Functional interaction between the DNA damage tolerance pathway and the DNA damage checkpoint: implications for genome stability and oncogenesis

Chames Kermi

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Interactions fonctionnelles entre le système de tolérance des lésions et le checkpoint des dommages à l'ADN : Conséquences sur la stabilité du génome et l'oncogenèse
“Ex ovo omnia”
“Everything comes from an egg”
William Harvey (1578-1657)

The important thing is not to stop questioning. Curiosity has its own reason for existing. Albert Einstein (1879-1955)
Functional interactions between the DNA damage tolerance pathway and the DNA damage checkpoint:

Implications for genome stability and oncogenesis
First of all, I would like to express my gratitude to my Ph.D. advisor Dr. Domenico Maiorano for welcoming me in his laboratory, for continuous support during my Ph.D, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and for writing this thesis. With him I spent four great years full of fascinating science and fun. I grew up personally and scientifically and learned a lot. I am glad to have been a member of his team. Domenico, thank you for believing in me, for your mentorship and constant encouragement. I appreciated very much the freedom you gave me to handle my projects and explore different aspects of science, expressing my personal opinion and curiosity in science. I have accumulated a lot of experience in your lab, and I believe I must be ready for new scientific challenges around the world.

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Last but not the least, I would like to thank my family: my parents, my brother Nassim and my sister Lynda Inesse for always supporting me spiritually throughout my adventures. Without you and your encouragement in the hardest moments, none of this would be possible. Thank you for putting so much trust in me.
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Abstract

Our genome is continuously exposed to DNA damaging agents. In order to preserve the integrity of their genome, cells have evolved a DNA damage signalling pathway known as checkpoint. DNA lesions that persist when cells enter the S-phase halt the progression of replicative DNA polymerases. This can cause prolonged replication forks stalling which threaten the stability of the genome. To preserve the integrity of replication forks, cells have developed a tolerance pathway which involves specialized DNA polymerases, called translesion DNA polymerases (TLS Pols) that have the unique ability to accommodate the damaged bases. In this process, the replication factor PCNA acts as a scaffold for many proteins involved in DNA metabolism. The mechanisms governing the exchanges between different PCNA partners are not well understood. Among the proteins that interact with PCNA, CDT1, p21 and PR-Set7/set8 are characterized by a high binding affinity. These proteins have a particular interaction domain with PCNA, called "PIP degron", which promotes their proteasomal degradation via the E3 ubiquitin ligase CRL4\(^{Cdt2}\). After UV-C irradiation, the replication initiation factor CDT1 is rapidly degraded in a PIP degron-dependent manner. During the first part of my work, we wanted to understand the functional role of this degradation. Our results have shown that inhibition of CDT1 degradation by CRL4\(^{Cdt2}\) in mammalian cells, compromises the relocalisation of TLS Pol \(\eta\) and Pol \(\kappa\) to nuclear foci after UV-C irradiation. We also found that only the proteins which contain a PIP degron interfere with the formation of Pol \(\eta\) foci. Mutagenesis experiments on CDT1 PIP degron revealed that a threonine residue conserved among PIP degrons is essential for inhibiting foci formation of at least two TLS polymerases. This results suggest that CRL4\(^{Cdt2}\)-dependent degradation of proteins containing PIP degrons regulates the recruitment of TLS polymerases at sites of UV-induced DNA damage.

During the second part of my thesis, we studied DNA damage checkpoint regulation during embryogenesis. Indeed, in early embryos, the DNA damage checkpoint is silent until the mid-blastula transition (MBT) due to maternal inhibiting factors. In this work, we have shown, both in vitro and in vivo, that the E3 ubiquitin ligase RAD18, a major regulator of translesion DNA synthesis, is a limiting factor for the checkpoint activation in \textit{Xenopus} embryos. We have also shown that RAD18 depletion leads to the activation of DNA damage checkpoints by inducing replication fork uncoupling in front of the lesions. Furthermore, we showed that the abundance
of RAD18 and PCNA monoubiquitination (PCNA\textsubscript{monoUb}) is regulated during embryonic development. Near the MBT, the increased abundance of DNA limits the availability of RAD18, thereby reducing the amount of PCNA\textsubscript{monoUb} and inducing the de-repression of the checkpoint. Moreover, we have shown that this embryonic-like regulation can be reactivated in somatic mammalian cells by ectopic expression of RAD18, conferring resistance to DNA damaging. Finally, we found high RAD18 levels in glioblastoma cancer stem cells highly resistant to DNA damage. All together, these data propose RAD18 as a critical factor that inhibits DNA damage checkpoint in early embryos and suggests that dysregulation of RAD18 expression may have an unexpected oncogenic potential.
List of Abbreviations

53BP1: p53-Binding Protein 1
5-FU: 5-Fluoro Uracil
8-oxoG: 8-oxo-7,8-dihydroGuanine
9-1-1: RAD9-Hus1-Rad1
AAD: ATR Activating Domain
AAF: N-2-Acetyl-2-AminoFluorene
AEP: Archaeo-Eukaryotic Primase
ALDH: ALdehye DeHydrogenase
AML: Acute Myeloid Leukaemia
A-NHEJ: Alternative Non-Homologous End Joining
APC/C: Anaphase-Promoting Complex/Cyclosome
APC: Anaphase Promoting Complex
ARF: Alternative Reading Frame
ARS: Autonomously Replicating Sequences
ATM: Ataxia Telangiectasia Mutated
ATR: Ataxia Telangiectasia mutated and Rad3 related
ATRIP: ATR Interacting Protein
BER: Base Excision Repair
BMPs: Bone Morphogenetic Proteins
CDC6: Cell Division Cycle 6 protein
CDKs: Cyclin-Dependent Kinases
CDT1: CDC10 dependent transcript 1
CFSs: Common Fragile Sites
CIMP: CpG Island Methylator Phenotype
CIN: Chromosomal INstability
Cisplatin: Cis-diamminedichloroplatinum
CMG: CDC45-MCM2-7-GINS
C-NHEJ: Canonical Non-Homologous End Joining
CPD: Cyclobutane Pyrimidine Dimer
CPT: Camptothecin
CRC: ColoRectal Cancer
CRL4\textsuperscript{Cdt2}: Cullin 4-RING E3-ubiquitin Ligase Cdt2
CS: Cockayne Syndrome
CSCs: Cancer Stem Cells
CTCF: CCCTC-binding Factor
DA: Damage Avoidance
DDK: DBF4-Dependent Kinase
dHJs: double Holliday Junctions
D-loop: Displacement-loop
DNA-PK: DNA-dependent Protein Kinase
DPCs: DNA-Protein Crosslinks
DSBs: Double Strand Breaks
dsDNA: double stranded DNA
DUEs: DNA Unwinding Elements
E. coli: Escherichia coli
EGT: Early Gastrula Transition
FA: Fanconi Anemia
FBH1: F-Box DNA Helicase protein 1
FEN1: Flap Endonuclease 1
FISH: Fluorescence In Situ Hybridization
GG-NER: Global Genomic NER
HECT: Homologous to E6-AP C-Terminus
HLTF: Helicase-Like Transcription Factor
HR: Homologous Recombination
ICLs: Inter Strand Crosslinks
ID1: Inhibitor of Differentiation protein 1
IDH1: Isocitrate DeHydrogenase 1
iPSCs: induced Pluripotent Stem Cells
IR: Ionising Radiations
IRIF: IR-Induced nuclear Foci
JNK: c-Jun N-terminal Kinase
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOH</td>
<td>Loss Of Heterozygosity</td>
</tr>
<tr>
<td>MBT</td>
<td>MidBlastula Transition</td>
</tr>
<tr>
<td>MCM</td>
<td>Mini-Chromosomes Maintenance</td>
</tr>
<tr>
<td>MDC1</td>
<td>Mediator of the Checkpoint 1</td>
</tr>
<tr>
<td>MEF2A</td>
<td>Myocyte specific Enhancer Factor 2A</td>
</tr>
<tr>
<td>MGMT</td>
<td>Methyl Guanine MethylTransferase</td>
</tr>
<tr>
<td>MIN</td>
<td>Microsatellite INstability</td>
</tr>
<tr>
<td>MMC</td>
<td>MitoMycin C</td>
</tr>
<tr>
<td>MMEJ</td>
<td>Microhomology-Mediated End-Joining</td>
</tr>
<tr>
<td>MMR</td>
<td>MisMatch Repair pathway</td>
</tr>
<tr>
<td>MMS</td>
<td>Methyl MethaneSulfonate</td>
</tr>
<tr>
<td>MRN</td>
<td>MRE11, RAD50 and NBS1 proteins</td>
</tr>
<tr>
<td>MZT</td>
<td>Maternal-Zygotic Transition</td>
</tr>
<tr>
<td>N/C ratio</td>
<td>Nuclear to Cytoplasmic ratio</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide Excision Repair</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear Factor κB</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-Homologous End Joining</td>
</tr>
<tr>
<td>NSPC</td>
<td>Neural Stem and Progenitor Cells</td>
</tr>
<tr>
<td>NSTCs</td>
<td>Non-Stem Tumour Cells</td>
</tr>
<tr>
<td>ORC</td>
<td>Origin Recognition Complex</td>
</tr>
<tr>
<td>Oris</td>
<td>Origins of replication</td>
</tr>
<tr>
<td>PARP1</td>
<td>Poly(ADP-Ribose) Polymerase 1</td>
</tr>
<tr>
<td>PBD</td>
<td>Polymerase-Binding Domain</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferation Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PCNA\textsubscript{monoUb}</td>
<td>PCNA monoubiquitination</td>
</tr>
<tr>
<td>PCNA\textsubscript{polyUb}</td>
<td>PCNA polyubiquitination</td>
</tr>
<tr>
<td>PDIP38</td>
<td>Pol Delta Interacting Protein 38</td>
</tr>
<tr>
<td>PIP</td>
<td>PCNA Interacting Peptide</td>
</tr>
<tr>
<td>PNKP</td>
<td>PolyNucleotide Kinase-Phosphatase</td>
</tr>
<tr>
<td>pre-IC</td>
<td>pre-Initiation Complex</td>
</tr>
<tr>
<td>pre-RC</td>
<td>pre-Replication Complex</td>
</tr>
<tr>
<td>R6B</td>
<td>RAD6-Binding domain</td>
</tr>
<tr>
<td>RAP80</td>
<td>Receptor-Associated Protein 80</td>
</tr>
</tbody>
</table>
Rb: Retinoblastoma protein
RF-B: Replication Factor B
RFB: Replication Fork Barriers
RING: Really Interesting New Gene
ROS: Reactive Oxygen Species
RPA: Replication Protein A
S. cerevisiae: Saccharomyces cerevisiae
S. pombe: Schizosaccharomyces pombe
SCE: Sister Chromatid Exchange
SCF: Skp1-Cul1-F box
SCJs: X-shaped Sister Chromatid Junctions
SDSA: Synthesis-Dependent Strand Annealing
SHPRH: SNF2 Histone linker Plant homeodomain RING Helicase
SIM: SUMO-Interaction Motif
SLE: Systemic Lupus Erythematosus
SLF1: SMC5-SMC6 complex Localization Factor protein 1
SLF2: SMC5-SMC6 complex Localization Factor protein 2
SNP: Single Nucleotide Polymorphism
SSBR: Single-Strand Break Repair
ssDNA: single stranded DNA
SUMO: Small Ubiquitin-like MOdifier
SV40: Simian Virus 40
TC-NER: Transcription Coupled NER
TGF-β: Transforming Growth Factor β
TLS: Translesion
TMZ: Temozolomide
TRIP: TNF Receptor associated factor (TRAF)-Interacting Protein
TS: Template Switch
TTD: TrichoThioDystrophy
TTF1: Termination factor 1
TUS: Terminus Utilization Substance
UAF1: USP1-Associated Factor 1
UBDs: Ubiquitin-Binding Domains
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBMs:</td>
<td>Ubiquitin-Binding Motifs</td>
</tr>
<tr>
<td>UBZs:</td>
<td>Ubiquitin-Binding Zinc fingers</td>
</tr>
<tr>
<td>USP1:</td>
<td>Ubiquitin-Specific Protease 1</td>
</tr>
<tr>
<td>UV:</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>WHO:</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WRN:</td>
<td>Werner syndrome helicase</td>
</tr>
<tr>
<td>WRNIP1:</td>
<td>WRN-Interacting Protein 1</td>
</tr>
<tr>
<td>XLF:</td>
<td>XRCC4-Like Factor</td>
</tr>
<tr>
<td>XP:</td>
<td>Xeroderma Pigmentosum</td>
</tr>
<tr>
<td>XRCC4:</td>
<td>X-Ray Cross Complementing protein 4</td>
</tr>
<tr>
<td>ZGA:</td>
<td>Zygotic Genome Activation</td>
</tr>
<tr>
<td>ZnF:</td>
<td>Zinc Finger</td>
</tr>
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Chapter I
Introduction
I. Introduction

1. DNA Damage: A Threat to Genome Integrity

Although DNA carries the genetic information essential for all organisms’ life, its structure is labile and has a limited chemical stability upon exposure to different agents. Cells are exposed to a large variety of DNA damage sources, including genotoxic agents, as well as endogenous damage generated by the cellular metabolism itself. Oxidation, hydrolysis and non-enzymatic DNA methylation are relatively frequent in vivo, and are normally counteracted by specific DNA repair pathways (Lindahl, 1993).

Modifications of the chemical composition of DNA are called DNA lesions or more generally DNA damage, and can be divided, depending on the source, into two distinct categories: a) endogenous damage arising from natural molecules present in cells that spontaneously react with the DNA, and b) exogenous damage resulting from the activity of external chemical and physical agents onto DNA molecule (Lindahl, 1993). During DNA replication, these alterations interfere with the stability, progression and restart of the replication forks thus challenging genome integrity (Ciccia and Elledge, 2010).

1.1. Endogenous DNA damage

Endogenous DNA damage is mainly a consequence of the cell metabolism in a microenvironment rich of water and oxygen that can generate reactive nucleophilic or electrophilic species. Spontaneous or actively induced hydrolytic attacks and oxidative damage constitute the major types of endogenous DNA damage, creating different kinds of lesions on DNA (Figure 1.1). When these lesions occur during DNA replication, they generate point mutations that constitute a threat for the integrity of the genome (Lindahl and Wood, 1999).

In the following section, I will describe the endogenous sources of DNA damage and their deleterious action on the DNA.
1.1.1. DNA damage through spontaneous nucleotide hydrolysis

A) Deamination of nitrogenous bases

Cytosine can be targeted by spontaneous hydrolytic deamination at physiological temperature and pH (Figure 1.1, reaction II), resulting in the conversion of cytosine to uracil (Frederico et al., 1990). This reaction is relatively frequent as it has been estimated that between 70 and 200 cytosine residues in a single cell are deaminated per day (Kavli et al., 2007). Cytosine deamination generates Uracil:Guanine (U:G) mispair that, if it persists, results in the substitution of the initial Cytosine:Guanine (C:G) pair with a Thymine:Adenine (T:A) one. It can also lead to C→T transition in vivo. In the absence of efficient DNA repair, mainly base excision repair (BER, see paragraph 2.2.2) (Kavli et al., 2007), the frequency of these transitions is higher (Duncan and Miller, 1980).

In a similar way but less frequently, guanine and adenine can undergo hydrolytic deamination, and be converted to xanthine and hypoxanthine respectively (Lindahl, 1993).

B) Formation of abasic sites

Hydrolytic cleavage can also target the N-glycosyl bond, resulting in the loss of the nitrogenous base and formation of an abasic site (Figure 1.1, reaction IV). Depending on the cleaved base (purine or pyrimidine), the reaction is called depurination (Adenine or Guanine) or depyrimidination (Cytosine or Thymine).

Whereas depyrimidination is a relatively rare event, spontaneous depurination occur at a rate of approximately 10 000 bases per day in a mammalian cell (Lindahl, 2000; Nakamura et al., 1998). Although error-free pathways using the complementary DNA strand can repair these abasic sites, they are particularly mutagenic during replication because the information carried by the damaged nucleotide is missing leading to a frameshift.
1.1.2. Oxidative DNA damage

A) Oxidative stress

During aerobic metabolism, oxygen is essential for producing energy, but it is also responsible for the formation of reactive oxygen-derived molecules capable of oxidising DNA, called Reactive Oxygen Species (ROS). ROS are an unavoidable natural by-product, generally produced in the cell at the electron transport chain of mitochondria when the reduction of molecular oxygen to water is inefficient (Riley, 1994). Cells have evolved antioxidant mechanisms in order to counter-balance ROS production (Hofmann et al., 2002; Wood et al., 2003). Oxidative stress arises when the equilibrium between ROS production and antioxidant activity is disrupted (Henle and Linn, 1997).

B) Reactive oxygen species (ROS) induced DNA damage

The most dangerous aerobic metabolism by-product is the hydroxyl radical (‘OH) due to its very high electrophilic reactivity. It can either drive an oxidation reaction of the nitrogenous bases, or an oxidative attack of the deoxyribose sugar residues, generating single-strand DNA breaks (Breen and Murphy, 1995). The most frequent lesions are the 8-oxo-7,8-dihydroguanine (8-oxoG) and the thymine glycol (Figure 1.1, reactions I and III). The 8-oxoG is highly mutagenic as it mispairs with adenine and forms a Hoogsteen 8-oxoG:A stable base pair without perturbing the DNA helix structure (Lipscomb et al., 1995), this make it unrecognizable by the MisMatch Repair pathway (MMR, see below paragraph 2.2.3) (Hsu et al., 2004). The replicative DNA polymerases preferentially insert an adenosine opposite to 8-oxoG without stalling (Furge and Guengerich, 1997; Shibutani et al., 1991). 8-oxoG induces mainly G→T transversion mutations (Moriya, 1993).

Other reactive species generated during alcohol metabolism or histone demethylations are aldehydes (Brooks and Theruvathu, 2005; Shi et al., 2004). They can bridge the two strands of DNA generating interstrand crosslinks and also DNA-Protein Crosslinks (DPCs). This kind of lesions are normally resolved by the Fanconi anemia pathway (see paragraph 2.2.6) (Kim and D’Andrea, 2012; Langevin et al., 2011; Rosado et al., 2011).
Figure 1. Spontaneous DNA damage linked to DNA reactivity (adapted from Lindhal, 1993; Tsanov, 2012)

(Left) The sites on DNA susceptible to react with water (red arrows) or reactive oxygen species (ROS) (blue arrows) at physiological conditions are shown. (Middle and right) Examples of common chemical reactions that modify DNA chemical structure and the potential produced lesions. Water attacks the exocyclic amino groups of the nitrogenous bases resulting in deamination, and the N-glycosyl bonds producing abasic sites. The hydroxyl radical (·OH) can add to the double bonds of bases, generating lesions such as 8-oxoG and thymine glycol.

1.1.3. Hard-to-replicate sequences and common fragile sites (CFSs)

Hard-to-replicate sequences and Common Fragile Sites (CFSs) are non-canonical DNA structures that challenge DNA replication and thus can generate genetic stability. One example of hard-to-replicate sequences is G-quadruplex structures formed in GC-rich regions of the genome and interfere with the progression of the replication machinery (Zeman and Cimprich, 2014). Another example are nucleotides repeats that can form hairpins structures. In order to ensure the replication of such structures, cells have different proteins such as the PIF1 helicase
that helps G4 replication in budding yeast (Paeschke et al., 2011) and RIF1 at telomeres (Yamazaki et al., 2013).

CFSs are unstable genomic regions that break under replication stress. They are characterized by a paucity of replication origins (Letessier et al., 2011). When replication at common fragile sites is compromised because forks have to move through long distance to complete the duplication of these late replicating domains, mitosis can begin with unreplicated DNA (Mankouri et al., 2013). The stability of these sequences seems to depend on the activity of the checkpoint kinase ATR (Cimprich and Cortez, 2008).

