also shown in a previous study that repair of all the lesions on rDNA, and hence the presence of a functional XPC protein, was necessary for RNAP1 return (re-entry phase) into the nucleolus (Daniel *et al*, manuscript submitted). One of the XPC partners, the protein Centrin2 (CETN2) belongs to a superfamily of Calmodulin (Schiebel and Bornens, 1995) proteins which, together with Calcium, modulate movements in cells. Therefore, we investigated the role of Centrin2 in RNAP1 displacement, alongside with the role of Calcium in this process.

We carried out IF experiments on wild type cells knocked-down for CETN2 (siCETN2) *via* small-interfering RNAs (siRNAs). A non-targeting siRNA (siMock) was used as control. Transfected cells were mock-irradiated (NT) or UV-irradiated at least 8h after the second siRNA transfection and fixed at the appropriate time after UV exposure (3h; 40h) (Figure 3A). SiRNA efficiency was confirmed by quantification of western blots (Figures S2A-B). We observed that in both siMock and siCETN2 knocked-down cells, RNAP1 relocated to the nucleolar periphery 3h after UV exposure. Strikingly, 40h after UV, RNAP1 remained at the nucleolar periphery in the siCETN2 knocked-down cells, whereas it returned into the nucleolus in the siMock-treated cells. In addition, we performed RNA FISH assays in the same conditions (Figures 3B & S2C). SiMock-treated cells displayed a decrease of RNAP1 transcription that restarted 40h after UV exposure (Figure 3B). Likewise WT cells, siCETN2-treated cells show a decrease of the 47S pre-rRNA level as well as a slower resumption of transcription 40h after UV (Figure 3B). In brief, siCETN2 knocked-down cells behave similarly to XPC cells (Daniel *et al*, manuscript submitted).

In parallel, we performed IF assays on wild type cells cultured in a medium with or without calcium (Ca<sup>++</sup>) and mock-irradiated or UV-irradiated (Figure 3A).

In presence or absence of  $\text{Ca}^{++}$ , in non-irradiated cells (NT), RNAP1 was located inside the nucleolus, whereas 3h after UV it was situated at the nucleolar periphery, demonstrating that  $\text{Ca}^{++}$  does not play a role in the displacement of the RNAP1 during DNA repair. However,  $\text{Ca}^{++}$  seems to play a role in RNAP1 relocation during transcription blockage because the only removal of  $\text{Ca}^{++}$  from the culture medium induced a decrease in ribosomal transcription but no relocation at the nucleolar periphery (Figures S1A & 3B) even though RNAP1 transcription blockage is known to send RNAP1 to the nucleolar periphery. This decrease was comparable with the reduction observed in UV irradiated cells (3h post-UV) (Figure 3B). RNAP1 transcription was not greatly inhibited when both UV and Ca-free medium were applied to the cells. These results suggest that there are two different mechanisms that regulate RNAP1 relocation at the nucleolar periphery. One depends on RNAP1 transcription blockage and is regulated by calcium; the other one depends on and is regulated by DNA lesions.

### **Nuclear actin and myosin required for RNAP1 return into the nucleolus**

We have demonstrated that RNAP1 relocation was dependent on XPC (Daniel *et al*, manuscript submitted) and the XPC-partner CETN2 but was independent of calcium. Because both XPC and CETN2 are involved in the repair of UV-lesions, we directly investigated two proteins that are involved with RNAP1 transcription, cell migration and organelle movements: ACT $\beta$  and NMI.

We carried out IF experiments on wild type cells knocked-down for NMI (siNMI) or ACT $\beta$  (siACT $\beta$ ) using siRNAs. A non-targeting siRNA (siMock) was used as control. Transfected cells were mock-irradiated (NT) or UV-irradiated at least 8h after the second siRNA transfection and fixed at the appropriate time after UV exposure (3h; 40h). SiRNA efficiency was confirmed by quantification of western blots (Figures S2A-B). In siMock-treated cells, RNAP1 behaved as previously described in wild type cells. Namely, RNAP1 relocated at the nucleolar periphery 3h after UV and returned into the nucleolus 40h after irradiation (Figure 4A). SiACT $\beta$  and siNMI transfection did not affect RNAP1 location without UV. As in siMock-treated cells, RNAP1 relocated to the periphery of the nucleolus in both siACT $\beta$  and siNMI knocked-down cells 3h after UV exposure. However, surprisingly, RNAP1

remained at the nucleolar periphery in 100 % of siACT $\beta$  knocked-down cells, 40h after irradiation. Similarly, but in a lesser proportion, RNAP1 remained at the nucleolar periphery in 70% of siNMI knocked-down cells 40h after UV irradiation (Figure S2C summarises RNAP1 location for each condition). To monitor RNAP1 activity in siACT $\beta$  or siNMI knocked-down cells, we performed RNA FISH labelling as previously described (Figures S2D & 4B). As expected, quantification of RNA FISH assays carried out in siMock-transfected cell showed that RNAP1 transcription decreased 3h after irradiation and restarted 40h after UV exposure (Figure 4B). siACT $\beta$  and siNMI knocked-down cells behaved as siMock-treated cells demonstrating that ACT $\beta$  and NMI are not involved in transcription inhibition but are essential to allow the re-entry of RNAP1 within the nucleolus after DNA repair completion.

In order to discard the hypothesis that both proteins could be involved in NER, we performed Unscheduled DNA Synthesis (UDS) assay in siACT $\beta$  or siNMI knocked-down cells. We observed an intense EdU signal (green) at the site of DNA damage ( $\gamma$ H2AX labelling in red) in siMock-treated cell (Figure 4C) and siACT $\beta$  and siNMI knocked-down cells, while siXPF knocked-down cells show no UDS signal. These results demonstrate that ACT $\beta$  and NMI are not involved in DNA repair (Figure 4D).

## DISCUSSION

The nucleolus is a specific nuclear region where takes place the ribosomal DNA (rDNA) transcription by RNA Polymerase I (RNAP1) (Goodfellow and Zomerdijk, 2012; Sirri et al., 2008). Although some NER factors have been described in the nucleolus, e.g. they interact with RNAP1, (Assfalg et al., 2012; Bradsher et al., 2002; Iben et al., 2002; Koch et al., 2014), most of them have not yet been located in this nuclear area. This point has for consequences the relocation of certain molecular mechanisms, such as rDNA replication and repair, at the nucleolar periphery (Dimitrova, 2011; Franek et al., 2016; Larsen and Stucki, 2016; Sluis and McStay, 2015).