1.1.4. Replication errors

Before mitosis, accurate DNA replication is a prerequisite for preserving cells against mutagenesis and genomic instability. DNA polymerase have a remarkably high fidelity, estimated at one incorrect base for every $10^9$ to $10^{10}$ replicated nucleotides (Loeb, 1991). This results from a highly accurate base incorporation combined to an exonuclease proofreading activity by the replicative DNA polymerases Pol $\delta$ and Pol $\varepsilon$, in addition to post-replication surveillance by the MMR system. Despite this, sometimes DNA polymerases make mistakes such as incorporating an adenine facing an 8-oxoG residue without changing the helix structure, making the mismatch invisible to the MMR pathway. The X-family DNA Pol $\lambda$ is involved in the repair of the generated A:8-oxoG mispairs (van Loon and Hubscher, 2009), in a PCNA and RPA –dependent manner (Maga et al., 2008). During this process, DNA Pol $\delta$-Interacting Protein 2 (PolDIP2) increases the processivity of Pol $\lambda$ for the error-free bypass of an 8-oxoG lesion (Maga et al., 2013), and the WRN helicase/exonuclease promotes long-patch DNA repair synthesis by Pol $\lambda$ of 8-oxoG:A mispairs (Kanagaraj et al., 2012).

Moreover, ribonucleotides are frequently misincorporated into DNA by replicative polymerases instead of deoxynucleotides (Dalgaard, 2012). A recent study has shown that Pol $\beta$ and Pol $\lambda$ can incorporate rNMPs opposite normal bases or 8-oxoG, delaying the repair by DNA glycosylases (Crespan et al., 2016). These rNTPs modify the helix structure and can perturb DNA replication during the following round, and generate short deletions when they are processed by topoisomerase I into single-stranded nicks (Kim et al., 2011a; Nick McElhinny et al., 2010; Watt et al., 2011). Translesion DNA polymerases can bypass these misincorporated
ribonucleotides, and leave them behind the fork for post-replication repair (Lazzaro et al., 2012b; Reijns et al., 2012), involving the endonucleases FEN1 EXO1, and RNaseH2 to cleave the misincorporated ribonucleotides in a process called ribonucleotides excision repair or RER (Lazzaro et al., 2012b; Sparks et al., 2012).

1.1.5. Oncogene-induced replication stress

In precancerous lesions, activated oncogenes can deregulate the entry into S-phase by inhibiting the Retinoblastoma protein (Rb) (Malumbres and Barbacid, 2001), inducing stalling and collapse of DNA replication forks, which in turn leads to formation of DNA Double Strand Breaks (DSBs) (Halazonetis et al., 2008). These DSBs may contribute to the genomic instability, a general feature of human cancers. In addition, activated oncogenes can induce DNA replication stress by perturbing origin firing and increasing the number of active replication forks with a concomitant decrease of nucleotides pool (Bester et al., 2011). Furthermore, transcription is also hyperactivated, increasing the risk of transcription/replication conflicts (Jones et al., 2013). Therefore, the sum of the concomitant deregulations of origin firing, nucleotide deprivation and transcription/replication conflicts is likely responsible for the oncogene-induced replication stress.

1.2. Exogenous DNA damage

Exogenous DNA damage arises from natural exposure to sunlight and cosmic rays, two physical sources that can alter the chemical composition of DNA. In addition, human activities generate a lot a chemicals and radiations that can interfere with biological processes and inevitably generate DNA damage.
1.2.1. Physical damage of DNA

A) Ionizing radiation

Ionizing radiation (IR) are electromagnetic waves of high-energy such as X-rays and γ-rays that can ionize a molecule by removing an electron to produce ions and free radicals. Cosmic rays are the main natural source of ionizing radiations. In addition, a natural background radiation is generated by radioactive isotopes such as $^{14}$C and $^{40}$K that are naturally absorbed and deposited within organs (Thorne, 2003).

In the early forties, the ability of X-rays to induce breaks into chromosomes was already observed (Giles, 1940). IR can induce DNA double-strand breaks formation which are the most lethal among all DNA lesions (Bernhard et al., 2007). The effect of ionizing radiation on DNA can be direct, when the DNA molecule with its solvation layer absorb the energy of the radiation, or indirect, if the effect on DNA is due to molecules by-products of IR on other molecules (Bernhard et al., 2007). When the energy of IR is absorbed by the sugar residues of DNA, it generates deoxyribose radicals and destabilizes the DNA structure inducing breaks. Moreover, the water molecules surrounding the DNA in the solvation layer react with the radiations and undergo radiolysis reaction producing hydroxyl radicals (·OH) that contribute to DSBs formation by an electrophilic attack on DNA strands (Bernhard et al., 2007).

In addition, exposure ionizing radiation can also result in ROS production through water radiolysis generating oxidative damage of DNA and the accumulation of 8-oxoG and thymine glycol (Breimer and Lindahl, 1985; Gajewski et al., 1990).

B) DNA damage by UV light

The ultra-violet (UV: 10-400 nm) is one of the main three types of light that constitute the spectrum of sunlight. The two others are visible (400-700 nm) and infra-red lights (700-1000 nm). UV spectrum is subdivided by wavelength into UV-A (315 to 400 nm), UV-B (280 to 315 nm), and UV-C (100 to 280 nm). As wavelength is inversely proportional to the photon energy ($E=hC/\lambda$, where $E$: energy, and $\lambda$: wavelength), UV-C are the most energetic and thus
the most dangerous. Fortunately, a large proportion of UV-C light is absorbed by the stratospheric ozone layer, while UV-A and UV-B easily penetrate the atmosphere of Earth.

Ultra-violet light creates photochemical crosslinks between adjacent pyrimidines (Lober and Kittler, 1977) generating two major photoproducts, the Cyclobutane Pyrimidine Dimer (CPD) and Pyrimidine-Pyrimidone [6-4] photoproduct ([6-4]PP). Upon UV-light, two thymines form a CPD which is the most frequent UV-photoproduct. This dimer does is not capable of pairing very well with the opposite adenines, because one of the hydrogen bonds is lost at the 5’T (Park et al., 2002). [6-4]PP can rearrange to another photoproduct known as 6-4 Dewar isomer when exposed to UV-A (Rastogi et al., 2010). The [6-4]PP disrupts more the DNA structure than CPD (Kim and Choi, 1995) and is even around 10 times more mutagenic than a cis-syn thymine-thymine CPD (Kamiya et al., 1998). However, CPD is the major cause of UV-induced mutagenesis and tumorigenesis in vivo (Jans et al., 2005) due to its high frequency if compared with [6-4]PP. Both CPD and [6-4]PP form bulky helix-distorting lesions that halt the progression of replicative polymerases Polα and Pol ô in vitro (Johnson et al., 1999; Masutani et al., 1999). Translesion polymerases (TLS polymerases) can efficiently bypass these lesions, in a process known as translesion synthesis (see paragraph 4.1). Then, they are cleaved by enzymatic excision, in a DNA repair process known as Nucleotide Excision Repair (NER) (see paragraph 2.2.1) (Hanawalt et al., 2003). In addition, UV can introduce mutations. C→T transition are the most frequent in skin cancer and constitute a signature of UV (Brash, 1997), although the mechanism behind is not well understood (Ikehata and Ono, 2011).

Ultra-violet light can also activate chemicals generating reactive aromatic compounds that bind to pyrimidine and form inter-strand cross-links (Hearst et al., 1984). One example is psoralens which are vegetal organic compounds that can induce DNA damage in a UV-dependent manner trough intercalating between DNA strands (Averbeck, 1989).

1.2.2. Chemical damage of DNA

In addition to physical agents, different natural or industry-derived chemical compounds, usually electrophilic, can cause DNA damage trough reacting with the nucleophilic nitrogen atoms in nitrogenous bases at physiological conditions (Lindahl, 1993). Microorganisms and marine algae produce a natural alkylating agent, called the methyl
chloride, which can methylate DNA (Crutzen and Andreae, 1990). Methylations very often occur on nitrogen N3 of adenine and the nitrogen N7 of guanine (Lindahl, 1993).

Cis-diamminedichloroplatinum (cisplatin) is a synthetized alkylating-like agent that bridges two adjacent guanine residues (cisPt-1,2-GpG) and forms 1,2-intra-strand cross-link, it is used for its antitumor genotoxic properties (Roberts and Pascoe, 1972). Other antitumor DNA damaging compounds are the Methyl MethaneSulfonate (MMS) alkylating agent and the cross-linking agent MitoMycin C (MMC) (Kim and D'Andrea, 2012).

Car exhausts, cigarette smoke, and burned meat contain a highly carcinogenic polycyclic aromatic hydrocarbon, called Benzo[a]pyrene (Schoket, 1999), which is metabolized in the cell to a very reactive epoxide known as BPDE. This epoxide react with the exocyclic nitrogen N2 of guanine generating bulky DNA lesions called N2-BPDE-guanine (Phillips, 1983). The benzo[a]pyrene induces G→T transversion mutations in vivo (Denissenko et al., 1996).

Aromatic amines are another class of chemical carcinogens (Ames et al., 1973). For example, the N-2-Acetyl-2-AminoFluorene (AAF) is metabolized and produces a highly reactive electrophilic metabolite that can bind covalently the carbon C8 of guanine forming a G-AAF (Kriek et al., 1967) that distorts the DNA double-helix structure (Fuchs, 1975), blocking the replication machinery and inducing frameshift mutations (Belguise-Valladier et al., 1994; Thomas et al., 1994).

2. The cell response to DNA damage

In order to preserve the stability of the genome, it is primordial for cells to develop an appropriate response to DNA damage so as to sense the damage and repair it whenever it is possible.

2.1. Checkpoint activation

Cells have evolved surveillance mechanisms, also known as cell-cycle checkpoints which are crucial for maintaining genomic stability when the integrity of the DNA is compromised (see cell-cycle paragraph 3.7.1) (Hartwell and Weinert, 1989). This surveillance
system was first discovered in budding yeast by isolation of radiation-sensitive mutants (*rad*) as a feedback mechanism restraining mitosis in the presence of DNA damage. Sensing DNA damage is the first step of countering the catastrophic consequences. Checkpoint activation allows cells to detect and tag sites of lesions, thus initiating an appropriate repair response or apoptosis if the damage is irreparable. In so doing, checkpoints act as a barrier to preserve the genome from mutations. Checkpoint genes mutations have been associated with early stages of malignant transformation in sporadic tumours (Bartkova et al., 2005; Gorgoulis et al., 2005; Myung and Kolodner, 2002). The actors of this pathway have been classified into three distinct categories: a) sensors that detect the DNA lesion, b) transducers that amplify and transfer the activation signal, and c) effector proteins which act directly on the cell-cycle (Bartek and Lukas, 2001).

Triggering of the cell-cycle checkpoint pathway relies upon two main protein kinases activated by different sources of DNA damage. These include members of the PI3KK phosphatidylinositol-3-OH kinases such as ATM, ATR and DNA-PK proteins (Ciccia and Elledge, 2010) which sense the DNA damage and activate the two main transducer protein kinases, CHK1 and CHK2 (Abraham, 2001; Ciccia and Elledge, 2010). DNA-PK is also involved in Non-Homologous End Joining (NHEJ, see paragraph 2.2.4) of double strand breaks. Hundreds of potential PI3KK substrates have been identified and are involved in a wide range of pathways, including cell-cycle regulation, repair, apoptosis, and transcription (Matsuoka et al., 2007).

The ATM (Ataxia Telangiectasia Mutated) kinase was identified as mutated enzyme in an autosomal recessive disease characterized by immunodeficiency, neurological disorders, and high cancer incidence (Gatti et al., 1988). It is one of the first proteins to be recruited at double strand breaks (Shiloh, 2003). ATM plays a major role in DSBs signalling, together with a complex made of MRE11, RAD50 and NBS1 proteins (MRN complex), inducing CHK2 phosphorylation. DSBs repair occurs through two main pathways, Homologous Recombination (HR, see paragraph 2.2.5) and non-homologous end joining (NHEJ). NHEJ involves re-ligation of the two broken DNA ends, whereas HR uses the sister chromatid as a template.

The ATR (Ataxia Telangiectasia mutated and Rad3 related) kinase was initially as an ATM homologue (Sanchez et al., 1996). This protein is a major sensor involved in signalling DNA damage at replication forks. It recognizes a wide spectrum of DNA lesions, including stalled forks, single stranded DNA breaks, and CPD dimers (Brown, 2003; Cimprich and
Cortez, 2008). ATR, in a complex with a partner called ATRIP, and the TOPBP1 protein, constitutes the minimal ATR-activating complex.

During S-phase, ATR and ATM response triggers CDC25A phosphorylation via CHK1 and CHK2, promoting thereby its proteasomal degradation (Molinari et al., 2000). As a consequence, CDK2/Cyclin E remains phosphorylated and inactive and CDC45 is not loaded onto origins (Costanzo et al., 2003), inducing an S-phase arrest in order to repair DNA before G2 entry (Mailand et al., 2002).

2.1.1. The ATR-mediated checkpoint

When replications forks stall, cells activate an intra S-phase ATR-CHK1 checkpoint to delay cell-cycle and progression and give more time to replication (Branzei and Foiani, 2010). When activated, the ATR-mediated pathway stabilize pre-existing replication forks trough preventing their collapse or reversal (Lopes et al., 2001; Tercero et al., 2003), and, if needed, promote their restart. In addition, S-phase checkpoint inhibits the replication of late origins, which could be eventually used as backup for stalled fork to restart (Alvino et al., 2007; Santocanale and Diffley, 1998). However, a recent study in yeast propose that replication fork stability is not dependent on S-phase checkpoint activation (De Piccoli et al., 2012).

During DNA replication, the forks can encounter an obstacle such as intrastrand crosslinks, UV-induced distortions (Cimprich and Cortez, 2008) that halt or slow down the replication machinery. In this case, the CMG helicase is not blocked and continues DNA unwinding producing functional replication fork uncoupling (Byun et al., 2005; Tercero et al., 2003), or uncoupling of the leading and lagging strands (Sogo et al., 2002). Thereby, long stretches of single stranded DNA (ssDNA) accumulate (Byun et al., 2005; Walter and Newport, 2000). Certain DNA lesions such as protein-DNA complexes, unprocessed crosslinks, does not generate ssDNA as they block the helicase as well, thus the ATR checkpoint is not immediately activated until the cells attempt to recover arrested forks by recombination (Lambert et al., 2005). Completely consistent with this, following ATM activation (see next paragraph) stalled forks are processed by nucleases, such as EXO1, DNA2, CtIP and MRN (D'Amours and Jackson, 2002), to generate ssDNA (Cotta-Ramusino et al., 2005) and thus induce ATR activation.
It has been assumed that ATR activation during S-phase relies on the accumulation of RPA onto ssDNA that recruits the ATR Interacting Protein (ATRIP) (Zou and Elledge, 2003), and targets ATR to sites of damage (Cortez et al., 2001). However, these studies did not take into account that RPA is essential for establishing active replication forks, thus without RPA, S-phase checkpoint is silent because there is no S-phase. Further work in budding yeast (Lucca et al., 2004) and more recent evidences in Xenopus have shown that neither massive recruitment of RPA at stalled replication forks (Recolin et al., 2012) nor RPA phosphorylation (Recolin and Maiorano, 2012) is required for checkpoint activation, consistent with data in mammalian cells (Lindsey-Boltz et al., 2012). Moreover, another work in mammalian cells demonstrated that ATR activation can occur through RPA-independent mechanism as RPA-deficient cells can phosphorylate ATR upon hydroxyurea or UV (Dodson et al., 2004). In addition, ATRIP binding to RPA is dispensable for CHK1 phosphorylation (Ball et al., 2005), and checkpoint is activated in yeast with very little RPA accumulation at stalled forks (Sogo et al., 2002). Finally, very recent work in S. cerevisiae has shown that ATR activation can also occur in an RPA-independent way (Kumar et al., 2014). There is more and more evidence that ssDNA accumulation is rather the initial signal for checkpoint activation (Betous et al., 2013; Byun et al., 2005; Recolin et al., 2012; Van et al., 2010). When ssDNA is generated at stalled forks, short replication intermediates are produced by slow DNA replication sustained by Polα and δ replicative polymerases (Van et al., 2010), and the TLS polymerase Polκ (Betous et al., 2013). These intermediates serve as a substrate for loading of the checkpoint clamp complex 9-1-1 (RAD9-HUS1-RAD1) (Figure 2.1) (Cimprich and Cortez, 2008). The 9-1-1 clamp is essential for a fully activated ATR response and is loaded at stalled forks by an RFC clamp in which the RFC1 subunit is replaced by RAD17 (Bermudez et al., 2003; Ellison and Stillman, 2003).

In the presence of RPA, the 9-1-1 preferentially binds the 5’ end of ssDNA/dsDNA junctions. Although in yeast, MEC1 (ATR homologue) and DDC1 (RAD9 homologue) show direct interaction (Majka et al., 2006), in human cells ATR and the 9-1-1-clamp are loaded independently (Zou et al., 2002).

TOPBP1 is the third protein of the minimal ATR-activating complex (ATR-ATRIP-TOPBP1). In addition to its role in the pre-IC complex (see paragraph 3.3.3) via its N-terminal domain (Muramatsu et al., 2010), TOPBP1 is also involved in checkpoint activation via its C-terminal domain ((Hashimoto et al., 2006; Parrilla-Castellar and Karnitz, 2003; Yan et al., 2006). TOPBP1 plays the role of an adaptor during the checkpoint response, interacting at the same time with ATR-ATRIP via an ATR Activating Domain (AAD) (Kumagai et al., 2006;
Mordes et al., 2008), and with the RAD9 subunit of the 9-1-1 clamp via its BRCT domains, thereby bridging it to ATR-ATRIP (Delacroix et al., 2007; Lee et al., 2007). It interacts also with MDC1 (Mediator of the Checkpoint 1) the checkpoint transducer which is a necessary CHK1 activation (Wang et al., 2011). The MRN complex is required for TOPBP1 recruitment (Duursma et al., 2013; Lee and Dunphy, 2013). It further interacts with FANCJ helicase during DNA crosslink repair (Gong et al., 2010). Once activated, ATR phosphorylates several substrates among them the histone variant H2AX (Ward and Chen, 2001), this phosphorylation is subsequently amplified through MDC1 recruitment (Wang et al., 2011). Several proteins play the role of transducer, for example Claspin in association with Timeless and Tipin to amplify ATR signalling and sustain CHK1 phosphorylation (Lee et al., 2003; Naylor et al., 2009; Unsal-Kacmaz et al., 2007).

ATR pathway seems to be important for normal DNA replication as ATR (Hekmat-Negad et al., 2000), but also TOPBP1 (Van et al., 2010) are bound to replication forks in normal circumstances and cells deficient in checkpoint proteins display slow fork progression (Petermann et al., 2006; Unsal-Kacmaz et al., 2007). Consistent with this interpretation ATR is an essential gene in mice (Brown and Baltimore, 2000; de Klein et al., 2000).
Figure 2.1 ATR-dependent checkpoint activation

When the replicative polymerase encounter a lesion (such as a CPD dimer generated by UV in the cartoon), it get stalled and the helicase continue DNA unwinding, generating long stretches of ssDNA coated by RPA (purple circles). Consecutively, the DNA damage checkpoint protein complex is recruited and ATR phosphorylates many substrates (see text for more details). This response induce a cell cycle delay in order to allow the cells to activate transcriptional programs and DNA repair pathways. When the cell fail to repair the damage, a cell death programme (apoptosis) is activated.

2.1.2. The ATM checkpoint

When double strand DNA breaks are generated after exposure to ionizing radiations or chemicals such as bleomycin, the ATM-mediated checkpoint is activated (Costanzo et al., 2000). The MRN (MRE11-RAD50-NBS1) complex plays a central role in this pathway by binding the double strand breaks (Lee and Paull, 2004; Uziel et al., 2003). The MRE11 subunit of this complex is an endo/exonuclease that cleaves the broken ends to allow the repair reaction to take place (Buis et al., 2008; Paull and Gellert, 1998; Williams et al., 2008).