Few studies have described RNAP1 relocation at the nucleolar periphery during repair, e.g. Double Strand Break (DSB) (Franek et al., 2016; Larsen and Stucki, 2016; Sluis and McStay, 2015) and recently NER (Daniel et al, manuscript submitted), and during rDNA replication (Dimitrova, 2011). Although this displacement have been described in different

cellular functions, no studies have been performed to investigate the molecular mechanism and identify the proteins involved in this process.

We have previously demonstrated (Daniel et al, manuscript submitted) that after UV irradiation, RNAP1/rDNA is displaced at the periphery of the nucleolus (eviction phase). After DNA repair completion the RNAP1/rDNA returns back within the nucleolus (re-entry phase), concomitantly with the restart of RNAP1 transcription. We also could demonstrate that the re-entry is strictly dependent on the DNA repair of UV lesions in the proximity of rDNA genes (Daniel et al, manuscript submitted). In XPC cells, UV lesions on untranscribed DNA are not repaired and, although transcription is fully recover, the RNAP1 remains at the nucleolar periphery.

In order to clarify some mechanistic aspects of this displacement, we have performed experiments that have brought to light that re-entry phase is not UV-dependent but more precisely lesion-dependent (Figure 1). By treating cells with cisplatin, we could induce the same RNAP1 displacement as observed in UV-irradiated cells. However, cisplatin treatment in WT cells as in XPC cells hindered RNAP1 re-entry while transcription was fully restored. This result can be explained by the fact that in WT cells not all cisplatin-adducts on DNA are repaired (Moggs et al., 1997), confirming that persistent lesions inhibits RNAP1 re-entry. Nevertheless, we could confirm that transcription inhibitors can induce the eviction of the RNAP1 from the nucleolus (Figure 2). To perform this experiment we used Cordycepin, an analogue of adenosine, that induced the premature RNA transcription termination (Horowitz et al., 1976; Penman et al., 1970; Siev et al., 1969). However, cordycepin also induced DNA lesions, e.g. DSBs (Lee et al., 2012), that have been described to trigger the displacement of RNAP1 to the nucleolar periphery (Moore et al., 2011; Sluis and McStay, 2015). Thus, we cannot attest that the displacement we observed in presence of cordycepin was only due to the transcription blockage or also to the presence of DNA damage.

In order to identify proteins that could play a role in the RNAP1 displacement during DNA repair we explored the possibility that one of the partners of XPC could be involved in this process. XPC exists in complex with centrin-2 (CETN2) (Liang et al., 2006), a protein belonging to the calmodulin family. Our results demonstrate that CETN2 is required for the



re-entry of RNAP1 into the nucleolus after DNA repair. Indeed, RNAP1 remained at the nucleolar periphery 40h after induction of UV lesions in absence of CETN2, while the RNAP1 transcription restarted (Figures 3C-D). CETN2 enhanced the GG-NER mechanism, but has not been demonstrated to be essential for this mechanism (Nishi et al., 2005; Renaud et al., 2011). Indeed, in the absence of CETN2, UV lesion removal is slower, but not absent as observed in XPC deficient cell lines (Venema et al., 1990). CETN2 cells, as XPC cells, are also TC-NER proficient and for this reason RNAP1 transcription restarts as in WT and XPC cells. However, similar to the XPC protein, CETN2 seemed to be necessary for RNAP1 re-entry after UV irradiation. Because CETN2 has a calmodulin function, we investigated the role of calcium in RNAP1 displacement. We have shown that the absence of calcium did not induce the RNAP1 relocation although the transcription dramatically decreased (Figures 3A-B). Thus, we concluded that two mechanisms could regulate RNAP1 relocation at the nucleolar periphery. One mechanism would be regulated by transcription and calcium, another one by DNA lesions.

Among the proteins that regulate cellular movements, two drew our attention because of their cellular function:  $\beta$ -actin (ACT $\beta$ ) and myosin. It is common knowledge that myosin proteins are part of the motor protein superfamily interacting with actin proteins. Both are implicated in different cellular events such as cell migration, muscle contraction or organelle movement (De Lanerolle and Serebryanny, 2011; Pollard and Cooper, 2009). Furthermore, ACT $\beta$  and Nuclear Myosin I (NMI) have been identified in the nucleus where they display several functions such as chromosome locus movements during interphase (Bridger, 2011; Chuang et al., 2006; Mehta et al., 2010) and RNAP1 transcription (Philimonenko et al., 2004; Ye et al., 2008). For these reasons, we focused our work on ACT $\beta$  and NMI.

Interestingly, we observed that absence of ACT $\beta$  completely prevented re-entry of RNAP1 into the nucleolus after UV exposure, while transcription resumption occurred normally (Figures 4A-B). NMI was not absolutely essential for RNAP1 re-entry since less than 30% of the cells showed a re-entry of RNAP1 into the nucleolus. However, the absence of NMI still hindered RNAP1 re-entry into the nucleolus in more than 70% of the cells, while

the transcription restarted (Figure S4). Thus, both proteins are involved in RNAP1 re-entry from the nucleolar periphery to the inside of the nucleolus. Several myosins have been described in the nucleus and the Nuclear Myosin V (NMV) has been implicated in RNAP1 transcription and also carries a calcium-binding domain (De Lanerolle and Serebryanny, 2011; Lodish et al., 2000). All these similarities between NMI and NMV could explain the 30% of the cells in which RNAP1 returns inside the nucleolus, e.g. NMI and NMV would have redundant functions in the RNAP1 re-entry phase. Moreover, ACT $\beta$  and NMI were not implicated in NER since UDS experiment has shown any difference between siMock, siACT $\beta$  or siNMI-treated cells (Figures 4C-D). Namely, RNAP1 retention observed in siACT $\beta$  and siNMI-treated cells 40h after UV irradiation cannot be due to the presence of unrepaired lesions since DNA repair is fully functional (no unrepaired lesions remaining on DNA).

For the first time, in human fibroblast, we demonstrated a calcium-dependent regulation of RNAP1 transcription (Figure 3D). Although, a calcium effect on RNAP2 transcription has been previously described in neurons and cardiomyocytes (West et al., 2001; Zarain-Herzberg et al., 2011), these studies demonstrated an activating effect of calcium on transcription of cardiac genes as well as the Brain-Derived Neurotrophic Factor (BDNF) gene. However, NMI, that is involved in RNAP1/rDNA displacement, has been previously implicated in RNAP1 transcription and some studies demonstrated that it possesses a calcium-binding regulatory domain (De Lanerolle and Serebryanny, 2011; Lodish et al., 2000). Thus, RNAP1 transcription inhibition that we observed in the absence of calcium could be due to the inactivation of the calcium-mediated activity of NMI.

A specific relocation of RNAP1/rDNA at the nucleolar periphery has been previously described during ribosomal DNA repair and replication (Daniel et al, manuscript submitted; Dimitrova, 2011; Franek et al., 2016; Larsen and Stucki, 2016). However, no studies have focused on this displacement from a mechanistic point of view. We demonstrated a role for ACT $\beta$  and NMI in this event. Both proteins are required for the re-entry of RNAP1 into the nucleolus after DNA damage repair.

The discovery of ACT $\beta$  and NMI involved in the re-entry phase of the RNAP1/rDNA displacement is the starting point for further studies that will disclose in details the full

molecular mechanism of nucleolar motions. Obviously, many proteins remain to be discovered, as well as the chromatin remodelling during this displacement and the modifications of the genomic environment of the rDNA during and after this displacement.

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## **AUTHOR CONTRIBUTIONS**

GGM and LD designed the experiments. LD and EC performed the experiments and analysed the data. POM assisted with the microscopy imaging and analysis. GGM and LD wrote the paper.

## **CONFLICTS OF INTEREST**

The authors disclose no potential conflict of interest.

## **FIGURES**

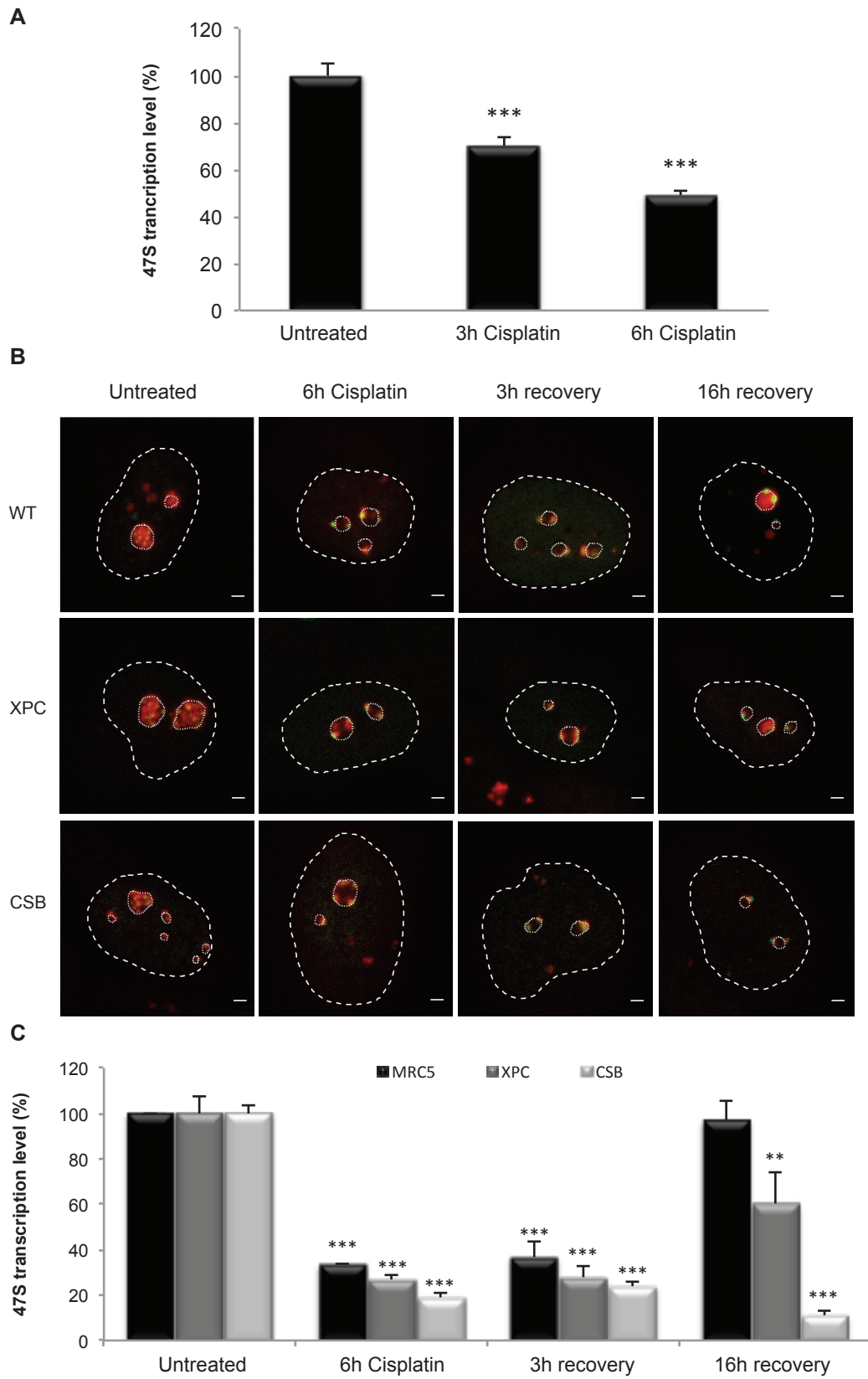
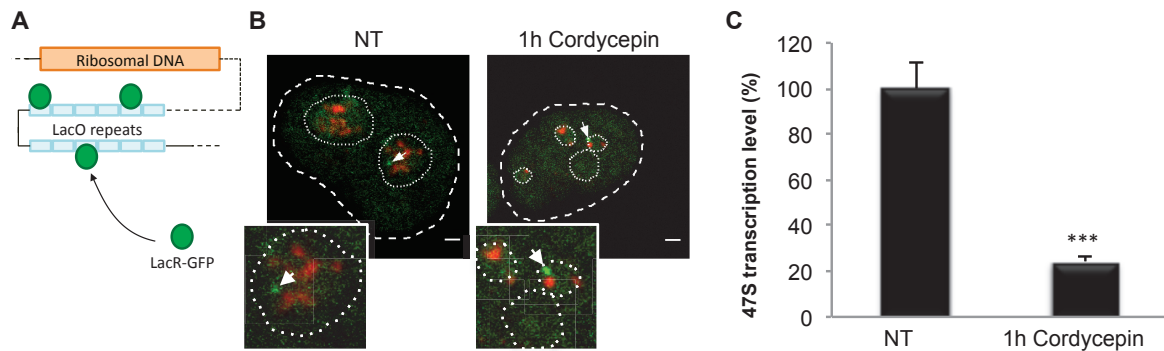