When activated, ATM dimer is autophosphorylated and acetylated by TIP60, giving an active monomer (Bakkenist and Kastan, 2003; Sun et al., 2007). DNA double strand break processing is initiated by ATM and MRN that recruits an endonuclease called CtIP (Clerici et al., 2005; Huertas et al., 2008) (Figure 2.2). Then, EXO1 and DNA2 nucleases (Cotta-Ramusino et al., 2005; Hu et al., 2012) with the help of helicases such as the RecQ family member BLM (Mimitou and Symington, 2008) continue the processing. This reaction generates 3’-ssDNA which activates ATR and serves as an intermediate for homologous recombination ((Cimprich and Cortez, 2008; Ira et al., 2004; Kondo et al., 2001). ATM phosphorylates numerous substrates, among them histone H2AX, MDC1 (Lou et al., 2006), 53BP1 (DiTullio et al., 2002), BRCA1 (Venkitaraman, 2002), and CHK2 which promotes S-phase arrest by phosphorylating CDC25A (Falck et al., 2001) (Figure 2.2). The histone H2AX is phosphorylated on serine 139 generating γH2AX (Rogakou et al., 1998) which amplifies and propagates the damage signal, and promotes the chromatin recruitment of downstream repair factors at sites of damage ((Fernandez-Capetillo et al., 2004; Paul et al., 2000; Stucki and Jackson, 2006). Furthermore, H2AX phosphorylation in also involved in facilitating homologous recombination in yeast through cohesins recruitment (Unal et al., 2004).

Some histones are also monoubiquitinated by RNF8 (Huen et al., 2007; Kolas et al., 2007), such as histone H2A and H2AX, generating H2A^Ub and H2AX^Ub. These are then
recognized and polyubiquitinated by the RNF168 E3 ligase (Stewart et al., 2009). Then, the Receptor-Associated Protein 80 (RAP80) detects ubiquitin chains and mediates BRCA1 recruitment onto damage sites (Sobhian et al., 2007).

Histones ubiquitination stabilizes p53-Binding Protein 1 (53BP1) on damaged chromatin, promoting non-homologous end joining of broken DNA ends (Panier and Boulton, 2014). 53BP1 sustains ATM signalling by interacting with RAD50 subunit of the MRN complex (Lee and Paull, 2007), and serves as a scaffold for the recruitment of more DNA double-strand breaks signalling and repair proteins (Panier et al., 2012). It has a major role in the choice of DSB repair pathway together with RIF1 protein, favouring non homologous end-joining through protecting DSB ends from end-resection machinery (Bunting et al., 2010; Chapman et al., 2013), and mutual exclusion with BRCA1 (Chapman et al., 2012; Escribano-Diaz and Durocher, 2013).

Figure 2. 2 ATM-dependent checkpoint activation (Lavin, 2008).

Upon DNA damage, DNA double-strand breaks (DSBs) are generated inducing the relaxation of chromatin that contributes to the activation of ataxia-telangiectasia mutated (ATM) pathway. First, the MRE11–RAD50–NBS1 (MRN) complex is recruited to the break, followed by the recruitment of ATM to regions that flank the break. In these flanking regions, the partial activation of ATM induces the
phosphorylation of p53 and possibly other substrates. ATM is recruited to the site of the break by the MRN complex and phosphorylates members of the complex and other downstream substrates. In response to DNA DSBs, the inactive ATM dimer is monomerized, and autophosphorylation occurs on at least three serine residues: Ser367, Ser1893 and Ser1981. ATM activity is regulated by phosphatases. For instance, protein phosphatase-2A (PP2A) constitutively associates with ATM, and prevents its inappropriate activation by autophosphorylation. In the presence of DNA DSBs, PP2A dissociates from ATM and loses its activity, therefore minimizing the risk of competition between phosphorylation and phosphatase activities. The phosphatase WIP1 and PP5 are also capable of removing phosphates from the three autophosphorylation sites. Acetylation (Ac) also contributes to the process of activation. The acetyltransferase TIP60 is constitutively associated with ATM, and in the presence of a DNA DSB it becomes activated and acetylates ATM at Lys3016 within the C-terminal FATC domain. Lys3016 mutants fail to upregulate ATM activity after DNA damage, prevent monomerization of ATM and inhibit downstream signalling through p53 and checkpoint kinase-2 (CHK2). After ATM recruitment and activation, the cofactor of DNA ligase 4 XRCC4, 53BP1 (p53 binding protein-1), and MDC1 (mediator of DNA-damage checkpoint protein-1) are detected at the break site.

2.1.3. DNA-dependent protein kinase (DNA-PK)

The DNA-dependent Protein Kinase (DNA-PK) is a serine/threonine protein kinase member of the PI3KK family that was discovered more than thirty years ago (Walker et al., 1985). It plays an important role in non-homologous end joining, the major pathway for repairing ionizing radiation-induced DNA double strand breaks in human cells. DNA-PK is composed of a large catalytic subunit (DNA-PKcs) and the Ku70/80 heterodimer (Jette and Lees-Miller, 2015).

Ku70/80 heterodimer was initially discovered as an autoimmune antigen that binds in a sequence independent manner and with high affinity dsDNA ends (Blier et al., 1993; Mimori and Hardin, 1986; Mimori et al., 1990). This dimer forms a basket shaped structure, surrounding double stranded DNA (dsDNA) with the arms allowing its sliding along the DNA in an ATP-independent manner (Walker et al., 2001). How Ku70/80 is removed from DNA is not completely understood although ubiquitination has been recently proposed using both Xenopus egg extracts (Postow, 2011; Postow et al., 2008) and human cells (Feng and Chen, 2012). Once bound to dsDNA ends, Ku70/80 recruits DNA-PKcs (Chan et al., 1996; Gottlieb and Jackson, 1993; Uematsu et al., 2007), and strongly stimulates its kinase activity by 5-10 folds.

The role of DNA-PK in non-homologous end joining (NHEJ) will be addressed in paragraph 2.2.4 below.
2.2. DNA repair

Exposing cells to DNA damaging agents generates lesions, and as for the checkpoint response, depending on the type of lesions different repair mechanisms are activated. Single stranded DNA damage is generally resolved by Nucleotide Excision Repair, Base Excision Repair (BER), or DNA Mismatch Repair. According to the cell-cycle phase, double stranded breaks can be repaired either by non-homologous end joining mainly during G1-phase, or homologous recombination when the sister chromatids are established during S and G2-phases. In addition, interstrand crosslinks are resolved by a multistep pathway, called Fanconi Anemia (FA) repair pathway. All these mechanisms strongly cooperate to maintain the integrity of the genome.

2.2.1. Nucleotide excision repair (NER)

The importance of NER for genome integrity is highlighted by extreme sensitivity to DNA damaging agents particularly sunlight in some rare autosomal recessive human disorders such as Xeroderma Pigmentosum (XP), Cockayne Syndrome (CS), and the photosensitive form of TrichoThioDystrophy (TTD) (de Boer and Hoeijmakers, 1999). When bulky DNA lesions that distort the DNA helix are generated, such as cisplatin adducts, benzo[a]pyrene, and 6-4PP photoproducts, NER proteins are recruited to DNA (Hanawalt et al., 2003). 6-4PP photoproduct are highly distorting lesions, and unlike CPDs they are not efficiently bypassed by the translesion synthesis polymerases. In this case, NER cleaves the 6-4PPs and resolves the replication fork block in an ATR-dependent manner (Auclair et al., 2008).

NER is a complex but highly conserved pathway that can be subdivided into five distinct steps (Figure 2. 3): The first one is recognition of a DNA lesion, depending on the chromatin context, NER can be divided into two sub-pathways: The Global Genomic NER (GG-NER) detecting lesions all over the genome using XPC protein together with its cofactors DDB1 and DDB2 (Hoogstraten et al., 2008; Nishi et al., 2009), and Transcription Coupled NER (TC-NER) involved in actively transcribed regions repair when the transcribing RNA polymerase II encounters a lesion that it cannot bypass, this sub-pathway uses CSA and CSB proteins (Le May et al., 2010). While the recognition step is different between GG-NER and TC-NER, they share all the following reactions (Figure 2. 3). The second step consists in a single strand
incision at both sides of the lesion where the 10 subunit-protein complex TFIIH is recruited and opens up the DNA (de Laat et al., 1999; Giglia-Mari et al., 2004). At this step RPA binds and stabilizes the generated ssDNA, and XPA protein binds TFIIH enabling the accumulation of NER proteins around the damaged site. Then, the lesion-containing single stranded DNA fragment of about 30 nucleotides is cut by the endonucleases XPG and XPF/ERCC1 on the 3’- and 5’- sides of the lesion respectively (Huang et al., 2006b). After this step, excised nucleotides are replaced by replicative DNA synthesis using the undamaged strand as a template. PCNA and RFC seem to be necessary for the gap filling reaction (Shivji et al., 1995). Finally, the remaining ssDNA nick is ligated by DNA ligase III/ XRCC1 (Moser et al., 2007).
During the global genome nucleotide excision repair (GG-NER; left), the damage is sensed by XPC, in complex with UV excision repair protein RAD23 homologue B (RAD23B) and centrin 2 (CETN2), which detect helix-distorting lesions (step 1, left), with the help of the UV–DDB (ultraviolet radiation...
DNA damage-binding protein) complex (step 2, left). RAD23B dissociates from the complex after binding of the XPC complex to the site of damage (step 3, left). In the transcription-coupled NER (TC-NER; right), the detection of the damage is indirect and occurs during transcript elongation by the stalling of RNA polymerase II (RNA Pol II) at a lesion. During this process UV-stimulated scaffold protein A (UVSSA), ubiquitin-specific-processing protease 7 (USP7) and Cockayne syndrome protein CSB transiently interact with RNA Pol II (step 1, right). Upon stalling at a lesion, the affinity of CSB for RNA Pol II increases (step 2, right) and the Cockayne syndrome WD repeat protein CSA–CSB complex is formed, which probably results in RNA Pol II backtracking (step 3, right), making DNA lesion accessible for repair. After damage recognition, the following steps are common between GG- and TC-NER. The TFIIH (transcription initiation factor IIH) complex is recruited to the lesion (step 4). Then, the XPG endonuclease, either associated with TFIIH or separately, binds to the pre-incision NER complex (step 4). The helicase activity of TFIIH further opens the double helix around the lesion, and the TFIIH subunit XPD verifies the existence of lesions with the help of the ATPase activity of the TFIIH XPB subunit and XPA, which binds to single-stranded, chemically altered nucleotides (step 4). In this step the single-stranded DNA binding protein replication protein A (RPA) is also recruited and coats the undamaged strand. XPA recruits a structure-specific endonuclease, called the XPF–ERCC1 heterodimer that interacts with RPA and cut the DNA 5′ to the lesion (step 5). Once this “point of no return” is reached, XPG is activated and cuts the damaged strand 3′ to the lesion, which results in the excision of the lesion within a 22–30 nucleotide-long strand (step 6). The trimeric proliferating cell nuclear antigen (PCNA) ring, which is directly loaded after the 5′ incision by XPF–ERCC1, recruits DNA Pol δ, DNA Pol κ or DNA Pol ε for gap-filling DNA synthesis that can begin immediately after the 5′ incision is made (step 7). DNA ligase 1 or DNA ligase 3 catalyses the final sealing of the nick (step 8).

2.2.2. Base excision repair (BER)

BER is the pathway responsible for removing small, non-helix-distorting base lesions from the genome that could otherwise cause mutations by mispairing or lead to DNA breaks during replication. Oxidized (notably the 8-oxoG), alkylated (3-methylAdenine, 7-methylGuanine) and deaminated (Xanthine and Hypoxanthine) bases account among the main BER substrates. Base excision repair is subdivided according to the size of damaged nucleotide tract into: short-patch BER when it is a single nucleotide, and long-patch BER removing between 2 to 13 nucleotides (Figure 2.4) (Wilson and Bohr, 2007). The first step of this pathway is the cleavage of the N-glycosyl bond by DNA glycosylase enzymes such as OGG1. Then, abasic sites or single stranded breaks are generated after removing the base(s) from the template DNA (van der Kemp et al., 1996). AP endonuclease-1 (APE-1) cleaves the 5′-end of phosphodiester bonds and a 5′ deoxyribose phosphate lyase (dRP) the 3′- end of the sugar residue to remove the rest of the nucleotide (Almeida and Sobol, 2007). For short-patch BER, the gap is filled by a specialized DNA polymerase Polβ which insert a single nucleotide, and
the remaining nick is ligated by DNA ligase III/XRCC1 complex (Campalans et al., 2005). In the situation of long patch-BER, DNA polymerases δ, ε or β, cooperate with a variety of other proteins including PCNA, FEN1, Poly(ADP-Ribose) Polymerase 1 (PARP1), and Ligase I to fill the gap (Almeida and Sobol, 2007).

![Figure 2. 4 Base Excision Repair (BER) pathway (Almeida and Sobol, 2007)](image)

BER is initiated by DNA glycosylase enzymes that cleave the N-glycosyl bond, followed by strand scission by APE1endonuclease. Then, a 5’ deoxyribose phosphate lyase (dRP) the 3’- end of the sugar residue to remove the rest of the nucleotide. During short-patch pathway (left), pol β incorporates a nucleotide and the resulting nick is ligated by a complex of XRCC1 and LigIIIα to complete the pathway. Long-patch pathway (right) is responsible for repair under conditions of 5’ lesions refractory to pol β cleavage. In this case, BER complex formation shifts, and nucleotide incorporation is conducted either by pol β or is transferred to pol δ or pol ε. The refractory 5’ moiety is removed by FEN1 and re-ligation is completed by LigI.
2.2.3. Mismatch repair (MMR)

The mismatch repair pathway is a system that recognizes and repairs small erroneous insertion, deletion, and also bases mis-incorporation that can escape to proofreading activity of polymerases and arise during DNA replication and recombination, as well as repairing some forms of DNA damage (Iyer et al., 2006). Mismatch repair is a strand-specific highly conserved process that recognizes the nascent DNA strand that, generally, includes errors. The actual model is eukaryotes is that the MMR machinery travels along the DNA together with the replication machinery. Initially, the MSH2-MSH6 heterodimer (MutSα homologue) and/or to a lesser extent the MSH2-MSH3 heterodimer (MutSβ homologue) recognizes and binds the mismatch. Then, the endonuclease MLH1-PMS2 heterodimer (MutLα homologue) that possesses a weak ATPase activity is recruited and introduces single-strand breaks at distal sites of the mismatch (Hong et al., 2008; Kunkel and Erie, 2005). After this ExoI exonuclease is loaded and will remove the mismatch (Schmutte et al., 2001; Tishkoff et al., 1997). The resulting single-stranded DNA gap is then filled by replicative polymerases together with its associated cofactors PCNA and RFC, and the resulting nick is sealed by DNA ligase I (Jiricny, 2006). MMR can also cooperate with translesion polymerase η to prevent 8oxoG-induced mutations (van der Kemp et al., 2009a).

2.2.4. Non homologous end joining (NHEJ)

Although homologous recombination is the most faithful way to repair DSBs using sister chromatid, NHEJ repairs the majority of DSBs throughout the cell-cycle in human cells (Rothkamm et al., 2003; Takata et al., 1998), whereas homologous recombination occurs in S-G2 when a donor template is available (Saleh-Gohari and Helleday, 2004). It is astonishing how such a low-fidelity pathway has evolved to dominate DSB repair and the molecular mechanism behind this choice remain unclear (Liu et al., 2014). There are two forms of NHEJ: canonical and alternative.

During Canonical Non-Homologous End Joining (C-NHEJ), the Ku heterodimer (consisting of Ku70 and Ku80 encoded by XRCC5 and XRCC6 genes respectively) recognizes and binds rapidly the broken DNA termini (Dynan and Yoo, 1998), protecting and stabilizing DNA ends and serving as a docking platform for the others NHEJ factors (Figure 2. 5). Then,
Ku heterodimer directly recruits the catalytic subunit DNA-PKcs to the damage sites (Chu, 1997; Critchlow and Jackson, 1998; Weaver, 1996), leading to the formation of a kinase complex DNA-PK (Collis et al., 2005), that has been shown to phosphorylate a wide range of substrates in vitro, but only Artemis protein (Soubeyrand et al., 2006) and DNA-PKcs itself (Uematsu et al., 2007) have been so far identified as in vivo substrates. In many cases, DNA ends cannot be directly re-joined, and need to be processed using several processing factors such as PNKP (PolyNucleotide Kinase-Phosphatase), Artemis and WRN (Werner syndrome) (Davis and Chen, 2013). Following this, the resulting DNA gaps are filled by DNA polymerases μ and λ, and then Ku promotes the recruitment of a complex composed of XRCC4 (X-Ray Cross Complementing protein 4), DNA ligase IV and XLF (XRCC4-Like Factor) (also called NHEJ1) (Buck et al., 2006; Wu et al., 2007) to ligate DNA ends (Critchlow et al., 1997; Modesti et al., 1999).

Alternative Non-Homologous End Joining (A-NHEJ) often uses microhomology in the vicinity of DSBs, this is why it has been frequently referred to as Microhomology-Mediated End-Joining (MMEJ) (Figure 2. 5), although not all A-NHEJ needs microhomology for activity (Mansour et al., 2010). It is called “alternative” because it was first described as a pathway activated when C-NHEJ is impaired, yet recent evidence showed that it can be substantially activated when HR and C-NHEJ are still functional (Truong et al., 2013). PARP1 initiates A-NHEJ by competing out Ku for DSB ends binding (Cheng et al., 2011; Wang et al., 2006). Then, MRN, CtIP and BRCA1 drive the ends resection (Zhong et al., 2002). AT this step, 53BP1 can block the resection to promote more accurate C-NHEJ (Bothmer et al., 2011; Bothmer et al., 2010). The ligation during A-NHEJ is different from C-NHEJ and utilizes instead of Ligase IV, either Ligase III/ XRCC1 complex (Della-Maria et al., 2011; Liang et al., 2008b), or Ligase I (Liang et al., 2008b).

2.2.5. Homologous recombination (HR)

When long DNA gaps are generated, or DNA undergoes DSBs or Inter Strand Crosslinks (ICLs), homologous recombination is the most faithful and non-mutagenic DNA repair pathway as it uses the non-damaged homologous sequence as a template for re-synthesis of damaged DNA. Hence, HR is restricted to S and G2-phases during the cell-cycle, when the sister chromatid is available and can be used as a template. Moreover, an open chromatin state
facilitates HR (Munoz-Galvan et al., 2013; Panier and Boulton, 2014). In addition to its role in DNA damage repair, HR is involved in replication fork stability, telomere maintenance and in recombining parental chromosomes crossover during meiosis.

HR starts with the processing of the DNA damage into a clean DSB because it often contains altered nucleotides especially when generated by Ionising Radiations (IR) or radiomimetic drugs (Figure 2. 5) (Paques and Haber, 1999). Then, the MRN complex resects the complementary strand at DSB in 5'- to 3' direction to expose the 3'-OH ssDNA overhanging tail (Mimitou and Symington, 2008). CtIP, DNA2 and EXO1 also participate to this step (Sartori et al., 2007; Zhu et al., 2008). Once generated, the 3'-OH overhanging tails are coated first by the ssDNA binding protein RPA and activates the DNA damage checkpoint (Byun et al., 2005). The next step is the ssDNA-RAD51 filament formation where RAD51 in a ternary complex with ATP (Conway et al., 2004) replaces RPA and forms a helical filament around the ssDNA (Sung, 1994). As RAD51 displays a lower affinity to ssDNA than RPA, it needs to cooperate with several cofactors such as the RAD51 paralogs RAD51B, RAD51C, RAD51D, XRCC2, XRCC3, and RAD52 (Mortensen et al., 2009). The BRCA2 tumour suppressor, thanks to its 9 RAD51-binding sites, promotes nucleation and stabilization of RAD51-ssDNA filaments, in addition to its protective role against excessive resection (Holloman, 2011; Schlacher et al., 2011). Then, the RAD51-coated ssDNA filament carrying the cofactors searches a homology in the sister chromatid and invades it by base-pairing with one of the strands and displacing the other strand, creating a Displacement-loop (D-loop) structure. DNA synthesis of the lacking fragment then starts using the newly annealed strand as template and the 3’-end of the invading strand as a primer. At this step different mechanism leading to different outcomes can be engaged. During Synthesis-Dependent Strand Annealing (SDSA), after a short elongation, the RTEL helicase induce RAD51 filament displacement and D loop dissociation (Barber et al., 2008) and the invading strand anneals back to the processed second end of the original break giving a localized recombination without crossover (West, 2003). Alternatively, the D-loop structure gives a complete replication fork that could replicate the entire distal chromosomal part, without forming crossovers (Figure 2. 5).

In another pathway, the invading strand could be ligated with the second end captured by D loop to form double Holliday Junctions (dHJs) (West, 2003). The second end capture is mediated by RAD52 which replaces RPA from the ssDNA and anneals the free DNA end to the D-loop. The dissolution of HJ intermediates occur through the action of BLM/TOPOIII complex (Bachrati et al., 2006), Otherwise they are cleaved by the endonucleases called
resolvases (Castor et al., 2013), including GEN1, MUS81/EME1, or SLX1/SLX4 to generate either crossover or non-crossover of sequences flanking the dHJ (Andersen et al., 2009; Ciccia et al., 2008; Fekairi et al., 2009). As crossovers can lead to loss of heterozygosity and genomic rearrangements in mitotic cells, they are tightly regulated.
**Figure 2. 5 Double strand breaks repair** *(Ciccia and Elledge, 2010)*

(A) Non-homologous end joining (NHEJ) is promoted by the rapid association of Ku to DSBs and the recruitment of DNA-PKcs. Sequential phosphorylation events on multiple DNA-PKcs amino acid clusters triggers the initial processing of DNA ends by ARTEMIS, followed by DNA-PKcs-dependent protection of DNA ends required for DNA ligation (see text for details). (B) During S and G2-phases, MRN complex is recruited to DSBs by PARP in competition with Ku, and mediates the initial stages of DSB resection together with CtIP and BRCA1 to promote homologous recombination (HR). 53BP1 has an inhibitory role on DSB resection and is negatively regulated by BRCA1. When NHEJ fails to resolve the break, the MRN/CtIP/BRCA1 complex can also promote DSB resection following DNA ends deprotection. EXO1 and BLM carry-out extensive DSB resection and formation of RPA-coated 3′-ssDNA ends. Then, BRCA2 induces the displacement of RPA from the 3′-ssDNA ends and assembly of RAD51 filaments, leading to strand invasion into homologous DNA sequences. D-loop structures formed after strand invasion can be cleaved by MUS81/EME1 or displaced by RTEL1 during SDSA to generate crossover or non-crossover events, respectively. Non-crossovers are generated also by dissolution of Holliday junctions (HJs) by the BLM/TOPOIII complex, whereas HJ resolution by the nucleases GEN1 and SLX1/SLX4, which associates with MUS81/EME1, can generate both crossover and non-crossover events. (C) Limited DSB resection carried out by CtIP and MRN in G1 results in alternative NHEJ. (D) Following DSB resection, 3′-ssDNA ends with homologous sequences can be directly annealed by RAD52.

2.2.6. Fanconi anemia pathway (FA)

Fanconi anemia is a rare human autosomal recessive genetic disorder characterized by bone marrow failure, genomic instability, and cancer predisposition. Cells derived from FA patients are extremely sensitive to DNA interstrand cross-linking agents such as cisplatin and mitomycin C due to interstrand cross-links repair defect. To date, nineteen genes involved in FA pathway have been identified, mutations in which cause FA. The proteins encoded by these 19 genes participate to different steps of ICL-repair. Based on their function, they can be theoretically sorted into 3 groups *(Wang, 2007)*. The first group includes FANCA, B, C, E, F, G, L, and M proteins that associate with three other proteins FAAP20, FAAP24 and FAAP100, forming together a large FA core complex with an E3 ubiquitin ligase activity *(Figure 2. 6, group I)*. Upon DNA damage or replication stress, the core complex binds to chromatin *(Kim et al., 2008; Qiao et al., 2001)*, where it mono-ubiquitinates the heterodimer ID2 complex made of FANCI and FANCD2 group II proteins *(Garcia-Higuera et al., 2001; Smogorzewska et al., 2007)* *(Figure 2. 6, group II)*. Then, Mono-ubiquitinated ID2 binds to chromatin and helps to resolve ICLs. During ICL repair, FANCM forms a heterotetrameric complex with FAAP24 and a dimer of MHF1 and MHF2 (also known as FAAP16 and FAAP10) *(Ciccia et al., 2007; Singh
This subcomplex recognizes specific DNA structures and allows the core complex recruitment to chromatin (Deans and West, 2009; Kim et al., 2008). In addition, it regulates the repair and the checkpoint signalling (Collis et al., 2008; Huang et al., 2010a). Using its RING domain, FANCL ubiquitinates ID2, with the help of the catalytic module formed by FANCB, FANCL, and FAAP100 (Ling et al., 2007; Meetei et al., 2003; Rajendra et al., 2014). This ubiquitination reaction requires also FANCT (UBE2T) E2 ubiquitin-conjugating enzyme (Alpi et al., 2008; Machida et al., 2006). FANCC, FANCE, FANCF and FANCA, FANCG, FAAP20 form two other subcomplexes helping catalytic subcomplex chromatin binding (Huang et al., 2014).

![Fanconi Anemia (FA) pathway protein complexes](Duxin and Walter, 2015)

The FA pathway comprises 19 proteins that have been classified into three groups. Upon detection of the crosslink, the FA core complex (group I, blue spheres) ubiquitinates the heterodimer FANCI-FANCD2 (ID2) (group II, green spheres). Ubiquitinated ID2 then coordinates processing by downstream repair factors (group III, orange spheres). Proteins in grey are important for ICL repair and can be classified as group I–III, but they have not been found to be mutated in patients with FA. Although BRCA1 and RAD51 are considered to fall into group III, they also have functions upstream of ID2 ubiquitination.
When ID2 is mono-ubiquitinated, it promotes group III proteins recruitment (Figure 2.7, step C). This group includes all the protein involved in the repair reaction, amongst them nucleases XPF (FANCQ) (Bogliolo et al., 2013) that induces incisions on either side of the ICL, SLX4 protein (FANCP) that serves as a scaffold (Figure 2.7, step D) (Kim et al., 2011b). After that, a two-ended double strand break is generated by the incisions on one of the sister chromatids whereas on the other it leaves a DNA adduct. TLS synthesis removes the adduct in a two-step reaction involving REV1 and pol ζ interaction with FA core complex and NER pathway (Figure 2.7, step E) (Kim et al., 2012a; Raschle et al., 2008). Finally, RAD51-mediated HR repairs the generated DSB using the intact sister chromatid as a homology donor (Figure 2.7, step F) (Long et al., 2011).
(A) DNA ICL that covalently links the two DNA strands and blocks the progression of two convergent forks (B) The FANCM–FAAP24–MHF1/2 complex recognizes the stalled replication fork structure and recruits the FA core complex to the ICL region. FANCM prevents the collapse of replication fork independently of FA pathway activation using its translocase activity. FANCM also initiates ATR-CHK1-dependent checkpoint response, which in turn phosphorylates multiple FA proteins, including FANCA/E/D2/I. (C) The FA core complex function as an E3 ubiquitin ligase and monoubiquitinites FANCD2 and FANCI, promoting the recruitment of the ID heterodimeric complex to the DNA lesion. (D) FANCD2UB serves as a platform to recruit nucleases that cut the flanking regions of the ICL. Many nucleases are involved in this process, including FANCP/SLX4, which interacts with ERCC1–XPF and MUS81–EME1 structure-specific nucleases, and FAN1 5'-flap endonuclease. (E) Unhooking leaves nucleotide adduct tethered to the complementary strand, which is bypassed by TLS polymerases such as REV1 and Pol ζ. (F) HR plays a role in repairing the DSB created by the incision. Downstream FA proteins promote RAD51-dependent strand invasion. Finally, NER removes remaining adducts and fills the gap. (G) At the end of this repair pathway, FANCD2-I are deubiquitinated by the USP1–UAF1 DUB complex.

3. DNA replication: Duplication of the Genome

3.1. Historical overview of DNA replication model

In 1953, Watson and Crick proposed a model to describe the molecular secondary structure of DNA based on X-ray crystallography data provided by Franklin (Franklin and Gosling, 1953). Shortly after, they also proposed a model to describe how DNA may be copied inside a cell. In their model, Watson and Crick proposed that the DNA double-helix unwinds and each strand serves as a template for semiconservative synthesis of a nascent strand (Watson and Crick, 1953). Watson and Crick figured that this model would result in two new double strands of DNA, each one with one strand of parent (or template) DNA and one strand of daughter (or newly-synthesized) DNA. They called this the semi-conservative model, because half of the parent DNA was conserved in each newly synthetized DNA molecule. However at that time, many scientists were sceptical about their model, and even doubted whether they were right about the structure as a double helix. Some scientists wondered how it could be possible that the double helix open itself up without getting tangled or torn apart. So they thought up some other ideas about how DNA replication works. One hypothesis, called the dispersive model, suggested that DNA only copied itself for short chunks at a time, producing new strands that alternated parent and daughter DNA (Forro, 1965; Williamson and Fennell, 1974). Another idea, called the conservative model, argued that DNA did not split open at all,
but somehow kept the parent strands intact while creating an entirely new and separate copy (Figure 3.1).

Five years later, Matthew Meselson and Franklin Stahl performed an ingenious experiment in bacterial cells from *Escherichia coli* (*E. coli*) that confirmed the model proposed by Watson and Crick. They analysed what happens to one parental DNA strand as it generates copies by incorporating the heavy isotope $^{15}$N into the cellular DNA. In so doing, they provided a demonstration for the semiconservative nature of DNA replication (Meselson and Stahl, 1958). During the following years, much of our understanding of the mechanism of DNA replication in eukaryotes was unveiled from studies of *in vitro* replication of the simian virus 40 (SV40) chromosome in mammalian *cell-free* systems (Li and Kelly, 1984; Stillman, 1989).

Nowadays, the semiconservative model applies to all organisms. DNA replication is bidirectional and semi-discontinuous. In this model, replication begins at several defined sites in the genome, called origins of replication (Oris). When these origins are fired, two DNA replication forks carrying all the replication machinery move away in opposite directions along the DNA molecule forming a replication bubble (Danna and Nathans, 1972; Huberman and Riggs, 1966; Kaguni et al., 1982; Masters and Broda, 1971). The DNA double helix is antiparallel, meaning that one strand is oriented in a 5'- to 3' direction and its complementary strand is hybridised to it in the 3'- to 5' orientation. Replicative DNA polymerases need a free 3'-OH end of a nucleotide chain to add further nucleotides and consequently DNA polymerization can occur only in the 3'- to 5' direction. This imperatively implies that only one strand from the antiparallel oriented original double stranded DNA molecule can be synthesized continuously (called the leading strand), giving rise to a continuous nascent strand, whereas the other strand (called the lagging strand) has to be replicated discontinuously from short RNA-primed DNA stretches, called Okazaki-fragments (Okazaki et al., 1968), which ultimately will be ligated to each other.

DNA replication process can be separated into three main different steps: the initiation step, by which replication origins are established and activated, the elongation corresponding to the synthesis and extension of DNA chains from these origins, and the final DNA replication termination step when the fork reaches a second fork that was replicating DNA from an adjacent origin.
Figure 3. 1 DNA replication models (PJ Russell, iGenetics 3rd ed. 2010).

(A) In the semi-conservative model, the two parental strands are separated and each serves as a template for making a copy of itself. After one round of replication, the two daughter molecules comprise each one old and one newly synthetized strand. Note that after two rounds, two of the DNA molecules consist only of new material, while the other two contain one old and one new strand.

(B) In the conservative model, the parental molecule directs synthesis of an entirely new double-stranded molecule, such that after one round of replication, one molecule is conserved as two old strands. This is repeated in the second round.

(C) In the dispersive model, material in the two parental strands is distributed more or less randomly between two daughter molecules. In the model shown here, old material is distributed symmetrically between the two daughter molecules. Other distributions are possible.

3.2. Origins of DNA replication

About fifty years ago, Jacob, Cuzin, and Brenner proposed the replicon model of DNA replication to describe chromosomal duplication in *E. coli* (Cuzin and Jacob, 1963; Jacob and Brenner, 1963). This model predicted that a positive *trans*-acting protein or protein complex, called the initiator, would activate DNA replication at or nearby a *cis*-acting sequence corresponding to replication origins, called the replicator. Subsequently, this model was validated by other studies, first in prokaryotes (Ogawa et al., 1985; van der Ende et al., 1985). Kornberg and Baker identified the dnaA protein as the initiator and oriC as the replicator for replication of the *E. coli* chromosome (Baker et al., 1986). The finding that a number of
bacteriophage and eukaryotic viruses use a similar mechanism to replicate their genomes supports the generality of this elegant and quite simple model (Challberg and Kelly, 1989; Stillman, 1989). In eukaryotic cells, the problem of chromosomal replication is much more complex and this model applies only partially. Instead of a single origin of replication, hundreds of origins (as in yeast) to many thousands (as in mammals) must be coordinately initiated and regulated. In addition, the timing and extent of replication are precisely controlled during this process. Interestingly, whereas eukaryotic initiator proteins are relatively well conserved throughout evolution, replicator sequences are not. Many features of eukaryotic origins are still unknown and no consensus motif with predictive value has been found yet, despite genome-wide studies of replication origins from multicellular organisms achieved recently (Cadoret et al., 2008; Cayrou et al., 2011).

DNA replication requires the coordinated action of a large number of enzymes and regulatory proteins forming a complex replication multifunctional machinery known as the “replisome” (Johnson and O'Donnell, 2005). In viruses and prokaryotes, origins contain specific DNA sequences containing several inverted GAGGC repeats and an AT-rich tract recognised by DNA-binding proteins that recruit initiator factors, leading to the assembly of the replisome (Borowiec et al., 1990).

In the budding yeast Saccharomyces cerevisiae (S. cerevisiae), replication origins were first isolated as Autonomously Replicating Sequences (ARS) that confer to a bacterial plasmid the ability to replicate autonomously in yeast (Stinchcomb et al., 1979). It was later shown that the ACS is the binding site for the Origin Recognition Complex (ORC, see below), a multiprotein complex that marks the origin and subsequently serves as a landing platform for the assembly of the other replication proteins. In the yeast S. cerevisiae there are around 400 origins of 100-200 bp characterised by well-defined sequences with a uniform distribution along the genome (Raghuraman et al., 2001). Interestingly, in the related yeast Schizosaccharomyces pombe (S. pombe) origins of DNA replication are larger (>500 bp) and AT-rich but are not defined by a strict consensus sequence, and at least half of the intergenic regions have potential origin activity (Dai et al., 2005), thus resembling more to those found in metazoans (Dubey et al., 1994; Maundrell et al., 1988; Segurado et al., 2003). In metazoans, thousands of origins are activated during replication (Huberman and Riggs, 1966). The structure of the eukaryotic origins is complex and remains elusive (Mechali, 2001; Mechali and Kearsey, 1984) with no consensus sequence and variable sequence composition and lengths. Several
signatures and features of metazoans origins have emerged recently (Figure 3. 2), including unmethylated CpG islands, AT-rich tracts, DNA loops, DUEs (DNA Unwinding Elements), histone modifications and G quadruplexes (Cayrou et al., 2011; Mechali, 2010). Furthermore, the position of origins may be also influenced by transcription initiation sites and chromatin structures, and can change during cellular differentiation (Mechali, 2010).

Figure 3. 2 Features of DNA replication origins in Metazoans (Méchali Nat Rev Mol Cell Biol, 2010).

Several features have been described for metazoan replication origins, but they are not widely distributed amongst all origins. In fact, they represent different marks or modules that can contribute to the selection of a given origin. At the sequence level, AT-rich elements and CpG islands have been reported as well as DNA regions that easily unwind (DNA unwinding elements (DUEs)), but their importance or role remains elusive. Concerning the structural characteristics, bent DNA (or cruciform DNA) and the formation of loops at matrix attachment region (MAR) has been described. At the chromatin level, histone acetylation, nucleosome-free regions, and DNase-sensitive sites have been seen, but their direct contribution in origin recognition is sometimes difficult to estimate. The possible links of transcription with replication origin recognition have been described, but evidence for direct interactions between replication origin factors and transcription factors are missing.

3.3. Initiation of DNA Replication

The initiation of DNA replication at origins is a tightly regulated process and must occur only once per cell-cycle in order to maintain constant the ploidy of a cell and thus ensure genome stability. For this, chromosomes are licensed to replicate only once during a cell-cycle by building up an unstable pre-replication complex (see below). In E. coli, the initiation of DNA replication is mediated by DnaA, a protein that binds to a region of the origin (OriC) known as the DnaA box. This is where the replication fork will form. Then, DnaA recruits a homohexameric helicase (DnaB proteins) to DNA. Recruitment of helicase requires six DnaC
proteins. DnaC is then released, DNA is unwound by the helicase forming the first replication bubble, and leading to assembly of the pre-priming complex. In order to stabilize the replication bubble, SSB protein is needed to prevent the single strands of DNA (ssDNA) from forming any secondary structures and to prevent them from reannealing. The unwinding of DNA by DnaB helicase allows for primase (DnaG) and RNA polymerase to prime each DNA template so that DNA synthesis can initiate and finally polymerase III starts the synthesis of the new DNA strands (Kaguni and Kornberg, 1984; Zakrzewska-Czerwinska et al., 2007).

In eukaryotes, the initiation step can be divided into two temporally distinct events (Diffley, 1994). First, the **origin licensing**, corresponding to recruitment of the DNA helicase MCM2-7 at origins in late M- and very early G1-phase (Diffley, 1996; Maiorano et al., 2000a; Nishitani et al., 2000; Tada et al., 2001), which corresponds to assembly of a **pre-Replication Complex** (pre-RC). However, the helicase is thought to remain inactive until the onset of S-phase. Then, the **origins firing** occurs at early S-phase, when the MCM2-7 complex is activated by cell-cycle kinases, unwinding the DNA double helix so as to allow recruitment of the replisome components (Mendez and Stillman, 2003). During this step, the pre-RC complex is converted into the **pre-Initiation Complex** (pre-IC). It is crucial to prevent a single origin from firing twice per cell-cycle. To do so sophisticated mechanisms including CDK activity and the ubiquitin proteasome system play a crucial role to ensure a temporal exclusion between origin licensing and origin firing (Blow and Dutta, 2005). These regulation mechanisms will be detailed in paragraph 3.7.

In this chapter, I will address the current knowledge concerning origin licensing and origin firing. Then, the different steps of DNA replication, including priming, elongation, and termination. Followed by a description of regulation mechanisms and specificities of DNA replication during early embryogenesis.

### 3.3.1. Recognition of origins by ORC

DNA replication starts with binding of the hetero-hexameric ORC complex to replication origins. This complex was originally identified as an ARS consensus sequence binding protein complex in budding yeast (Bell and Stillman, 1992; Diffley, 1992), and homologues of all six subunits (ORC1–6) have since been identified in all eukaryotic species examined, including humans (Bell and Dutta, 2002; DePamphilis, 2003). All these subunits are
relatively high conserved in eukaryotes, except the ORC6 subunit, which shows structural similarity to the other subunits (Duncker et al., 2009). Only ORC1 and ORC5 possess an ATPase activity and this activity is essential for ORC function (Bell and Dutta, 2002). In addition, ORC also plays a role in heterochromatin formation in mammalian cells and transcriptional silencing in budding yeast (Sasaki and Gilbert, 2007). ORC binds AT-rich DNA tracts in humans with no intrinsic sequence specificity explaining the absence of consensus origins sequences (Vashee et al., 2003).

3.3.2. Origin licensing: the pre-replication complex

Replication origins are licensed for replication by loading of the MCM 2-7 complex to form the pre-RC (Blow and Dutta, 2005; Maiorano et al., 2000a; Nishitani et al., 2000; Tanaka et al., 1997). Once bound to replication origins, the pre-RC remains without changes until the onset of S-phase when it will be eventually converted into a pre-IC.