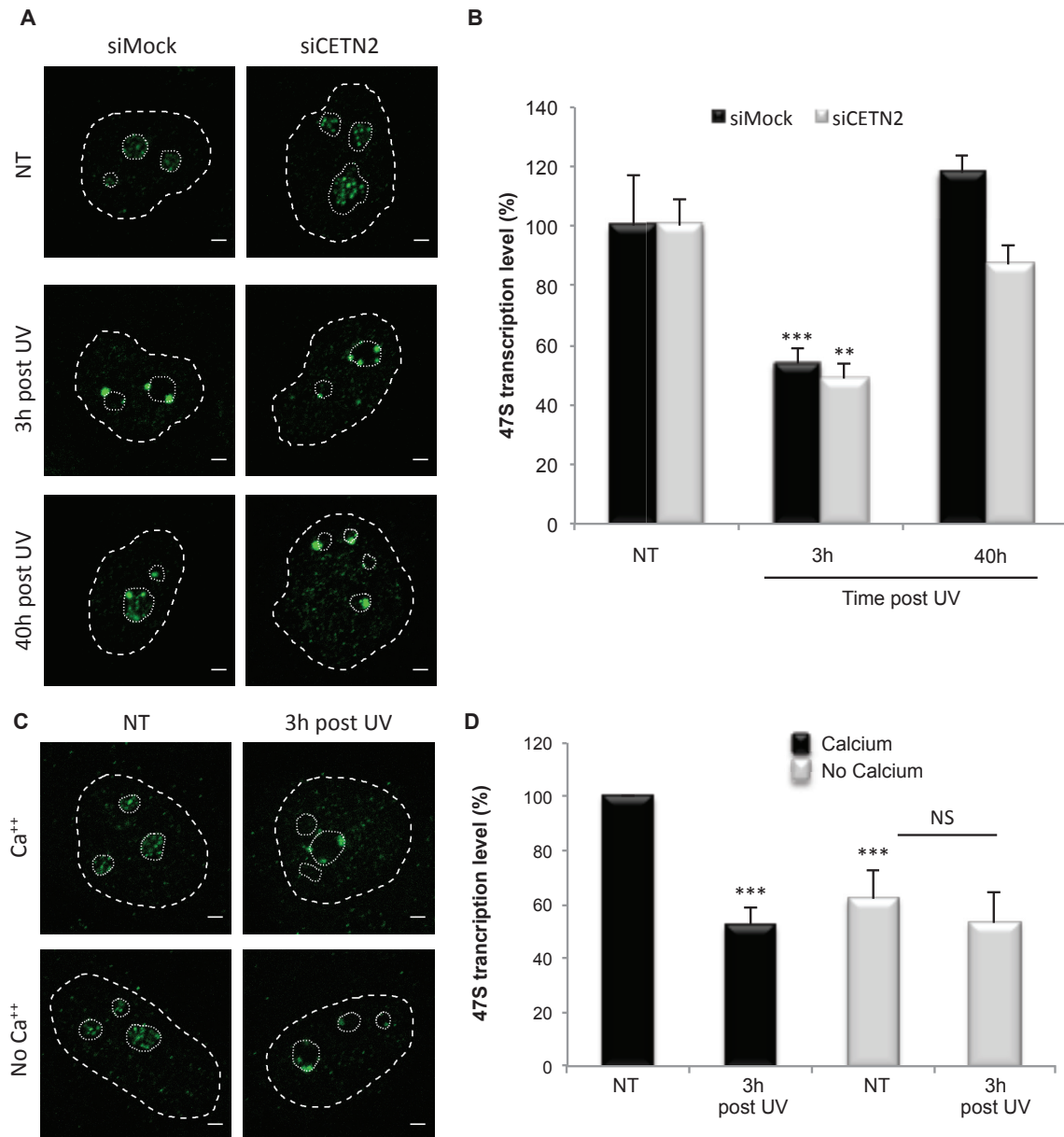
Figure 1. Cisplatin effect on RNAP1 during NER

**A)** Quantification of 47S pre-rRNA transcription level from confocal images of RNA FISH experiment performed on wild type cells incubated with Cisplatin (20 $\mu$ g/ml) for 3h or 6h. Error bars show the SEM of 30 cells. \*\*\*  $p < 0.001$ . **B)** Confocal composite images showing both 47S pre-rRNA (red) and RNAP1 (green) signals in WT, GG-NER deficient (XPC) and TC-NER deficient (CSB) cells. Cells were incubated for 6h with Cisplatin, and then allowed to recover for 3h or 16h before fixation. Nuclei and nucleoli are indicated by dashed and dotted lines respectively. Scale bar corresponds to 2 $\mu$ m. **C)** Quantification of 47S pre-rRNA transcription level from confocal images of RNA FISH experiment. Error bars show the SEM of 30 cells. \*\*  $p < 0.01$  \*\*\*  $p < 0.001$ .