ORC recruits the Cell Division Cycle 6 protein (CDC6) which is an ATPase. The ORC/CDC6 complex serves as an ATP driven helicase loader (Randell et al., 2006). The CDT1 protein (CDC10 dependent transcript 1, Hofman and Beach 1994) is an essential factor for MCM2-7 loading in yeast (Devault et al., 2002; Nishitani et al., 2000; Tanaka and Diffley, 2002) and Metazoans (Maiorano et al., 2000b; Wohlschlegel et al., 2000). It recruits two MCM2-7 hexamers via interaction with ORC6 (Chen et al., 2007; Remus et al., 2009; Takara and Bell, 2011). The activity of CDT1 is tightly regulated during the cell-cycle and is inhibited by the Geminin protein (Tada et al., 2001; Wohlschlegel et al., 2000). In Metazoans, CDT1 can also recruit HBO1 histone acetyl transferase at origins stimulating MCM2-7 loading via histone H4 acetylation (Miotto and Struhl, 2008, 2010). This acetylation could be part of a mechanism for origins selection in higher eukaryotes (Chadha and Blow, 2010) although histone acetylation is not always associated with active origins (Mechali, 2010).

The MCM2-7 complex consists of six closely related proteins that are highly conserved throughout the eukaryotic kingdom (Kearsey et al., 1996). Genes coding for this helicase complex were identified in 1984 as essential genes for minichromosome maintenance by genetic screen in budding yeast (Maine et al., 1984). The MCM2-7 complex is believed to be the eukaryotic replicative DNA helicase (Labib and Diffley, 2001; Labib et al., 2000; Pacek and Walter, 2004). It is required for both initiation and elongation of DNA replication. In Xenopus
and yeast, MCM2-7 forms a double-hexamer when bound to DNA origins prior to DNA replication and this configuration is compatible with a bidirectional replication fork (Remus et al., 2009) (Evrin et al., 2009; Gambus et al., 2006). Instead of two hexamers, around 10 to 40 MCM2-7 hexamers are loaded at each origin (Edwards et al., 2002). This excess of MCM2-7 complexes has been suggested to serve as backup helicases to activate dormant origins following replication stress (Woodward et al., 2006).

3.3.3. Origin firing: the pre-initiation complex

The MCM2-7 helicase is activated and the pre-RC is converted into the pre-IC thus allowing bidirectional DNA unwinding and producing a replication bubble (Figure 3. 4). This activation relies upon the concerted action of S-phase Cyclin-Dependent Kinases (CDKs) and the CDC7-DBF4/DRF1, also called the DBF4-Dependent Kinase (DDK) (Zou and Stillman, 1998, 2000). CDKs and CDC7 activities are tightly regulated during the cell-cycle and depend upon levels of their regulatory subunits: S-phase cyclins and DBF4/DRF1 respectively, whose expression increase at the onset of S-phase (Jares and Blow, 2000; Morgan, 1997). Once activated, CDKs and DDK promote recruitment at the pre-RC of several replication factors to form a large complex known as pre-initiation complex (Mendez and Stillman, 2003). In yeast and metazoans, MCM2-7 activity requires a larger complex formed together with CDC45 (Hopwood and Dalton, 1996) and the GINS complex (Sld5, Psf1-3) (Kubota et al., 2003; Takayama et al., 2003), called the CMG (CDC45-MCM2-7-GINS) complex that unwinds the DNA during DNA replication (Aparicio et al., 2009; Gambus et al., 2006; Moyer et al., 2006; Pacek et al., 2006). The pre-IC complex includes also other components, namely: MCM10 (a protein unrelated to the MCM2-7 family), TOPBP1, RECQL4, and TRESLIN (Bell and Dutta, 2002; Mechali, 2010; Schepers and Papior, 2010). RECQL4 (Sld2 in yeast) is necessary for Replication Protein A (RPA) loading, the ssDNA binding protein that stabilizes the replication bubble (Wold, 1997) before polymerase recruitment (Masumoto et al., 2002; Sangrithi et al., 2005). TRESLIN (Sld3 yeast orthologue) interacts with TOPBP1 BRCT domains when it is phosphorylated by CDKs (Tanaka et al., 2007; Zegerman and Diffley, 2007) and recruits TOPBP1 to CMG complex (Heller et al., 2011). TOPBP1 (Dpb11 in S. cervisiae or Cut5 in S. pombe), is required for DNA replication initiation and recruitment of the replicative polymerase
All the six subunits of the MCM2-7 contain conserved Walker A and Walker B motifs and thus are AAA+ ATPases. This complex has low enzymatic activity on its own, whereas the CMG has increased unwinding and ATPase activity (Ilves et al., 2010). Recent studies, using electron microscopy, have suggested that CDC45 and GINS within the CMG complex preserve the ring-like structure of the MCM2-7 helicase (Costa et al., 2011).

MCM10 is another DNA replication factor that interacts with MCM2-7 complex downstream of CDC45 and GINS and is required for Pol α, Pol δ and RPA recruitment (Heller et al., 2011; Kanke et al., 2012; van Deursen et al., 2012). It is also required for both initiation and elongation of DNA synthesis (Homesley et al., 2000; Ricke and Bielinsky, 2004). MCM10 does not share sequence similarities with the other MCM proteins, although it was one of the mutated genes in mini-chromosomes maintenance screen (Maine et al., 1984). MCM8, discovered first in mammalian cells (Gozuacik et al., 2003), is an MCM2-7-related protein that displays in vitro helicase activity required for normal progression of replication forks in Xenopus egg extracts (Gambus and Blow, 2013; Maiorano et al., 2005a), and Drosophila (Crevel et al., 2007). A study in mammalian cells proposed that MCM8 may be a component of the pre-RC by facilitating CDC6 chromatin loading (Volkening and Hoffmann, 2005). However this function has not been confirmed by other groups (Gambus and Blow, 2013; Park et al., 2013). Finally, another MCM2-7-related protein, MCM9, was identified (Lutzmann et al., 2005) and proposed to be a component of pre-RC in Xenopus (Lutzmann and Mechali, 2008, 2009). This function has not been confirmed in both Xenopus and mammalian cells (Gambus and Blow, 2013; Park et al., 2013). However MCM9 appears to be important to restart stalled or paused replication forks by homologous recombination in a complex with MCM8 (Lutzmann et al., 2012; Nishimura et al., 2012) and is also involved as a helicase in mismatch repair (Traver et al., 2015).
The figure shows three adjacent replication origins. (A) Licensing of replication origins during the G1-phase of the cell cycle by the sequential loading of pre-replication complex (pre-RC) proteins on all potential origins in the genome. First, the origin recognition complex (ORC, comprising the six subunits ORC1–6) is recruited to replication origins. Then, CDC6 and CDC10-dependent transcript 1 (CDT1) bind to the ORC. The last step of the licensing reaction is the loading of the mini-chromosome maintenance (MCM) helicase complex, containing the six subunits MCM2–7. (B) At the G1-S transition, origins are activated, and this step implicates the formation of a pre-initiation complex (pre-IC) and the activation of the MCM helicase complex. Assembly of the pre-IC is triggered by DBF4-
dependent kinase (DDK) and cyclin-dependent kinases (CDKs), and its activation into a functional replisome occurs in the S phase. DDK and CDKs phosphorylate several replication factors (of which MCM10, CDC45, ATP-dependent DNA helicase Q4 (RECQL4), TRESLIN, GINS, DNA topoisomerase 2-binding protein 1 (TOPBP1) and DNA polymerase ε (Pol ε) are the most important) to promote their loading on origins. Moreover, DDK and CDKs directly phosphorylate several residues within the MCM2–7 complex, resulting in helicase activation and DNA unwinding. (C) During origin firing, the helicase is activated and the MCM2–7 double hexamer divides into two hexamers that move in opposite directions. Then, other proteins are recruited, such as replication factor C (RFC), proliferating cell nuclear antigen (PCNA), replication protein A (RPA) and other DNA polymerases, converting the pre-IC into two functional replication forks that move in opposite directions from the activated origin, with a replisome at each replication fork. It is not yet clear whether one or two ORCs remain on the duplicated origin after initiation of DNA synthesis. The functional helicase at the forks is the CMG complex (which consists of CDC45, the MCM hexamer and the GINS complex). Although all the origins are licensed, only one out of three origins on average is activated, whereas the other adjacent origins are silent and the replisome is formed only at the activated origin. Inhibition of adjacent origins is controlled in part by the checkpoint kinases ATR and ATM that activate CHK1 and CHK2. However, the exact mechanisms that are responsible for the local inhibition of these flexible origins and how flexible origins are selected for activation or silencing remain unclear.

3.4. Priming and recruitment of DNA polymerases

In order to engage DNA replication, replicative DNA polymerases need an RNA primer, synthesized by the DNA primase subunits p48 and p55 of the DNA polymerase alpha (Pol α) holoenzyme (Frick and Richardson, 2001; Rowen and Kornberg, 1978). Pol α needs to bind ssDNA in order to prime DNA replication. Displaying this unique ability among DNA polymerases, Pol α is recruited at unwound DNA and incorporates about 12 ribonucleotides, thus forming an RNA primer on ssDNA template (Conaway and Lehman, 1982; Waga and Stillman, 1998). RPA is also essential for the priming reaction (Figure 1.4). The RPA complex (made of three subunits: 70, 32, and 11 kDa also known as RPA1, 2 and 3), binds ssDNA with high affinity and stabilises the unwounded DNA double-helix (Wold, 1997). Then, interaction with RPA stimulates the two polymerase subunits of Pol α (p180 and p66) extending the RNA primer by a stretch of about 20 bases of DNA forming a DNA/RNA hybrid that serves as a starting point for DNA polymerases activity (Dornreiter et al., 1992; Tsurimoto and Stillman, 1989).

At this stage, the 3'-end of the newly synthetized DNA strand is recognized by a heteromeric complex made of five subunits, called Replication Factor C (RFC) (Tsurimoto and Stillman, 1990). Using its ATPase activity, RFC loads Proliferative Cell Nuclear Antigen
PCNA (Miyachi et al., 1978) on nascent DNA (Sakato et al., 2012). PCNA is a homotrimeric sliding clamp that serves as a docking platform for a large number of proteins involved in many processes, including replication, repair, chromatin remodelling and cell-cycle control (Moldovan et al., 2007). PCNA binds DNA topologically by embracing the double-helix (Hingorani and O'Donnell, 2000) and functions as a processivity factor for the replicative DNA polymerase delta, stimulating its activity (Tsurimoto and Stillman, 1991). PCNA was first identified as a highly expressed human auto-antigen in the nuclei of cells undergoing proliferation in an autoimmune disease called Systemic Lupus Erythematosus (SLE), and was therefore called PCNA (Miyachi et al., 1978). The function of PCNA was unveiled ten years later when a 36 kDa protein, called initially Replication Factor B (RF-B), was shown to be an essential factor for DNA synthesis elongation (Bravo et al., 1987; Prelich et al., 1987a; Prelich et al., 1987b). Pol α can prime both DNA strands, and is even capable of extending both strands in the presence of CMG (Yurieva and O'Donnell, 2016) (Figure 3. 4).

Figure 3. 4 Initiation of DNA replication (Recolin et al., Genes, 2014)

When the pre-IC complex is formed and origins fired, Polα is recruited at unwound DNA and incorporates thanks to its two primase subunits p48 and p55 about 12 ribonucleotides forming an RNA primer on ssDNA template (red waves). The ssDNA binding protein RPA is also essential for the priming reaction through binding single-stranded DNA with high affinity and stabilising it at unwound DNA double-helix. Then, the interaction with RPA stimulates the two polymerase subunits of Polα (p180 and p66) extending this primer by a stretch of about 20 bases of DNA (black arrows) forming a DNA/RNA hybrid that serves as a starting point for DNA polymerases activity.
3.5. Elongation of DNA replicon

DNA replication is initiated by DNA polymerase alfa (Pol α). This heterotetrameric enzyme consists of four subunits in eukaryotes: the catalytic p180 subunit dispensable for both the catalytic activity and the assembly of the tetrameric complex, two small subunits with a DNA primase activity (p49 and p55) (Arezi and Kuchta, 2000), and the B subunit which has no detectable enzymatic activity, but appears to have a role in maintaining a functional heterotetrameric complex (Arezi and Kuchta, 2000). Pol α initiates DNA replication by synthesising primers from which the replicative DNA polymerases extend DNA, in a process known as elongation of DNA synthesis. After initiating DNA replication, Pol α is displaced from the complex and replaced with elongating polymerase (Pol ε and Pol δ). This process is known as polymerase switch (Tsurimoto et al., 1990; Waga, 1994). When associated to PCNA, these polymerases continue replication of the leading and the lagging strands (Prelich and Stillman, 1988). Pol δ discontinuously replicates the lagging strand (Nick McElhinny et al., 2008) and participates to the maturation of Okazaki fragments, whereas Pol ε synthesises in a continuous way the leading strand (Kunkel and Burgers, 2014). In yeast, although Pol ε cells display serious defects in DNA replication, the catalytic active domain is dispensable for growth (Dua et al., 1999), suggesting that another polymerase, probably Pol δ, can substitute for Pol ε. Recent biochemical and structural studies from O’Donnell and colleagues suggest an asymmetric recruitment of these polymerases on the leading and lagging strands. Pol ε can processively replicate the leading strand in vitro, yet it is inactive on the lagging strand. Whereas Pol δ is highly active on the lagging strand in vitro, it has a weak but significant activity with CMG on the leading strand (Yurieva and O’Donnell, 2016). Hence, it is unclear how the labour between DNA polymerases is shared at replication forks, but it seems that DNA polymerase δ can function on both lagging and leading strand.

Polδ is tetrameric complexes containing a large catalytic subunit (p125) and three regulatory small subunits (p66, p50, p12). The Pol δ complex was originally isolated as a dimer of p125 (POLD1) and p50 (POLD2), with POLD2 playing an essential structural role (Lee et al., 1984; Ng et al., 1991). Two additional subunits were identified later, POLD3 (p66) and POLD4 (p12), both of them stimulates the catalytic activity of the complex in vitro (Hughes et al., 1999; Liu et al., 2000; Mo et al., 2000). Recent studies has shown that POLD3 regulates the repair of broken replication forks through break-induced replication (BIR) and promotes repair-
associated DNA synthesis in mitosis (Costantino et al., 2014; Lydeard et al., 2007; Mayle et al., 2015; Minocherhomji et al., 2015). POLD3 is essential for mouse development and viability of adult animals and POLD3 deficiency lead to replication stress and cell death, which could be explained by the destabilization of the Pol δ complex (Murga et al., 2016). The human POLD2 and POLD3 and also their yeast orthologues Pol31 and Pol32 has also been shown to be essential subunits for the translesion polymerase Pol ζ (Baranovskiy et al., 2012; Johnson et al., 2012; Makarova et al., 2012). Recently, Pol η TLS polymerase was shown to interact with the p50 subunit of Pol δ and this interaction is required for efficient TLS activity (Baldeck et al., 2015). POLD4 is required for the in vitro Pol δ activity, and maintaining genomic stability in human cells (Huang et al., 2010b).

Pol ε has a similar structure as other B-family polymerases where p261 is the catalytic subunit and p59, p17, p12 displaying regulatory functions (Hubscher et al., 2002). Apart from the large catalytic subunit, the second largest, subunit B (p59), is also essential for viability in yeast (Araki et al., 1991). The DPB3 and DPB4 genes encode the two smallest, nonessential subunits of budding yeast Polε, corresponding to the p12 and p17 subunits respectively in human (Araki et al., 1991; Li et al., 2000; Ohya et al., 2000). Pol ε differs from Pol δ in that it does not require the DNA sliding clamp PCNA for high processivity (Hogg and Johansson, 2012). Recent data showed that the large palm domain of Pol ε contain a processivity domain named the P domain (Hogg et al., 2014; Jain et al., 2014a; Jain et al., 2014b). Pol δ and Pol ε are high fidelity replicative polymerases that extend DNA according to base complementarity in the 5’→3’ direction by adding deoxynucleotides to the 3’-hydroxyl group (3’OH) of the primer. Both polymerases possess a 3’→5’ proofreading exonuclease activity localized within the catalytic subunit (Johnson and O’Donnell, 2005), ensuring high-fidelity DNA replication by allowing removal of misincorporated nucleotides (Fortune et al., 2005; Shcherbakova et al., 2003). Replicative polymerases are very processive being able to replicate DNA at a speed of around 2.0 - 3.0 kb/min (Huberman and Riggs, 1968). Recent work has shown that the polymerase activity of Pol ε is enhanced by the checkpoint complex TIM-TIPIN (Aria et al., 2013).

Due to the antiparallel structure of the DNA double-helix, one of the two strands has to be synthesized discontinuously. In the DNA replication model, Pol ε synthesizes continuously the leading strand in the direction of the replisome sliding, whereas the lagging strand is synthesized by Pol δ in short discontinuous DNA stretches of about 100-200 nucleotides in
eukaryotes, known as Okazaki fragments (Okazaki et al., 1968). The maturation of these fragments into a single stretch of DNA requires two other enzymes; the Flap Endonuclease 1 (FEN1) (Liu et al., 2004) which cuts the 5’ overhanging RNA flaps left by Pol δ while moving along template DNA and displacing RNA primers. Sometimes these flaps are long, in this case the DNA2 endonuclease/helicase shortens them to allow FEN1 to cut efficiently (Gloor et al., 2012). Flap cutting generates nicks that are finally ligates by DNA Ligase 1 (Garg and Burgers, 2005). This enzyme is recruited to replication sites through its PCNA-binding motif.

PCNA plays a crucial role maturation of Okazaki fragment and the coordination between all the actors of this process (Moldovan et al., 2007). Posttranslational modifications of FEN1 regulates its recruitment and PCNA partners switch during Okazaki fragment maturation (Guo et al., 2010). Whereas FEN1 methylation by PRMT5 methyltransferase (Guo et al., 2010) increases its affinity to PCNA and competes out Pol δ, FEN1 phosphorylation by CDK1-cyclin A (Henneke et al., 2003) induce its displacement from PCNA by DNA Ligase 1 to terminate the maturation of Okazaki fragments (Guo et al., 2010). In this way, FEN1 posttranslational modifications regulate the timing for PCNA association of Okazaki fragments maturation factors.

In addition to Pol δ, Pol ε, PCNA, and the loader clamp RFC, Topoisomerase II is required for the elongation of DNA replicon. This enzyme segregates the two intertwined daughter DNA molecules produced by DNA replication and plays an important role in termination of DNA replication (Prelich et al., 1987b; Sundin and Varshavsky, 1981; Uemura and Yanagida, 1984; Yang et al., 1987).

3.6. Termination of DNA replication

Many different hypothesis for the replication termination process have been described for the three phylogenetic kingdoms. While in eubacteria, DNA replication termination is site specific, in archaea and eukaryotes termination is thought to occur randomly when two replication forks converge (Zhu et al., 1992). This process is poorly understood and the molecular mechanisms behind replisome unloading from the DNA are largely unknown. However, replication termination have been associated in eukaryotes to some site-specific replication barrier factors (Dalgaard et al., 2009).
In prokaryotes, at the opposite site of replication origin on the circular chromosome, there are sequences called Replication Fork Barriers (RFB), also known as TER-sites (termination sites). These are sequence-specific replication barriers that are bound by the TUS (Terminus Utilization Substance) protein close to where replication terminates (Hill et al., 1989; Kobayashi et al., 1989; Sista et al., 1989). TER-sites has been associated to replication termination of specific sites in eukaryotes, including ribosomal DNA (rDNA) (Linskens and Huberman, 1988; Little et al., 1993), transfer RNA genes (tRNA) (Deshpande and Newlon, 1996), and the yeast mating-type switch locus (Dalgaard and Klar, 2001). Since rDNA and tRNA gene are highly transcribed, the role of these TER-sites is preventing replication/transcription head-on collisions generating genomic instability, as it was shown in yeast (Takeuchi et al., 2003).