**Figure 2. rDNA location during RNAP1 transcription arrest**

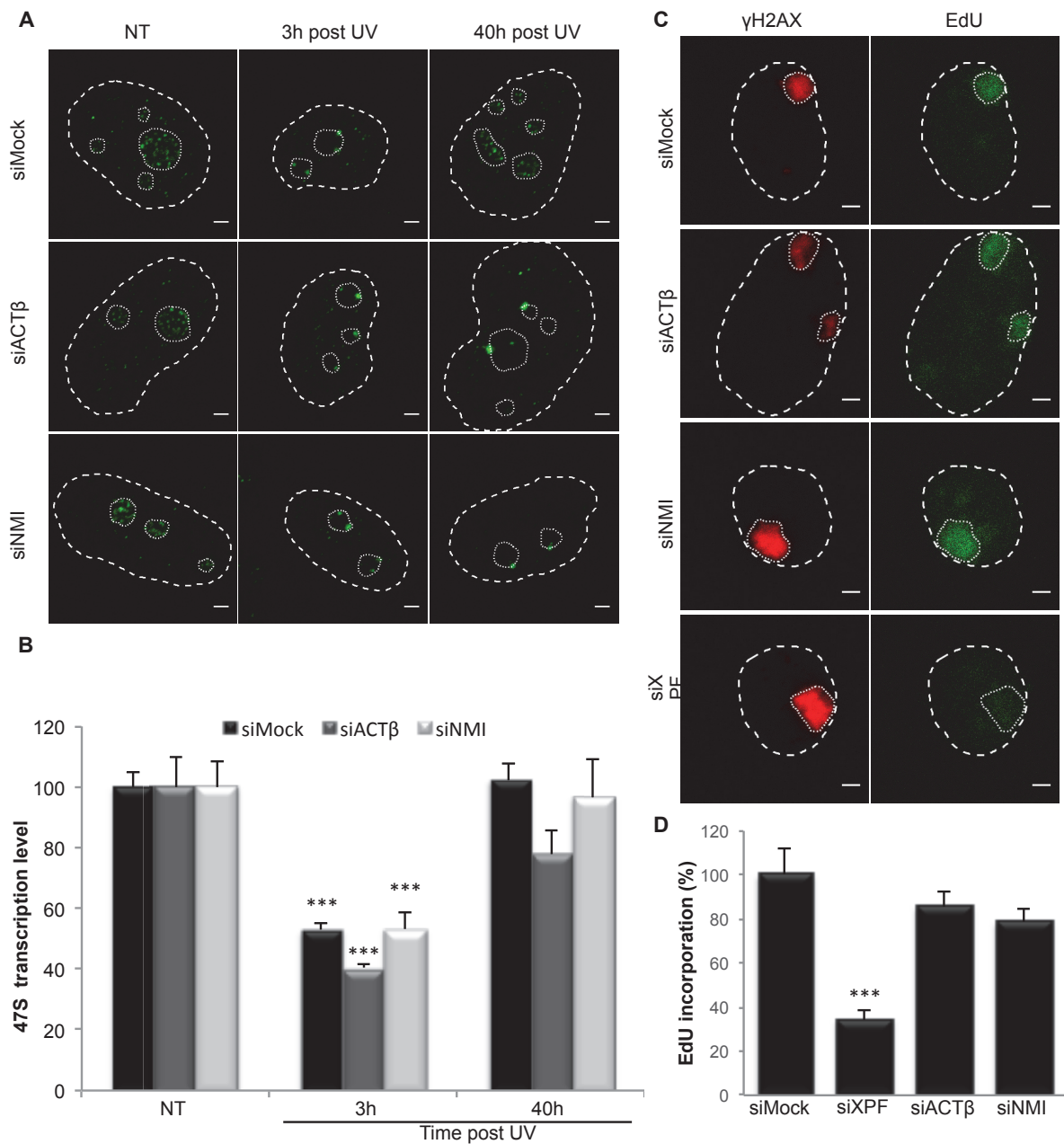
**A)** Schematic representation of the LacO/LacR system used to visualize the ribosomal DNA. Several Lac Operon genes were integrated downstream of the rDNA of the cells, which were also transfected with a plasmid carrying the Lac Repressor gene tagged with the GFP coding sequence. **B)** Confocal images of immunofluorescence assay against RNAP1 (red) in LacR-GFP (green) expressing cells. Cells were treated with Cordycepin (50 $\mu$ g/ml) for 1 hour before fixation. Mock-treated (NT) cells were used as control. Nuclei, nucleoli and LacO array (GFP green dot) are indicated by dashed lines, dotted lines and white arrows respectively. Scale bar 2 $\mu$ m. **C)** Quantification of 47S pre-rRNA transcription level from confocal images of RNA FISH experiment performed in cells in the conditions described above. Error bars show the SEM of 30 cells. \*\*\*  $p < 0.001$ .



**Figure 3. Calcium effect and Centrin2 role on RNAP1 displacement after DNA damage**

**A)** Confocal images of immunofluorescence staining against RNAP1 (green) performed on WT cells. Cells were transfected with small-interfering RNAs (siRNA) against Centrin2 (siCETN2) or with a non-targeting siRNA (siMock), exposed to  $16\text{J}/\text{m}^2$  of UV-C (3h post UV) or not (NT) 24h after the second transfection, and fixed 3h or 40h later. Nuclei and nucleoli are indicated by dashed and dotted lines respectively. Scale bar:  $2\mu\text{m}$ . **B)** Quantification of confocal images from RNA FISH experiments. The graph represents the 47S pre-rRNA level in WT cells treated as previously described. Error bars show the SEM of 3 independent experiments. \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . **C)** Confocal images of immunofluorescence staining against RNAP1 (green) performed on WT cells. Cells were exposed to  $16\text{J}/\text{m}^2$  of UV-C (3h post UV) or not (NT), incubated with ( $\text{Ca}^{++}$ ) or without calcium (No  $\text{Ca}^{++}$ ), and fixed 3h later. Nuclei and nucleoli are indicated by dashed and dotted lines respectively. Scale bar:  $2\mu\text{m}$ . **D)** Quantification of confocal images from RNA FISH experiments. The graph represents the 47S pre-rRNA level in WT cells grown with ( $\text{Ca}^{++}$ ) or without calcium (No  $\text{Ca}^{++}$ ), exposed to UV-C light (3h post UV) or not (NT). Error bars show the SEM of 3 independent experiments. \*\*\*  $p < 0.001$ .





**Figure 4. Nuclear factors involved in RNAP1 retention at the nucleolar periphery after repair**

**A)** Confocal images of immunofluorescence staining against RNAP1 (green) performed on WT cells transfected with siRNA (siMock; siACTβ; siNMI or siFbl). Transfected cells were exposed or not (NT) to  $16\text{J}/\text{m}^2$  of UV-C and fixation was performed 3h or 36h after UV exposure. Nuclei and nucleoli are indicated by dashed and dotted lines respectively. Insets zoom into the nucleoli indicated with arrows. Scale bars correspond to  $2\mu\text{m}$ . **B)** Quantification of the RNA FISH experiments performed on WT cells that have been treated the same way as for the IF experiment (A). Error bar represents the SEM of 3 biological replicates. \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . **C)** Confocal images of Unscheduled DNA Synthesis (UDS) assay after local UV-irradiation ( $100\text{ J}/\text{m}^2$ ). Site of damage is labelled with the antibody against  $\gamma\text{H2AX}$  (red) and DNA synthesis is revealed by the incorporation of 5-Ethynyl-2-deoxyUridine (EdU) stained with the alexa488 antibody (green). SiMock and siXPF are used as



positive and negative controls respectively. **D)** Quantification of the UDS experiment ( $n > 10$  cells). \*\*\*  $p < 0.001$ .