Many factors are involved in the function of the replication barriers. Among them, the protein FOB1 which was isolated as an essential gene for the RFB activity in budding yeast (Kobayashi and Horiuchi, 1996). The FOB1-defective mutants lose RFB replication fork blocking activity, and recombination in the rDNA repeats is much less efficient (Defossez et al., 1999; Johzuka and Horiuchi, 2002; Kobayashi et al., 1998; Merker and Klein, 2002). In fission yeast, the orthologue proteins RTF1, REB1, and SAP1 (Eydmann et al., 2008; Sanchez-Gorostiaga et al., 2004) play a similar role. Deletion of the transcription Termination factor 1 (TTF1) was shown to abolish RFB activity (Gerber et al., 1997). Ku proteins may also participate to replication termination as shown in HeLa cell extracts (Wallisch et al., 2002).

In S. cerevisiae, the PIF1p helicase and the highly related RRM3p regulates rDNA replication fork progression through RFB sequences but in opposite ways. Whereas RRM3p serves as helicase for rDNA as it promotes fork progression throughout rDNA, PIF1p blocks the fork at RFB (Ivessa et al., 2000). Similarly to this, the checkpoint protein complex TOF1p-CSM3p in S. cerevisiae protects replications forks at TER-sites and counteracts RRM3 helicase (Mohanty et al., 2006).

At the end of DNA replication, the MCM2-7 helicase is removed by a protein called MCM-BP, whose silencing in human cell or immunodepletion from Xenopus egg extract delays MCM2-7 dissociation in late S-phase (Nishiyama et al., 2011). Recently, it has been shown in both yeast and Xenopus that the p97/CDC48 segregase removes polyubiquitylated MCM7 from chromatin thus inducing the CMG helicase unloading at the end of replication (Maric et al., 2014; Moreno et al., 2014).
3.7. Regulation of DNA replication

Cells have to replicate their entire genome once, and only once, during the S-phase of each cell-cycle before cell division begins (Nurse, 2000a). This is needed to maintain the integrity of the genome and avoid parts of the genome from being replicated more than once, in a process called re-replication (Blow and Dutta, 2005; Machida et al., 2005). To do so, cells possess redundant regulatory mechanism that control origin licensing during the cell-cycle.

3.7.1. Cell-cycle regulation of DNA replication

A) An overview of the cell cycle

The cell-cycle consists in a series of successive events that allows the cell to accomplish four essential tasks in order to duplicate itself: growth, chromosomal duplication, chromosomal segregation, and finally cell division (Mitchison and Creanor, 1971). In eukaryotes, these events are separated in time; DNA replication occurring during the S-phase early in the cell-cycle, and chromosome segregation or mitosis during the M-phase at the end of cell-cycle. The phase before S-phase is called G1-phase, and the one before mitosis is called G2-phase (Nurse, 2002).

Cell-cycle has to be tightly controlled during cellular proliferation in order to ensure a unidirectional progression of the cell through G1 into S-phase and from G2 to M-phase. The regulation of cell-cycle transitions requires the concerted action of CDKs and their regulatory subunits, the Cyclins. Most of these mechanisms are conserved among eukaryotes. CDKs are essential for the initiation of DNA replication and coordinate progression through cell-cycle in eukaryotes (Blow and Nurse, 1990; Nurse, 2000b). These small serine/threonine protein kinases drive the progression of the cell-cycle by phosphorylating their substrates at a specific motif S/T-P-X-K/R (Morgan, 1997). The first CDK isolated was CDK1 in a yeast screen for cell-cycle division mutants, named initially CDC2 in *S.pombe* and CDC28 in *S.cerevisiae* (Dutcher and Hartwell, 1983; Nurse and Thuriaux, 1980), and it was later shown to be a conserved kinase from yeast to humans (Lee and Nurse, 1987). Cyclins regulate the activation of CDKs by inducing a conformational change allowing substrate proteins to enter the active site (Jeffrey et al., 1995). They were discovered first as fluctuating proteins during the cell-cycle of sea urchins (Evans et al., 1983). While in yeast, a unique CDK (CDK1) regulates cell-cycle progression via
interaction with different cyclins, in metazoans there are several, although CDK1 could substitute other CDKs indicating that CDK function may be redundant (Fisher, 1996; Satyanarayana and Kaldis, 2009; Sclafani and Holzen, 2007) (Fig 1.5).

B) Fluctuating CDK activity during cell cycle

In mammalian cells, CDK 4/6 interacts with cyclin D in early G1-phase, inducing the phosphorylation of the retinoblastoma protein Rb. This phosphorylation triggers the release of E2F transcription factors that will promote cyclins A and E transcription (Frolov and Dyson, 2004) and restricts ORC1 expression to late M / early G1-phase (Ohtani et al., 1996) and likewise E2Fs regulate expression of CDT1, CDC6, and MCM members (Yoshida and Inoue, 2004). In late G1, cyclin E activates CDK2 and promotes the G1/S transition. CDK2 and DDK (CDC7-DBF4) promotes recruitment at the pre-RC of several replication factors to form a large pre-IC (Mendez and Stillman, 2003). CDK2 associate to cyclin A during S-phase, playing an important role in S-phase progression and DNA replication. At G2/M transition, CDK1 is activated by Cyclin A and initiates the mitotic prophase, and later it associates with cyclin B to complete mitosis (Murray, 2004) (Figure 3. 5). CDC6 induces CDK1 inhibition, thus controlling the timing of the first M-phase entry and progression in embryos (El Dika et al., 2014b).

CDK activity is also tightly regulated during cell-cycle progression by interaction with protein inhibitors members of the INK4 family (p16INK4a, p15INK4b, p18INK4c, p19INK4d) and CIP/KIP family (p21CIP/WAF1/SDI, p27KIP1, p57KIP2) (Morgan, 1997). The INK4 proteins specifically bind to CDK4/6 and block cyclin D activity, and the CIP/KIP proteins inhibit CDK2/cyclin E, CDK2/cyclin A, CDK1/cyclin A, and CDK1/cyclin B activities (Figure 3. 5) (Aprelikova et al., 1995; Toyoshima and Hunter, 1994). The M-phase entry is also controlled by the equilibrium between CDK1 kinase and Greatwall-PP2A axis (Figure 3. 5) phosphatase that regulates the dynamics of cyclin B (El Dika et al., 2014a; Wang et al., 2014). In addition to this regulatory mechanisms of CDK activities, the cyclins are also targeted for proteasomal degradation by the Anaphase Promoting Complex (APC) and the Skp1-Cul1-F box (SCF) E3 ubiquitin ligase (Cardozo and Pagano, 2004).
In early G1-phase, CDK 4/6 interacts with cyclin D, and phosphorylates the retinoblastoma protein Rb, this phosphorylation triggers the release of E2F transcription factors that promotes cyclins A and E transcription and restricts ORC1 expression to late M / early G1-phase. E2Fs regulates also the expression of CDT1, CDC6, and MCM members. Then, ORC, CDC6 and CDT1 recruit MCM2-7 helicase to replication origins to form pre-replication complexes (pre-RC). At this point ORC1 is active and Geminin is not bound to CDT1. Once the pre-RC is assembled, the origin is licensed to replicate and CDK2 phosphorylates components of ORC and the licensing factors CDC6 and CDT1 to prevent re-replication, concomitant with the re-association of Geminin to CDT1. Upon entry into the S-phase, CDC45 and GINS are recruited to replication origins in a manner dependent upon TOPBP1 (Dpb11 is the orthologue in yeast as shown in the figure), RecQL4 (sld2 is the orthologue in yeast as shown in the figure) and TRESLIN (sld3 is the orthologue in yeast as shown in the figure) under the regulations of CDK2/cyclin E and the Dbf4-dependent kinase CDC7 (DDK). The phosphorylated MCM2-7 helicase, together with CDC45 and GINS, forms a CMG complex that functions in unwinding the DNA replication origin site. Subsequently, Pol ε and Pol δ are recruited to the replication fork, and DNA replication initiates. During S-phase, CDK2 associate to cyclin A, playing an important role in DNA replication and progression through S-phase. At G2/M transition, CDK1 is activated by Cyclin A and initiates the mitotic prophase, and later it associates with cyclin B to complete mitosis. CDK activities are regulated by the action of their respective cyclin, as well as by the CDK inhibitors, the INK4 and the CIP/KIP families.
C) Preventing re-replication by CDT1 inhibition

In eukaryotes, DNA replicates only once per cell-cycle, no re-replication occurs until the cell passes through mitosis to the next S-phase. Using cell-free extracts derived from activated *Xenopus* eggs, Laskey and colleagues proposed the existence of a factor that license DNA for replication binding to the chromatin when the nuclear membrane breaks down during mitosis (Blow and Laskey, 1988). Later, biochemical purification experiments from *Xenopus* egg extracts have identified this factor as the CDT1 protein (Tada et al., 2001).

In order to prevent re-replication, CDT1 has to be inactivated at the end of origin licensing. Several laboratories have shown that re-replication is induced when active, recombinant CDT1 is added to G2 nuclei formed in *Xenopus* extracts (Arias and Walter, 2005; Blow and Dutta, 2005; Maiorano et al., 2005b; Yoshida et al., 2005). In metazoans, CDT1 reactivation is prevented through binding of Geminin (Tada et al., 2001; Wohlschlegel et al., 2000). Geminin binds to chromatin in early S-phase until the end of M-phase, preventing re-licensing by CDT1 (Maiorano et al., 2004). During G1, Geminin levels are kept low by proteasomal degradation via the E3-ubiquitin ligase APC\(^{\text{CDC20}}\) (McGarry and Kirschner, 1998). In S-phase, CDT1 is degraded via the SCF\(^{\text{Skp2}}\) pathway in a CDK2/cyclin A dependent manner (Li et al., 2003), and also via the Cullin 4-RING E3-ubiquitin Ligase Cdt2 pathway (CRL4\(^{\text{Cdt2}}\)) that targets PCNA bound-CDT1 for proteasomal degradation (Arias and Walter, 2007; Tsanov et al., 2014).

Geminin and CRL4\(^{\text{Cdt2}}\)-mediated CDT1 inactivation seem to be redundant pathways that restrict origin firing to strictly once per cell-cycle (Li and Blow 2005; Maiorano et al. 2005a; Arias and Walter 2006). Very recently, USP37, a ubiquitin hydrolase that deubiquitinates CDT1 has been identified, although its role (if any) in preventing re-replication has not yet been clarified (Hernandez-Perez et al., 2016).

D) The proteasome-dependant cell-cycle regulation

The cell-cycle is regulated through proteolytic degradation of regulatory proteins, including CDK inhibitors and cyclins (Machida et al., 2005). This regulation is ensured by the proteasome which is an ATP-dependent protease (Ravid and Hochstrasser, 2008). In eukaryotes, the 26S proteasome is a large protein complex composed of two subunits: the 19S
regulatory subunit that recognises the substrate and unfold it in an ATP-dependant manner thus transferring the substrate to the proteolytic chamber of a cylindrical-shaped 20S core particle (Elsasser and Finley, 2005; Groll et al., 1997).

3.7.2. The ubiquitin-proteasome system

A) The ubiquitin-conjugating system

In order to be targeted for proteasomal degradation, substrates are tagged by a chain made of several monomers of a highly conserved small protein, called ubiquitin (Figure 3. 6) (Hershko and Ciechanover, 1998; Ravid and Hochstrasser, 2008). This 76 amino acids protein was first described as a polypeptide of unknown function expressed in all eukaryotic cells (Schlesinger et al., 1975). Later, the role of ubiquitin in ATP-dependent protein degradation was discovered and the main enzymatic reactions of this system elucidated using a cell-free proteolytic system from reticulocyte lysates (Ciehanover et al., 1978; Hershko et al., 1979). The ubiquitin-substrate ligation requires the sequential action of three enzymes: the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme (Ubc), and the E3 ubiquitin-ligase (Figure 3. 6) (Ravid and Hochstrasser, 2008). This reaction consists in the formation of an amide isopeptide linkage between the C-terminal carboxyl group of ubiquitin (Ub-COOH) and the ε-amino group of a lysine amino acid in the substrate and requires ATP as source of energy. First, the ubiquitin forms with ATP a ubiquitin adenylate intermediate that binds to cysteine residue in the active site of the E1 with a high-energy thioester bond (Ciechanover, 1994; Hershko and Ciechanover, 1992). This step resembles to amino acids activation during protein synthesis, and involves an aminoacyl adenylate intermediate (Ub-COO~AMP) and the release of PPI (pyrophosphate) (Hershko and Ciechanover, 1982). Then, the activated ubiquitin is transferred from E1 to a cysteine residue in the active site of an ubiquitin-carrier protein, the E2 enzyme. In the third step, an E3 enzyme ubiquitin-protein ligase links ubiquitin to its substrate. In this reaction, the ATP energy, temporally stored in thioester linkage, is used to power the formation of an isopeptide bond between ubiquitin and the substrate (Hershko and Ciechanover, 1982).
There are few E1s, but there are several E2s and many E3s or E3 multiprotein complexes. In the ubiquitin reaction, the E3 ligases usually provide substrate specificity by binding protein substrates that contain specific recognition sites. Sometimes, the combination of E3 ligases with E2 enzymes is necessary to recognise the specific substrate (Hershko and Ciechanover, 1998). In certain circumstances, an adaptor protein could be necessary to target the E3 binding to its substrate. Many different E3s could carry out the ubiquitin transfer to the substrate protein. They belong to two main families of multiprotein E3 ubiquitin ligases, depending on their active domain; RING (Really Interesting New Gene) finger domain or HECT (Homologous to E6-AP C-Terminus) domain (Ardley and Robinson, 2005). RING and HECT E3 ubiquitin ligases have different ubiquitination mechanisms. The RING finger domain possesses two zinc ions in the protein pocket that interacts with the E2 Ubc (Deshaies and Joazeiro, 2009) to transfer directly the ubiquitin residue from the E2 to substrate. With the HECT domain E3 enzymes, ubiquitin is first transferred from an appropriate E2 to a conserved cysteine residue at the active site of the E3 enzyme before ubiquitinating the final substrate (Ardley and Robinson, 2005).

**B) Polyubiquitination as a signal for proteasomal degradation**

After the monoubiquitination of the substrate protein, the E2-E3 ligase complex can build a polyubiquitin chain, in which the C-terminus of each ubiquitin unit is linked by an isopeptide bond to a specific Lysine residue (generally Lys48) of the previous ubiquitin in the same way as in ubiquitin-substrate reaction (Pickart, 1997; Pickart and Fushman, 2004).

The function of polyubiquitin chains is dependent on the Lys residue used to polymerize the chain and the number of residues (Pickart and Fushman, 2004). The receptor subunits of the 19S regulatory particle of the proteasome recognizes a chain of a minimum four ubiquitins linked through Lys48, and transfers the substrate into the protease catalytic site of the proteasome (Chau et al., 1989; Thrower et al., 2000). Interestingly, polyubiquitination on Lys63 is involved in DNA damage tolerance (Ulrich and Walden, 2010).

The proteasomal degradation generates different kinds of products: free peptides, short peptides linked to ubiquitin, and polyubiquitin chains (Hershko and Ciechanover, 1998). The two latter ones are cut by ubiquitin-C-terminal hydrolases or isopeptidases and recycled to free and reusable ubiquitin. Some of these isopeptidases may have a regulatory role by
deubiquitination and salvaging the incorrectly ubiquinated substrates (Hershko and Ciechanover, 1998).

Figure 3.6 The ubiquitin modification pathway (Bergink and Jentsch, 2009)

Ubiquitin (A) and SUMO (B) modifiers are small polypeptides that form isopeptide bonds between their C-terminal glycine residues and the internal lysine residues of target proteins. During ubiquitination or SUMOylation reaction, either one residue of modifier or a chain of covalently linked modifiers can be added (polyubiquitylation or polySUMOylation). Both reactions involve E1, E2 and E3 ubiquitin or SUMO conjugating enzymes. The number of implicated enzymes varies between the yeast Saccharomyces cerevisiae and in humans, and they are shown between brackets respectively. Ubiquitination and SUMOylation are both reversible, and many UBPs (ubiquitin-specific proteases) and ULPs (ubiquitin-like protein (SUMO)-specific proteases) have been described.
C) The SUMO regulation system

Besides ubiquitination, another post-translational modification that regulates the protein function is SUMOylation. SUMO (Small Ubiquitin-like MOdifiers) is a small protein of about 100 amino acids in length and 12 kDa mass in humans. There are four SUMO isoforms in humans; SUMO-1, SUMO-2, SUMO-3 and SUMO-4 (Hay, 2005). SUMOylation substrates are mainly nuclear proteins, although cytosolic SUMOylated proteins have been also described (Hannich et al., 2005; Panse et al., 2004; Psakhye and Jentsch, 2012).

The SUMOylation pathway shares a lot of similarities with ubiquitination. The first step during the conjugation reaction is catalysed by SUMO activating enzymes, called E1s: a heterodimer in the case of budding yeast (AOS1-UBA2). E1 enzymes initially catalyse adenylation of the C-terminal glycine residue of SUMO in an ATP-dependent reaction. The resulting SUMO-AMP adducts are then transferred onto cysteine residues within the internal catalytic domain of the enzymes, forming thioester bond between the SUMO residue and E1 enzyme (Figure 3.6). Once activated, the SUMO modifiers are then transferred onto internal cysteine residues of SUMO-conjugating enzymes (termed E2s), forming thioester-linked complexes between the SUMO and the E2 enzymes (Geiss-Friedlander and Melchior, 2007). The final step is substrate modification, where the ε-amino groups of lysine residues of substrates are covalently conjugated to the C-terminal glycine residue of SUMO, catalysed by E3 ligases enzymes. SUMOylation of substrates may also yield poly-SUMO chains. Similar to ubiquitination, SUMOylation can be reversed by specialized enzymes that remove the SUMO modifications (Figure 3.6) (Geiss-Friedlander and Melchior, 2007).

As with the ubiquitin system, the SUMO pathway targets a wide range of proteins and fulfils numerous functions. Three different mechanisms can explain the biological roles of SUMOylation. The first one is competition with other lysine-directed post-translational modifications like acetylation or ubiquitination. For instance, SUMOylation prevents Myocyte specific Enhancer Factor 2A (MEF2A) acetylation, which is necessary for the transactivation activity of MEF2A (Shalizi et al., 2006). Another mechanism is steric hindrance of protein-protein interactions because SUMO is a bulky modification. One example is SUMOylation of PCNA at residue K127 on the surface of PCNA, preventing the binding of PIP-box containing proteins such as the yeast Ecol required for establishment of sister chromatid cohesion during S-phase (Moldovan et al., 2006). The third mechanism of SUMO action and the most commonly used, is the promotion of protein-protein interactions (Bergink and Jentsch, 2009;
SUMOylated proteins interact with each other through increasing a specific motif called SUMO-Interaction Motif (SIM) \((\text{Kerscher, 2007})\). This termed as SUMO glue property \((\text{Psakhye and Jentsch, 2012})\). An example of this mechanism is the binding of the anti-recombination SRS2 helicase to SUMOylated PCNA on K164 \((\text{Papouli et al., 2005; Pfander et al., 2005})\). This is achieved because SRS2 harbours in its C terminal tail a SIM and a nearby PIP-box for PCNA binding \((\text{Armstrong et al., 2012; Papouli et al., 2005; Pfander et al., 2005})\).

In contrast with the primordial role of ubiquitin in the regulation of DNA replication, SUMO appears to exert more subtle regulatory effects on replication initiation. In \textit{Xenopus laevis} cell-free egg extracts, inhibition of SUMOylation was shown to induce more origins firing and to increase replication rates \((\text{Bonne-Andrea et al., 2013})\). This is likely due to the modification of cyclin E following recruitment of the cyclin E-CDK complex to pre-RCs. A very recent study in budding yeast showed a cell-cycle regulated SUMOylation of the MCM2-7 helicase, increasing at the origin licensing stage when the pre-RC is loaded onto origins, but declining upon origin firing at the G1-to-S transition \((\text{Wei and Zhao, 2016})\). Furthermore, upon inducing SUMOylation, CMG assembly and origin firing were inhibited, most likely through recruiting a phosphatase that removes essential phosphorylations necessary for CMG activation \((\text{Wei and Zhao, 2016})\). Moreover, other components of the pre-RC have also been identified as SUMOylation targets, among them the subunits of ORC \((\text{Golebiowski et al., 2009})\).