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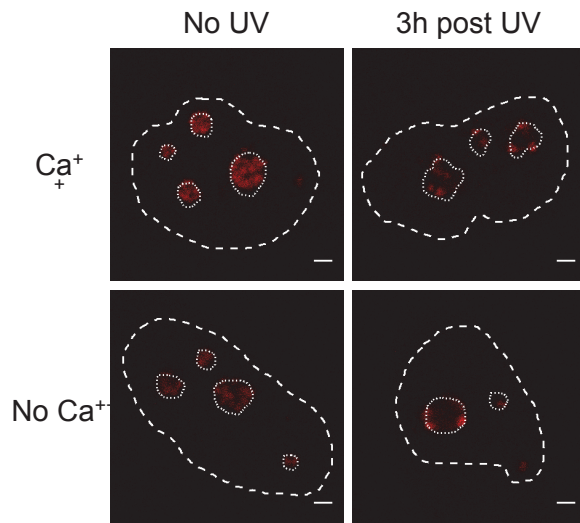
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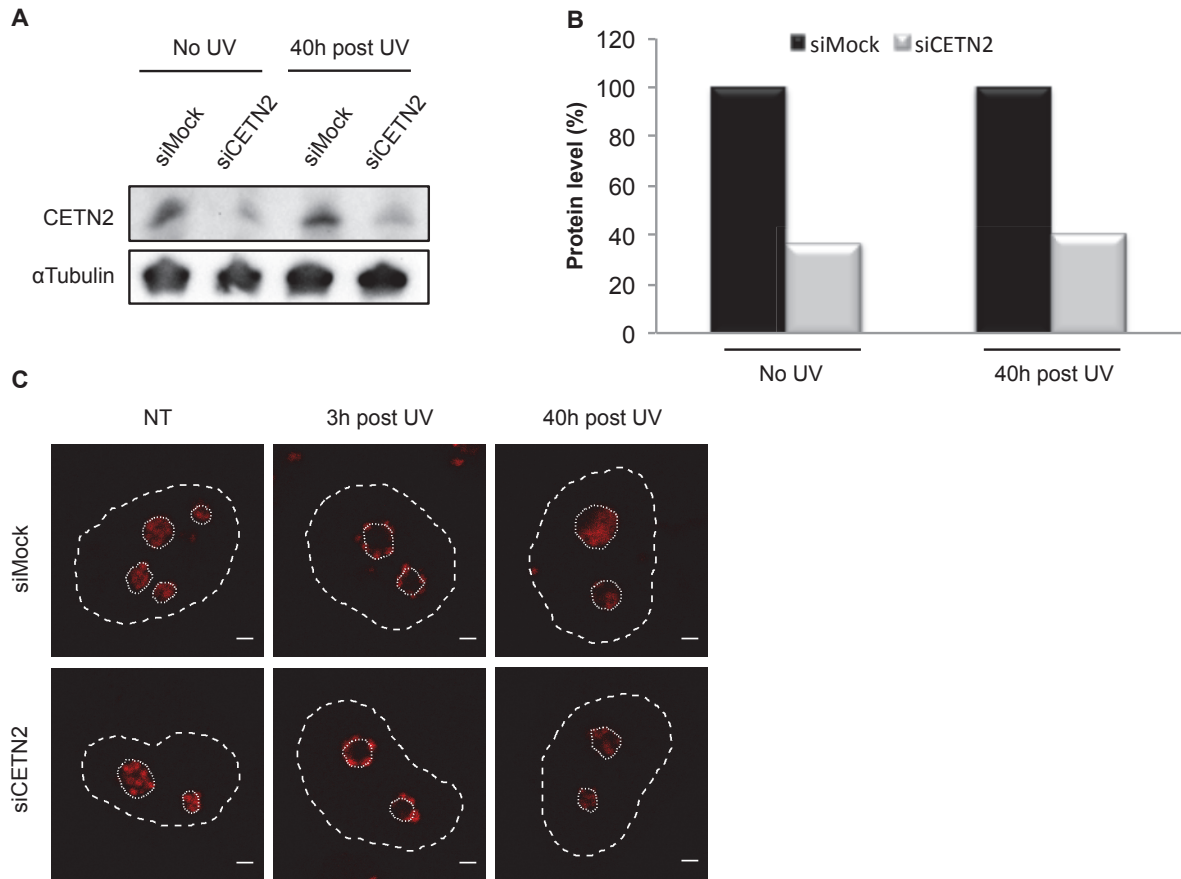


## SUPPLEMENTAL DATA



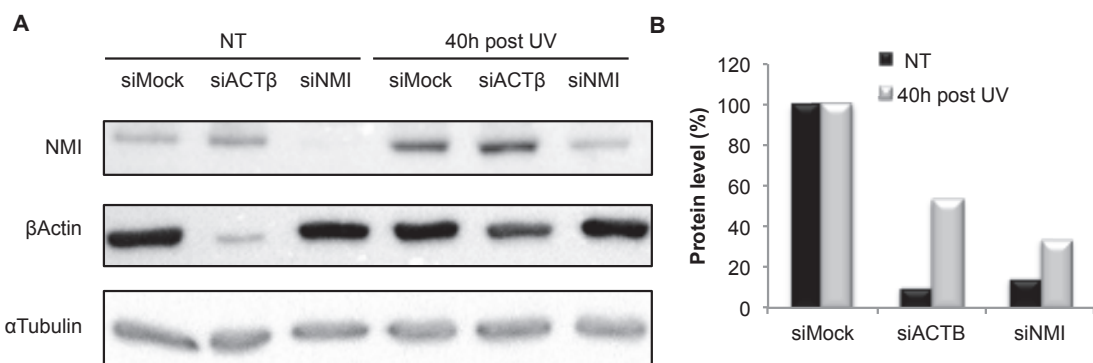
**Figure S1. Calcium effect on RNAP1 transcription level**

*Confocal images of RNA FISH labelling (red) performed on WT cells. Cells were exposed to 16J/m<sup>2</sup> of UV-C (3h post UV) or not (NT), incubated with (Ca<sup>++</sup>) or without calcium (No Ca<sup>++</sup>), and fixed 3h later. Nuclei and nucleoli are indicated by dashed and dotted lines respectively. Scale bar 2μm.*



**Figure S2. SiCETN2 efficiency and RNA FISH images**

Confocal images of RNA FISH assays (red) performed on WT cells. Cells were transfected with small-interfering RNAs (siRNA) against Centrin2 (siCETN2) or with a non-targeting siRNA (siMock), exposed to  $16\text{J}/\text{m}^2$  of UV-C (3h post UV) or not (NT) 24h after the second transfection, and fixed 3h or 40h later. Nuclei and nucleoli are indicated by dashed and dotted lines respectively. Scale bar  $2\mu\text{m}$ .



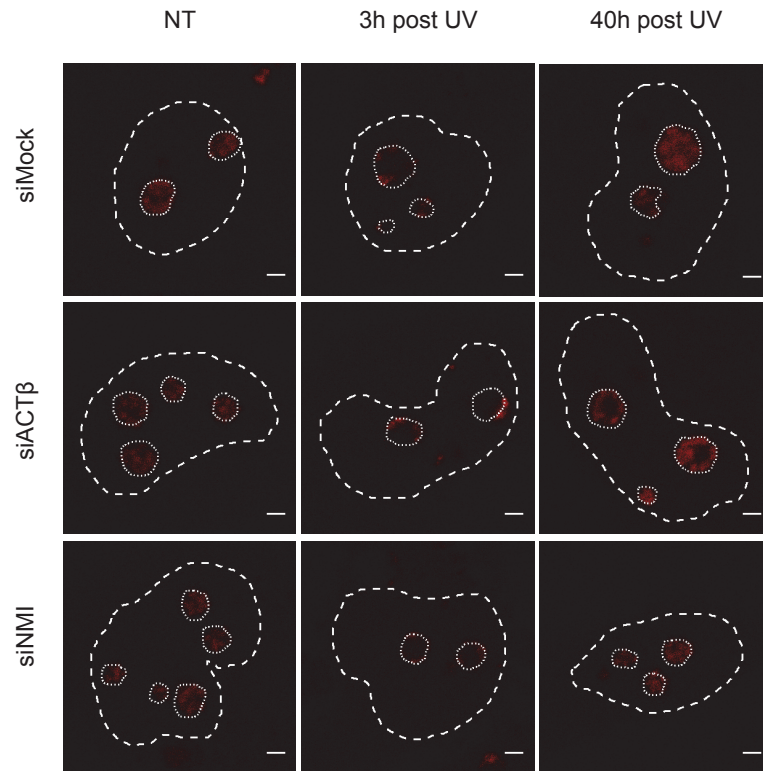
**Figure S3. SiRNA efficiency for RNA FISH**

**A)** Western blot performed on whole cell extracts against Nuclear Myosin I (NMI),  $\beta$ Actin and  $\alpha$ Tubulin.  $\alpha$ Tubulin signal was used as loading control. Whole cell extracts were performed at the

time of the 40h-post-UV irradiation (40h post UV) and at fixation time (NT). **B)** Quantification of the western blot.

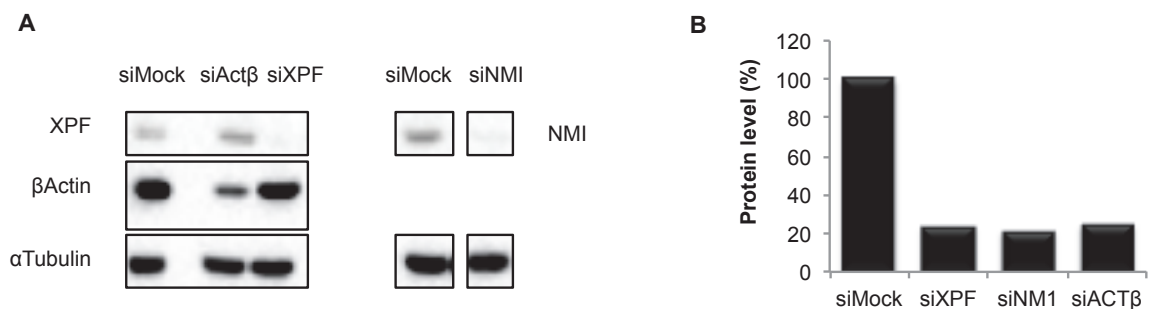
RNAP1 location in the nucleolus				
	siMock	siAct $\beta$	siNMI	
NT	In	In	In	
3h	Out	Out	Out	
16h	Out	Out	Out	
40h	In	Out	In 28%	Out 72%

Figure S4. RNAP1 location summary



**Figure S5. SiACT $\beta$  and siNMI effect on RNAP1 transcription level**

Confocal images of RNA FISH labelling (red) performed on WT cells transfected with siRNA (siMock; siACT $\beta$  and siNMI). Cells were transfected twice with the siRNAs, exposed to 16J/m<sup>2</sup> of UV-C (3h post UV) or not (NT) and fixed 3h later. Nuclei and nucleoli are indicated by dashed and dotted lines respectively. Scale bar 2 $\mu$ m.



**Figure S6. SiRNA efficiency for UDS**

**A)** Western blot, against Nuclear Myosin I (NMI),  $\beta$ Actin, XPF and  $\alpha$ Tubulin, performed on whole cell extracts.  $\alpha$ Tubulin signal was used as loading control. **B)** Quantification of the western blot.