3.8. Regulation of DNA replication during early embryonic cleavages

3.8.1. Early embryonic cell cycles

In most organisms, immature oocytes are arrested in Prophase I of meiosis. This can last for decades as in humans. In most vertebrates, upon maturation and after one meiotic division, eggs are again arrested at Metaphase of meiosis II (MII arrest). During this MII arrest maintained by a high CDK1 activity, chromosomes are aligned on the bipolar mitotic spindle. In so doing, eggs are ready to respond rapidly to fertilization by segregating sister chromatids. After fertilization, the first mitosis is relatively slow in comparison with the following cell-cycles. This extra time is necessary in order to complete the second meiotic division of the egg and ensure decondensation of sperm chromatin and the fusion of male and female pronuclei to
give a diploid genome (Hormanseder et al., 2013). This fusion occurs in interphase before to the first mitosis in sea urchin, Caenorhabditis elegans, and Xenopus laevis (Longo and Anderson, 1968; Strome and Wood, 1983; Ubbels et al., 1983), whereas in mammals, the nuclear envelope breaks down after the two pronuclei undergo DNA replication independently and then chromosomes can associate during the first mitosis (Bomar et al., 2002; Ciemerych and Czolowska, 1993; Das and Barker, 1976; Mayer et al., 2000).

The early embryonic cell-cycles of most metazoans (such as in Xenopus) are usually very fast and highly synchronous, including a replication phase (S-phase) and a division phase (M-phase) with virtually absent intermediate gap phases (G1 and G2) (Farrell and O'Farrell, 2014). However, these divisions can be asynchronous as in mammals and nematodes (Hormanseder et al., 2013). This amazingly fast early cell-cycles could be explained as a kind of adaptation in order to ensure the subsistence of the laid egg in the hostile external environment. Because the egg cannot eat and relies only on the limited maternal nutrients, and it is also devoid of escape or active defence means until hatching, it needs to proceed to hatching as quickly as possible. Hence, after duplicating the DNA, embryos divide the large pre-existing cytoplasm in the absence of significant growth (O’Farrell, 2004).

During the earliest cycles of Drosophila melanogaster, nuclear divisions are exceptionally rapid with a duplication every 8.6 minutes (Foe and Alberts, 1983), meaning that a Drosophila embryo divides 14 times in only 1.5 hours, forming a syncytium without cell division, whereas an average tissue culture takes 8-24 hours to go through a single cell-cycle (Blumenthal et al., 1974; Shermoen et al., 2010). In this contracted cell-cycle, the entire genome of the fly is replicated simultaneously in as fast as 3.4 minutes. It is only following the MidBlastula Transition (MBT), corresponding to the onset of massive Zygotic Genome Activation (ZGA) and gap phases introduction, that the cell-cycle slows down to a duration of 50 minutes. Two mechanisms have been put forward to explain the lengthening of embryonic S-phase after the MBT. The first one is the inter-origins distance, from 7.9 kb in the preblastoderm embryo (Blumenthal et al., 1974), to 10.6 kb in a cycle 14 Drosophila embryo (McKnight and Miller, 1977) because replication origins spacing is short in early embryos. Thus, it would take longer for a replication fork to move along DNA and replicate between origins after MBT. However, this could not explain alone the enormous increase (∼15 fold) of S-phase length between the first cell-cycles and the 14th one.

Replication timing of different genomic sequences seems to play a major role in S-phase lengthening during embryogenesis. During S-phase of a somatic cell, as in post-MBT embryos,
DNA sequences replicate at different time points. Upon the onset of S-phase, the euchromatin genes begin to replicate immediately, whereas heterochromatin sequences are replicated later in the S-phase and are then called late-replicating sequences (Shermoen et al., 2010). An example of these late-replicating genes in *Drosophila* is the long stretches of highly repetitive DNA that represent approximately 30% of the genome, namely the satellite sequences (Lohe et al., 1993; Shermoen et al., 2010). In contrast, all regions of the DNA including both euchromatin and heterochromatin sequences replicate at the same time in the preblastoderm embryos resulting in a replication task quickly completed. While the embryos is developing, the satellite sequences progressively shift from being early replicating to late replicating sequences, then after the MBT clusters of satellite sequences dramatically turn to late-replicating sequences (McCleland et al., 2009; Shermoen et al., 2010; Yuan et al., 2014). In the pre-MBT cycles, the shift is gradual and subtle, and the replication of euchromatin and satellite sequences still largely overlaps. The change is dramatic after MBT, when different clusters of satellite sequences replicate in late S-phase (McCleland et al., 2009; Shermoen et al., 2010; Yuan et al., 2014). For instance, certain late sequences start replicating 15 to 30 min after the beginning of S-phase in cell-cycle 14, a period of time longer than the entire 13th cycle S-phase (Shermoen et al., 2010).

In *Xenopus* embryos, the first 12 cell-cycles are fast and synchronous, alternating between DNA replication and division at 30 min intervals until the MBT (Newport and Kirschner, 1982a), where cell-cycles slowdown progressively (50, 99, and 253 min for 13th, 14th, and 15th cell-cycle respectively) (Howe et al., 1995) (Figure 3. 7). The 15th cell-cycle corresponds to the onset of gastrulation. As in many species, during the first cell divisions, early *Xenopus* embryos are incompetent for *de novo* transcription. They rely on maternally inherited factors (mRNAs and proteins) for accomplishing their different tasks until the Maternal-Zygotic Transition (MZT), when the control of cell-cycle and development is transferred from the maternal genome to the zygotic genome. Furthermore, embryos at this early stages lack checkpoints that halt the cell-cycle progression in response to DNA damage (O’Farrell et al., 2004) (see paragraph 3.8.2). This transition is more like a succession of progressive events rather than a single point sudden change (Farrell and O’Farrell, 2014). Because embryos progression in divisions is accompanied by an increase in transcriptional activity, molecular assays measuring ZGA have been used as a measure of MZT. Very often, the first phenotypes of zygotic transcripts appear later in the division than the onset of zygotic transcription. This led to a controversy in defining the MZT. For example, in *Drosophila melanogaster*, radioactive
precursors incorporation experiments into mature transcripts have shown that the MZT occurs at the 10th cell-cycle, even though the phenotypic changes are visible starting from stage 14 (Edgar and Schubiger, 1986; Zalokar, 1976). This is probably related to the sensitivity of the method used to detect zygotic transcripts. More recently, using sensitive techniques such as single-embryo RNA-seq, it has been demonstrated that ZGA commences before the 10th cell-cycle (Ali-Murthy et al., 2013; De Renzis et al., 2007; Lott et al., 2011). It seems as there is a progressive increase of transcription over development. Similarly, in Xenopus laevis, transcription, which was previously thought to begin at the MBT (Newport and Kirschner, 1982a, b), was detected at prior stages (Kimelman D, Cell 1987).

The MBT was defined in Xenopus as the initial slowing of the cell-cycle concomitant to the onset of zygotic transcription, and cellular movements (Newport and Kirschner, 1982a, b). Nevertheless, these three events have been shown subsequently to be temporarily uncoupled in Xenopus (Kimelman et al., 1987) as well as in Drosophila (Edgar and Schubiger, 1986). In addition, another dramatic change in the cell-cycle, related to cyclin A regulation, occurs in the embryo after the MBT and just prior to gastrulation, called the Early Gastrula Transition (EGT) (Howe et al., 1995). In Drosophila, the MBT corresponds conventionally to the cycle 14. However, in comparison to Xenopus, similar MBT changes are observed at cycle 10 in Drosophila embryos, which is called pre-MBT slowing, and it would be more appropriate to compare the Drosophila cycle 14 embryos with the EGT changes in Xenopus (Farrell and O’Farrell, 2014).

The MBT timing in Xenopus, as in Drosophila, is dependent on the Nuclear to Cytoplasmic ratio (N/C ratio), and not on zygotic transcription as it was shown by performing α-amanitin injection experiments in the embryos to inhibit RNA synthesis (Newport and Kirschner, 1982a). Whether gap phases are introduced at MBT or EGT is still controversial (Frederick and Andrews, 1994; Graham, 1966; Iwao et al., 2005). However, the different studies agree that the first change is S-phase lengthening, then by the time of gastrulation, G1 and G2-phases are acquired.

During Zebrafish embryos development, similar events occur. The embryo goes initially through 9 rapid and synchronous cell-cycles, starts slowing down slightly in 10th and 11th cycle, before undergoing massive cell-cycle changes, zygotic transcription and initiating cell movements. Cell-cycle asynchrony appears first in cycle 11 (Kane and Kimmel, 1993).

As in Drosophila and Xenopus, the MBT onset is also timed by the N/C ratio in Zebrafish as suggested by partial enucleation experiments (Kane and Kimmel, 1993). Similarly,
injection of α-amanitin in Zebrafish embryos did not delay the MBT, showing that it is independent from zygotic transcription (Dalle Nogare et al., 2009; Kane et al., 1996). The G1-phase is introduced in the cell-cycle for the first time at the MBT in a transcription-dependant manner, suggesting that G1 introduction is not responsible for the slowing of the cell-cycle at the MBT (Zamir et al., 1997).

The timing of zygotic transcription activation in other species is variable. For example, during mouse embryonic development zygotic transcription starts at the two-cell stage (Hamatani et al., 2006). Despite some differences, transcriptional quiescence during early embryonic development is an evolutionarily conserved phenomenon in almost all species.

Figure 3. Cell cycle regulation during Xenopus early development (adapted from Hörmanseder et al., 2013)

The cartoon shows the cell cycle regulation during Xenopus early development. Cell cycles are different between the fertilization until the mid-blastula transition. After fertilization, the first mitosis is relatively slow in comparison with the following cell cycles. This extra time is necessary in order to complete the second meiotic division of the egg and ensure the decondensation of sperm chromatin and the fusion of...
male and female pronuclei during the interphase to give a diploid genome. The 12 following cell cycles are fast and synchronous, alternating between DNA replication and division at 30 min intervals until the MBT where gap phases (G1 and G2) are introduced. The curves represent the oscillations of CDK1–cyclin B and APC/C E3 ubiquitin ligase activity during development. Bars in the lower half depict activity levels for the APC/C inhibitor XErp1/Emi2, the proto-oncogene c-Mos and the MAP kinase pathway (c-Mos/MAPK), Emi1 (Early meiotic induction 1) a major modulator of APC activity, and the spindle assembly checkpoint (SAC), as well as inhibitory Thr-14/Tyr-15 phosphorylation of CDK1 (CDK1-pY). At the bottom of the figure, major developmental transitions and oocyte/embryo stages are represented (from left to right; oocyte, first division, MBT stage, and gastrulation).

3.8.2. Cell-cycle regulation of replication during early embryogenesis

A) MBT timing regulation

In both *Xenopus* and *Drosophila*, the CHK1 kinase is activated at MBT stage (12th cleavage) and plays an important role in cell-cycle lengthening (Shimuta et al., 2002). Once activated, CHK1 can promote the inhibitory phosphorylation of CDK1 by activating WEE1 and MYT1 kinases and inhibiting CDC25 phosphatase (Figure 3. 8) (Ferrell et al., 1991). Phospho-CDK1 accumulates between the MBT and EGT (Shimuta et al., 2002). Supposedly, CHK1 activation in *Xenopus* delays mitosis as S-phase extends. Whereas in *Xenopus* CHK1 phosphorylation suppresses CDC25 activity and promotes its destruction (Figure 3. 8) (Shimuta et al., 2002; Uto et al., 2004), in *Drosophila* Twine (CDC25 orthologue) degradation does not require CHK1 activity (Farrell and O'Farrell, 2013). During early embryonic cleavages in *Xenopus*, cyclin B1 and B2 oscillate and their accumulation drives entry in S-phase via formation of a complex with CDC2, while cyclin B degradation leads to mitotic exit (Hartley et al., 1996; Murray and Kirschner, 1989). Cyclin E/CDK2 shows a different oscillation pattern. It accumulates during the first mitotic cycle and remains stable until MBT (Hartley et al., 1996; Rempel et al., 1995). Despite the fact that cyclin E levels do not vary, cyclin E/CDK2 activity changes, with two peaks at S-phase and M-phase for mitosis initiation (Guadagno and Newport, 1996). However, cyclin A/CDC2, which is also present in early embryos, is more involved in DNA replication than cyclin E/CDK2 as demonstrated in cell-free *Xenopus* extracts (Strausfeld et al., 1996; Strausfeld et al., 1994). Cyclin E1 is degraded during MBT (Hartley et al., 1996; Rempel et al., 1995) and this degradation is independent from N/C ratio, cell-cycle regulation, zygotic transcription, or *de novo* protein synthesis (Howe and Newport, 1996). Using the
Xenopus CDK inhibitor Xic1 (Su et al., 1995), Hartley and colleagues suggested that cyclin E/CDK2 regulation in early embryogenesis is linked to “an autonomous maternal timer” driving the early embryonic cleavages until the MBT (Hartley et al., 1997). A more recent study has suggested that WEE1 kinase disrupts cyclin E/CDK2 activity near MBT (Wroble et al., 2007).

A recent study in Xenopus laevis has proposed that four DNA replication factors (Cut5/TOPBP1, RECQL4, TRESLIN, and the DBF4 orthologue DRF1,) are limiting for MBT onset, thus the slowing down of cell-cycle, inducing developmental activation of CHK1, and zygotic replication initiation at increasing N/C ratios. Upon overexpression of these four replication factors, additional short pre-MBT-like cycles are introduced without accelerating the pre-existing pre-MBT cycles (Collart et al., 2013). However the specificity of these factors in inducing extra cycles of replication after MBT has not been tested. Hence it remains unclear whether destabilization of these factors is critical for cell-cycle lengthening at MBT. More recent work in C. elegans (Butuci et al., 2015) and Drosophila (Blythe and Wieschaus, 2015) suggest that the activation of transcription triggers CHK1 phosphorylation through activation of the replication checkpoint. In this model, conflicts between replication and transcription would be responsible for developmental activation of CHK1.

In Zebrafish, no connection between the N/C ratio and S-phase lengthening or between the N/C ratio and CDC25/CDK1 destabilization is clearly established. Using high-resolution imaging experiments, it has been shown that upregulating CDC25A activity or expressing an inhibitory phosphorylation-resistant CDK1 causes continued rapid divisions (Dalle Nogare et al., 2009), pointing out to a role of CDC25a and CDK1 inhibition in cell-cycle lengthening and asynchrony between the 9th and 12th cycles. Of note, zygotic transcription initiation is not required for cell-cycle lengthening In Zebrafish (Dalle Nogare et al., 2009).

Genetic studies in Drosophila melanogaster further support involvement of Twine and String (CDC25 orthologues) and CDK1 regulation in cell-cycle elongation at the MBT. Embryos from mother with germ cells having two extra copies of Twine gene can present a one-cycle delayed MBT. Moreover, embryos heterozygous for Twine and deficient for String (having one copy of CDC25 orthologues instead of four) display a lower number of early embryonic mitoses which is 12 (Edgar and Datar, 1996). Additional mutation in the CDK Inhibitor gene Fruhstart dramatically increases the frequency of extra early embryonic mitosis (Gawlinski et al., 2007), confirming the role of CDC25 and CDK1 downregulation in MBT timing and cell-cycle slow-down. This slowing occurs in two phases: a pre-MBT gradual one, and an abrupt slowing at MBT. The first phase is linked to the gradual activation of CHK1
pathway. Prior MBT, DNA replication activates the replication checkpoint progressively from one cell-cycle to a later one, giving the impression of a gradual division lengthening. Consistent with this, grapes (CHK1 orthologue) mutant flies embryos never hatch and undergo mitotic catastrophe in mitosis 13 due to a premature entry in M-phase with incompletely replicated chromosomes (Fogarty et al., 1997; Yuan et al., 2012). These embryos fail de delay mitosis until completion of replication because, in the absence of Grapes, CDK1 is not phosphorylated and inhibited (Fogarty et al., 1997; Sibon et al., 1997). Furthermore, grapes-mutated embryos fail to prolong pre-MBT cycles as in normal embryos (Sibon et al., 1997) suggesting a major role of grapes (CHK1)-driven inhibitory phosphorylation in pre-MBT interphase lengthening.

**Figure 3. 8 CDK1 regulatory mechanisms** (Hörmanseder et al., 2013)

CDK1 regulatory mechanisms. Inactive CDK1 and inhibitory phosphorylation (P) are depicted in Red background colour, while green colour denotes active CDK1 or activating phosphorylation. CycB and Cyc denote cyclin B and any cyclin, respectively; CKI, CDK inhibitory protein; CAK, CDK-activating kinase. For detailed mechanisms see the text.

Whereas the cell-cycle lengthening is progressive and gradual in pre-MBT embryos, cycle 14 shows dramatically increased duration, going from a 15 minutes S-phase to 50 minutes and additionally G2-phase is introduced (Edgar and O'Farrell, 1990; Shermoen et al., 2010) (Figure 3. 8). Moreover, cells loose cycle synchrony and enter mitosis at different timings (Foe, 1989; Hartley et al., 1997). CDK1 seems to be responsible for these changes (Edgar and
but in a different way than pre-MBT slowing. WEE1 and MYT1 kinases are present in excess during all embryonic stages including MBT. It looks like CDC25 phosphatase activity is the limiting factor for CDK1 stability and interphase lengthening at MBT (Dunphy and Kumagai, 1991; Edgar and O'Farrell, 1990; Russell and Nurse, 1986). During S-phase of cell-cycle 14, CDK1 is completely converted to an inactive phosphorylated form while in the previous cycles very few of CDK1 is phosphorylated (Stumpff et al., 2004). CDC25 mRNA microinjection in embryos in early MBT triggers a premature exit from G2 and induce early replication of the satellite sequences thus shortening the S-phase (Farrell et al., 2012). This data suggests that an abrupt destabilization of CDC25 at the onset of MBT induce CDK1 inactivation with as a consequence G2 introduction and DNA replication slowing. The two CDC25 orthologues in *Drosophila* String and Twine have high levels during the pre-MBT cycles (Di Talia et al., 2013; Edgar et al., 1994; Farrell and O'Farrell, 2013). Twine level remains high until early MBT, when it is rapidly destroyed, whereas String level progressively declines until disappearing prior MBT (Edgar et al., 1994). Therefore, Twine protein appears to be responsible for CDK1 inhibitory phosphorylation that lengthen S-phase, and add G2-phase at MBT in *Drosophila melanogaster*.

**B) Zygotic transcription activation**

As the cell-cycle slowing, the onset of zygotic transcription seems to be a gradual process in which genes initiate expression at different times. Genes can be divided in two categories: those whose transcription is dependent on N/C ratio and time dependent genes, based on a high-throughput study comparing the expression of many genes in wild-type versus haploid embryos. Some genes were expressed one cycle later in haploid embryos, whereas others kept normal transcription timing independently from DNA amount (Lu et al., 2009). Accordingly, given that cell-cycle slowing requires transcription activation, Twin (CDC25) degradation could be dependent on expression of N/C dependent genes. This model is supported by the fact that haploid embryos show delayed Twine degradation (Farrell and O'Farrell, 2013).

How the N/C ratio could control transcription and induce cell-cycle remodelling leading to MBT trigger is still puzzling. Several models have been proposed to explain the onset of zygotic transcription in early embryos. One model is the titration, on the exponentially increasing DNA, of some maternal components present in a limited amount in the embryo.
These components serve as a sensor for N/C ratio and trigger transcription of N/C dependent transcripts inducing zygotic genome activation and cell-cycle remodelling. This suggests the existence of transcription repressors in the early embryos that maintain silent the genomic DNA and that are subsequently titrated allowing zygotic transcription to begin. Consistent with this model, increasing the DNA content of an embryo by inducing polyspermy or injecting large amount of DNA, can induce earlier transcription onset (Newport and Kirschner, 1982a).