# CONCLUSIONS & PERSPECTIVES



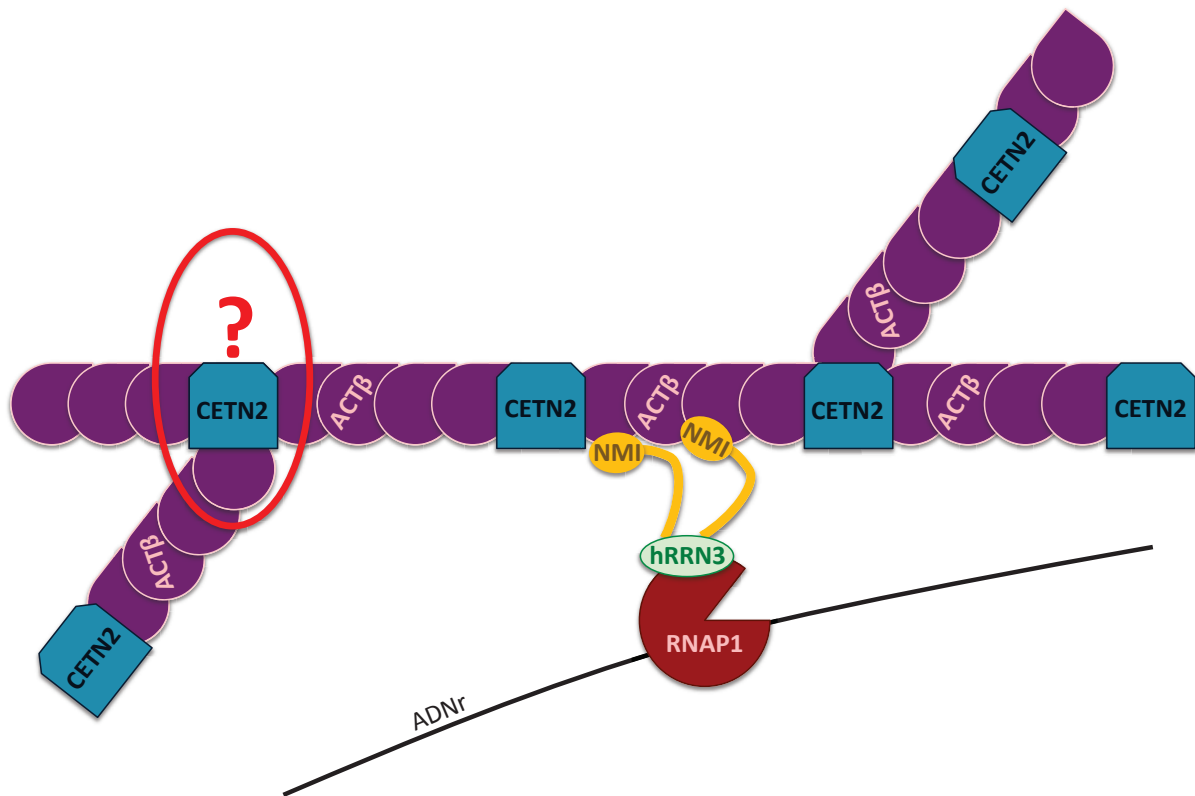
My PhD project gave unexpected and interesting results concerning the repair process of UV-damaged ribosomal DNA. We have demonstrated that NER was involved in repair of bulky adducts induced by UV irradiation or cisplatin incubation. In particular, we implicated the complete TC-NER mechanism in the repair of active rDNA. These results are concomitant with previous studies performed in yeast, even though some differences have been demonstrated, e.g. a CSB-independent TC-NER mechanism has been described in yeast (Conconi et al., 2002; Verhage et al., 1996).

Moreover, we observed a specific behaviour for both RNAP1 and rDNA after UV irradiation. Firstly, RNAP1 accumulated on rDNA after UV light exposure. Interestingly, this result is in contradiction with others obtained in yeast that show the RNAP1 dissociation from the rDNA (Tremblay et al., 2014). Secondly, RNAP1 together with rDNA relocates at the nucleolar periphery after UV irradiation. We demonstrated that the relocation was dependent on both transcription inhibition and/or presence of DNA lesions, whereas the re-entry of RNAP1 inside the nucleolus was dependent only on the presence of unrepaired lesions on the untranscribed rDNA, despite the RNAP1 transcription resumption. Previous studies about DSBs repair have described a similar relocation of rDNA and RNAP1 in nucleolar caps at the nucleolar periphery (Franek et al., 2016; Larsen and Stucki, 2016; Moore et al., 2011; Sluis and McStay, 2015). Thus, the displacement of RNAP1 seems to be part of the repair process allowing some proteins, which have been excluded from the nucleolus, to access the rDNA.

In addition, we identified several proteins implicated in RNAP1 re-entry into the nucleolus when repair was completed. CETN2, which is a partner of XPC, is required to RNAP1 return into the nucleolus but not for rDNA transcription restart. We identified two other proteins required for RNAP1 re-entry into the nucleolus: NMI and  $\beta$ -Actin. Both proteins have been described as RNAP1-transcription-like factors in several studies (Almuzzaini et al., 2016; Fomproix and Percipalle, 2004; Gettemans et al., 2005; Grummt, 2006; Philimonenko et al., 2004). Furthermore, they both interact with each other and NMI is part of the motor protein superfamily (Lodish et al., 2000). Hence, these results are consistent with our findings about the implication of NMI and  $\beta$ -Actin in RNAP1



displacement during repair. Because no proper actin filaments have been observed yet in the nucleolus, we suggest the following model, involving CETN2, NMI and  $\beta$ -Actin in RNAP1 return into the nucleolus after complete repair of UV-induced lesions, to help the understanding of this displacement observed during DNA repair.



**Figure 23: Diagram of RNAP1 return in the nucleolus after DNA repair of UV-induced lesions**

This project gives rise to a wide range of perspectives. Intriguingly, a methyltransferase, fibrillarin (FBL), has been found to specifically add a methyl group only to the histone H2A present on rDNA. It would be interesting to study the RNAP1 displacement in the absence of FBL, using the same method previously described (combination of siRNA transfection, IF and RNA FISH assays). To further identify new factors required for RNAP1 displacement during repair (eviction or re-entry), we will set up a siRNA bank screening (transcription factors, chromatin remodelling and DDR factors) at different time after UV exposure, with a read out based on IF against RNAP1. Once new factors will be identified, we will verify whether they have an impact either on transcription resumption, via the RNA

FISH protocol, or on repair, through UDS. We will also study the epigenetic marks on rDNA, i.e. methylation and SUMOylation, during repair. In addition, rDNA repeats are encompassed by Distal and Proximal Junctions (DJ/PJ) on the short arm of each acrocentric chromosome (see .1.3 Floutsakou and co-workers (2013) demonstrated that DJs are located at the periphery of the nucleolus. Since nucleolar caps induced by ActinomycinD (ActD) were formed exactly next to the DJs at the nucleolar periphery, the authors suggested that DJs, as an anchor, could be responsible for the location of rDNA at the nucleolar periphery after transcription inhibition by ActD (Floutsakou et al., 2013). Therefore, we will focus on DJs in order to verify their implication in RNAP1 displacement and potentially identify new factors that could bind to any domain of these DJs. Finally, we would like to investigate the genomic environment of the rDNA before, during and after repair. To do so, we will use one of the 4C technologies which combines the 3C (Chromosome Conformation Capture) technique with chromatin ImmunoPrecipitation (ChIP) assay: the ChIA-PET (Chromatin Interaction Analysis with Paired-End-Tag sequencing) method (Fullwood et al., 2009). This method enables the unbiased genome-wide screening for DNA contacts made by our region of interest: the rDNA.

Since rDNA has been involved in aging, tumorigenesis and severe diseases such as ribosomopathies, it is important to focus on how rDNA is repaired to better understand the defect and identify potential therapeutic targets.



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