Previous studies showed that chromatin assembly is limiting for activation of transcription in Xenopus, suggesting that histones can actually be the repressors of transcription (Almouzni and Wolffe, 1995; Prioleau et al., 1994). A recent work indeed demonstrates that histones H3/H4 titration sets the N/C ratio threshold for the MBT. The authors identified histones H3 and H4 as concentration-dependent transcription inhibitors and cell-cycle remodeler. By adding or depleting H3/H4 from the Xenopus egg extract, the amount of DNA required for transcriptional activation in shifted accordingly. Moreover, addition of H3/H4 shortened post-MBT cell-cycles (Amodeo et al., 2015). Nevertheless, in Drosophila CDC25 expression in MBT embryos, which is actively degraded in cycle 14 (Farrell et al., 2012), is sufficient to introduce extra short cell-cycles arguing that the titration is not directly responsible for cell-cycle remodelling.

Another model proposes that just after fertilisation an autonomous molecular maternal timer is triggered and regulates the events preceding MZT. This is confirmed by the fact that Cyclin A and El proteins degradation is independent from the N/C ratio and depends on time after fertilisation (Howe et al., 1995; Howe and Newport, 1996; Stack and Newport, 1997). Furthermore, work in Drosophila favours the “maternal timer” model rather than titration (Lu et al., 2009).

A third model links transcription silencing to the DNA replication machinery that induce transcripts abortion during the first rapid cell-cycles. This model comes from experiments showing premature zygotic transcription in Xenopus and Drosophila embryos when blocked at cell cycles before normal zygotic transcription onset (Edgar and Schubiger, 1986; Kimelman et al., 1987).

Additional regulations does exist implicating other proteins. Among them: Zelda and Smaug. Zelda (Vielfaltig) is a zinc-finger DNA-binding protein which binds specific Zelda binding site on the genome and is highly enriched at genes that are expressed during the pre-MBT and the 14th cycle in Drosophila (Harrison et al., 2011; Nien et al., 2011). It is possible that Zelda serves as a binding platform for other transcription factors (Fu et al., 2014; Satija and
Increasing the number of Zelda binding domains induce premature transcription of the target gene. Conversely, removing Zelda binding sites near a gene delays onset of its transcription (ten Bosch et al., 2006). Some zelda-mutated embryos show an extra pre-MBT rapid cell-cycle, supposedly one or several genes involved in MBT timing are regulated by Zelda (Liang et al., 2008a; Sung et al., 2013).

Smaug is an RNA-binding protein that promotes RNA destruction by shortening their poly (A) tail through recruiting the CCR4/POP2/NOT deadenylase complex and thereby exposing them to degradation (Semotok et al., 2005; Smibert et al., 1996; Tadros et al., 2007). It has been also proposed as a timer of the MBT (Benoit et al., 2009). Smaug mutated embryos fail to activate efficiently the DNA replication checkpoint and do not show cell-cycle slowing and MBT onset. As replication checkpoint plays an important role in regulating the embryo cell-cycles, Smaug role could be indirect through Grapes (CHK1) pathway. In addition, these embryos present a defect in the onset of zygotic transcription (Benoit et al., 2009). However, the molecular basis of Smaug role in DNA replication checkpoint and transcription and its regulation by the N/C ratio are not well understood. Smaug is involved in the destabilization of maternally supplied mRNA near the MBT (Rouget et al., 2010) but there is no connection clearly established between this function and its role in controlling the onset of zygotic transcription or the activity of the DNA replication checkpoint.

C) DNA damage checkpoint in early embryos

During the first cell-cycles, the S-phase checkpoint (see paragraph 2.1) that halts DNA replication upon damage (Anderson et al., 1997; Hensey and Gautier, 1997) on unreplicated chromatin (Dasso and Newport, 1990; Kimelman et al., 1987) is, despite its critical functions, silent until the MBT. As previously mentioned, this could be a kind of early developmental adaptation in order to ensure rapid proliferation in an environment not always propitious. Early embryos are incredibly resistant to DNA damaging agents, for instance, wild-type C. elegans embryos are not sensitive to high doses of both the alkylating agent Methyl Methane Sulphonate (MMS) and UV light (Hartman and Herman, 1982; Holway et al., 2005).

The molecular mechanisms responsible for checkpoint inhibition in early embryos are poorly understood. Using in vitro and in vivo experiments in Xenopus (Conn et al., 2004; Dasso and Newport, 1990; Kappas et al., 2000), checkpoint activation has been shown to be
independent of transcription or translation, and it seems to pertain to the N/C ratio as the amount of DNA doubles every cell-cycle without significant cell growth, suggesting titration of maternal limiting factors of unknown identity. Adding of a threshold amount of undamaged DNA plasmid or sperm chromatin allows a DNA damage checkpoint response to be activated confirming the titration model (Peng et al., 2008). Few years ago, genetic studies in the worm C. elegans (Holway et al., 2006; Ohkumo et al., 2006; Roerink et al., 2012b) have involved rad-2, gei-17 sumo E3 ligase, and the translesion DNA polymerase polh-1 (TLS Polη) specialized in the replication of damaged DNA (see (Sale et al., 2012), for a review and paragraph 4.1.1).

4. DNA damage tolerance pathways

In some circumstances, when cells initiate DNA replication, they can tolerate and bypass the lesion, leaving it behind the progressing fork to be repaired afterwards in a process known as Post-Replication Repair (PRR). The first evidence of PRR were provided by early studies showing that DNA synthesis is discontinuous in the presence of damage, which suggests that gaps might be left behind the fork, and repaired later (Rupp and Howard-Flanders, 1968). More recent data in S. cerevisiae defective strain for NER, showed a functional uncoupling of leading- and lagging-strand DNA synthesis upon arresting replication forks by psoralen UV-cross-links, resulting in the formation of long stretches of primed ssDNA (Lopes et al., 2006), consistent with previous study in E. coli (Pages and Fuchs, 2003). Using a combination of two-dimensional gel electrophoresis and electron microscopy, Lopes and colleagues showed the accumulation of short ssDNA gaps (less than 400 bp) on both strands and also at far distance from the forks (~2.5-5.0 kb). Moreover, deleting of REV1, REV3, and RAD30 Pol η in this strain resulted in more frequent gaps without affecting the average gap size, ruling out the possibility of slow replication-coupled TLS after damage, and supporting the PRR hypothesis in which TLS is required for gap sealing (Lopes et al., 2006). In this scenario, possibly DNA replication is reprimed downstream of the block. However, this model cannot completely exclude the possibility that TLS can be coupled to replication during the S-phase. Consistent with this, PCNA monoubiquitination upon DNA-damage is maximal in S-phase followed by a rapid decrease in G2/M phase in S. cerevisiae (Daigaku et al., 2010; Davies et al., 2008).
Stalling replication forks for a long period may expose the cells to more deleterious lesions and generate genome instability. Damage tolerance pathways do not mediate the removal of the lesion as they often use error-prone DNA polymerases with low fidelity that contribute to the damage-induced mutagenesis. This pathway is subdivided into two main sub-pathways: error-prone translesion synthesis and error-free template switching.

4.1. Translesion DNA Synthesis: error prone damage tolerance pathway

Replicative DNA polymerases are very accurate and possess a proofreading activity that allows them to recognize mis-incorporated nucleotides which are not correctly base-pairing with the template strand. This high fidelity makes every DNA lesions not repaired before S-phase become a replication fork block as replicative polymerases cannot go through bulky lesions. In order to bypass these lesions, cells have evolved specialized DNA polymerases, known as translesion (TLS) polymerases that carry out a tolerance process called translesion DNA synthesis (Friedberg et al., 2002).

4.1.1. The Y-family of DNA polymerases: enzymes specialised for TLS

Among all translesion polymerases, those from the Y-family seem to play the main role in translesion DNA synthesis. In eukaryotes, the Y-family DNA polymerases includes four polymerases: REV1, Pol κ, Pol η, and Pol ι (Ohmori et al., 2001). Unlike replicative DNA polymerases, these enzymes are characterized by a large catalytic site capable of accommodating bulky lesions but in an error-prone way. They also have a low processivity and lack of 3’→5’ proofreading exonuclease activity, reducing their fidelity while replicating undamaged DNA template. REV1 gene was the first identified in a genetic screen of DNA damage tolerance defective mutants in budding yeast (Lemontt, 1971), then the function was determined later in the nineties (Nelson et al., 1996a, b). Few years later, other evolutionary-related DNA polymerases specialised in replication DNA through blocking lesions were discovered (Friedberg et al., 2001). The Y-family of TLS DNA polymerases can be subdivided into four families: UmuC, DinB, REV1 and Rad30. The UmuC family, exclusively prokaryotic,
includes the *E. coli* pol V. The human pol kappa (Polκ) and the *E. coli* pol IV are members of the ubiquitous DinB family. However, DinB genes are found neither in the budding yeast *S. cerevisiae* nor in *Drosophila* (Ohmori et al., 2001). REV1 and Rad30 families are only found in eukaryotes. The Rad30 family comprises the Rad30A subfamily including Pol eta (Polη) which exists in both yeast and humans, and the Rad30B subfamily represented by Pol iota (Polι) is found exclusively in higher eukaryotes (Ohmori et al., 2001).

When replication forks stall in front of a lesion present in the template DNA strand, long stretches of ssDNA coated by RPA are generated resulting from the uncoupling between polymerase and helicase activities (Byun et al., 2005). In order to bypass the block and continue DNA synthesis, specialized TLS polymerases can be recruited. How these polymerases are acting to resolve the block is still controversial. In the “polymerase switch” model TLS polymerases are recruited to the replication forks where they replace replicative polymerases and efficiently bypass the lesion. Then, error-prone TLS polymerases are disassembled, switching back to more accurate and more processive replicative polymerases to resume DNA replication (Napolitano et al., 2000; Pages and Fuchs, 2003). Another model suggest that the replication fork reinitiates downstream the lesion as demonstrated in bacteria and yeast (Branzei and Foiani, 2010), leaving behind the replication fork ssDNA gaps, which will be subsequently filled by either TLS synthesis or error-free post-replication repair (Branzei and Foiani, 2010; Daigaku et al., 2010; Karras and Jentsch, 2010). Mis-incorporated nucleotides are subsequently removed by several mechanisms, including NER and BER (Lehmann, 2011). Recent data suggest that in mammalian cells both repriming and lesion bypass at the fork are employed to overcome replication blocks (Despras et al., 2010; Martinez-Jimenez et al., 2015; Quinet et al., 2014; Rudd et al., 2014).

Polη is the only enzyme capable of error-free bypass of the major UV-photoproduct CPD *in vitro*, however the highly distorting UV-photoproduct [6-4]PP blocks Pol η bypass (Johnson et al., 2001). Moreover, it efficiently bypasses in an error-free manner different types of lesions, including 8-oxoG and thymine glycol generated by oxidative stress, and cisplatin intra-strand cross-links (Haracska et al., 2000; Kusumoto et al., 2002; Masutani et al., 2000; Nilforoushan et al., 2015; Vaisman et al., 2000). In an error-prone manner, Pol η can also bypass the BPDE-N2-guanine (Chiapperino et al., 2002). The mis-incorporation rate of Pol η is high compared to replicative polymerases (10^6), and varies from 10^-2 to 10^-3 depending on the
type of lesion (Haracska et al., 2000; McCulloch et al., 2004). This is due to the absence of intrinsic proofreading exonuclease activity (Matsuda et al., 2000).

Polκ is the human homologue of the bacterial DinB gene. This polymerase is specialised for the bypass of the benzo[a]pyrene adduct BPDE-$N^2$-guanine and other $N^2$-guanine adducts. Its mis-incorporation rate is very similar to that of Polη ($10^{-2}$ to $10^{-3}$), and depends on the substrate (Zhang et al., 2002).

Pol τ is another Y-family TLS polymerase which can bypass relatively accurately 8-oxoG by inserting preferentially a cytosine (Vaisman and Woodgate, 2001; Zhang et al., 2001) and is thus necessary for oxidative stress tolerance (Petta et al., 2008). It has a lower fidelity than Pol η or Pol κ with a frequency of errors 100 times higher on undamaged DNA. For instance it incorporates more frequently G opposite to a template T rather than A (Johnson et al., 2000; Tissier et al., 2000). It is also capable of bypassing helix-distorting DNA lesions in vitro with yet a low fidelity (Johnson et al., 2000).

REV1 is a deoxycytidyl transferase, which can only insert C opposite to a template DNA across abasic sites in vitro (Nelson et al., 1996a). For this reason it is not considered as a true DNA polymerase.

4.1.2. Other TLS polymerases

In addition to Y-family TLS polymerases, several other DNA polymerases possess a TLS activity. Pol θ and Pol ν are both members of the A-family DNA polymerases that display a TLS activity (Lange et al., 2011).

Pol zeta (polζ) is another TLS polymerase that belongs to the B-family of DNA polymerases, including the replicative polymerases Pol α, Pol δ, and Pol ε. Pol ζ contains two subunits: a regulatory subunit (Rev7) and the catalytic core (REV3). Although capable of CPDs bypassing in vitro, this enzyme is believed to extend primer-template termini synthesised by other translesion polymerases (Johnson et al., 2000). Moreover, the X-family member Pol β was also implicated in TLS across CPDs in mammalian cells (Bergoglio et al., 2003; Servant et al., 2002).
4.1.3. Regulation of translesion synthesis

   i) TLS polymerases bind to PCNA through PIP box-mediated interaction

   In both eukaryotes and prokaryotes, Y-family translesion polymerases require a physical and functional interaction with the sliding clamp PCNA. This interaction is primordial to fully activate translesion polymerases. Unlike REV1 which interacts with PCNA through a BRCT domain (Guo et al., 2006), Pol η, Pol κ and Pol τ possess a conserved C-terminal PCNA Interacting Peptide (PIP) box (Haracska et al., 2001a; Haracska et al., 2001b; Haracska et al., 2001c; Haracska et al., 2002).

   Whereas Pol η and Pol τ interact with PCNA by docking their PIP-box into a hydrophobic pocket within PCNA, Polκ PIP-box may not be sufficient on its own to mediate a functional interaction with PCNA as it has a lower affinity (Haracska et al., 2005; Haracska et al., 2001a; Haracska et al., 2001b; Hishiki et al., 2009). PCNA, together with RPA and RFC, stimulates the enzymatic activity of Pol η, Pol τ, and Pol κ in vitro (Haracska et al., 2001b; Haracska et al., 2001c; Haracska et al., 2002). When it interacts with the sliding clamp, Pol η has more affinity for dNTPs in vitro (Haracska et al., 2001c) but its processivity remains low in contrast to the E. coli pol IV (Lenne-Samuel et al., 2002). Consistent with this, the UV sensitivity of XP-V cells is only partially rescued by a Pol η mutant lacking the PIP-box motif (Bienko et al., 2005; Schmutz et al., 2010), suggesting that Pol η PCNA binding is necessary for its complete activation both in vitro and in vivo.

A) Reversible posttranslational modifications of PCNA regulate TLS

   In the current model of Y-family DNA polymerases functional regulation, PCNA ubiquitination plays the most important role (Figure 4. 1). As they have lower affinities in comparison to other PCNA interactors, TLS polymerases are recruited to chromatin-bound PCNA through an interaction with ubiquitin residue on PCNA that increases their affinity (Kannouche and Lehmann, 2004; Kannouche et al., 2004). This is confirmed by the fact that Polη C-terminal deletion mutants display a normal polymerase activity in vitro while they are inactive in vivo (Broughton et al., 2002; Kannouche et al., 2001; Masutani et al., 1999). This
suggests that the C-terminal domain of Pol η contains regulatory sequences, essential for its activity in vivo but not in vitro.

In many Y-family DNA polymerases, the C-terminal region is well conserved and contains, in addition to a PIP box motif, two other regulatory sequences; a nuclear localisation signal, and an ubiquitin-binding domain interacting with the monoubiquitinated form of PCNA (Bienko et al., 2005; Haracska et al., 2001c; Kannouche et al., 2004; Watanabe et al., 2004). In eukaryotes, Y-family DNA polymerases display two types of Ubiquitin-Binding Domains (UBDs): the Ubiquitin-Binding Zinc fingers (UBZs), and the Ubiquitin-Binding Motifs (UBMs) (Bienko et al., 2005). Pol η contains a classical zinc finger UBZ characterised by a central zinc ion coordinated by two cysteine and two histidine residues, while in the UBZ of Pol κ one histidine is replaced by a cysteine (Bomar et al., 2007; Ogi et al., 2005). REV1 and Pol ι contain an UBM that interacts with a hydrophobic surface of the ubiquitin residue (Bomar et al., 2010). The Pol ι UBM has a higher affinity to PCNAmonoUb than that the UBZ of Pol η (Bomar et al., 2010; Bomar et al., 2007). In budding yeast as well as in mammalian cells, the UBZ of Pol η and the UBM of REV1 are required for the complementation of UV-sensitivity and mutagenesis regulation (Bienko et al., 2005; Bomar et al., 2010; Guo et al., 2006; Parker et al., 2007; Schmutz et al., 2010).

In eukaryotes, the RAD6-RAD18 ubiquitin ligase complex is mainly responsible for the reversible monoubiquitination of PCNA that regulates the function of Y-family polymerases in TLS (Figure 4.1) (Lehmann et al., 2007). When replication fork arrests in front of a lesion, PCNA is monoubiquitinated and this increases the affinity of Y-family DNA polymerases for chromatin-bound PCNA, by providing an additional interaction site for their UBDs on the trimeric docking platform (Bienko et al., 2005; Kannouche and Lehmann, 2004; Watanabe et al., 2004). Translesion polymerases form microscopical subnuclear foci when recruited to stalled replication forks. However formation of these foci appear not to be essential for TLS polymerases function (Akagi et al., 2009; Gourdin and Vermeulen, 2009; Soria et al., 2009).

B) The RAD6-Rad18 complex catalyses PCNA monoubiquitination

Almost fifteen years ago, it has been shown that PCNA is the main substrate of RAD6-mediated ubiquitination and DNA damage response (Hoege et al., 2002). As RAD18 physical interaction with RAD6 was previously demonstrated and it contains a RING finger domain, a
hallmark of E3 ubiquitin ligases (Bailly et al., 1994; Ulrich and Jentsch, 2000), the link between $\text{PCNA}^{\text{monoUb}}$ and RAD18 E3 ubiquitin ligase was established (Hoege et al., 2002). Therefore, RAD18 and RAD6 were identified as an E3/E2 ubiquitin ligase complex responsible for $\text{PCNA}^{\text{monoUb}}$. Furthermore, using mutagenesis experiments, the Lys164 residue has been identified as the exclusive site of PCNA monoubiquitination, which is highly conserved from yeast to humans (Hoege et al., 2002). In mammalian cells, PCNA is monoubiquitinated by RAD6/RAD18 upon exposure to different DNA damaging agents, such as UV light, benzo[a]pyrene, cisplatin, H$_2$O$_2$, and methyl methanesulfonate (MMS) (Albertella et al., 2005; Bi et al., 2006; Hoege et al., 2002; Kannouche et al., 2004; Watanabe et al., 2004; Zlatanou et al., 2011), but unlike in yeast (Davies et al., 2008; Frampton et al., 2006), ionising radiation does not induce $\text{PCNA}^{\text{monoUb}}$ in mammalian cells. Moreover, a deubiquitinating isopeptidase (DUB) called USP1 counteracts the PCNA monoubiquitination reaction (Huang et al., 2006a).

PCNA can also be SUMOylated predominantly on Lys164 and to a minor extent on Lys127 (Hoege et al., 2002). This reaction requires a unique SUMO-specific E2, Ubc9. Depending on SUMOylation site, SUMO E3 Siz1, and Siz2 ensure the transfer of SUMO residue on Lys164 and Lys127 respectively (Hoege et al., 2002; Parker et al., 2008). PCNA SUMOylation is thought to induce the recruitment of the helicase PARI (SRS2 related protein in higher eukaryote) which can remove RAD51 filaments from single-stranded DNA and thus eliminates eliminating toxic recombination intermediates (Moldovan et al., 2012).

C) Other ubiquitin ligases that catalyse PCNA monoubiquitination

In response to DNA damage, the RAD18 E3 ubiquitin ligase cooperates together with the E2 RAD6 to monoubiquitinate PCNA. However, PCNA has also been reported to be monoubiquitinated in unperturbed cells by the CRL4$^{\text{Cdt2}}$ E3 ubiquitin ligase in a RAD18 independent manner. CRL4$^{\text{Cdt2}}$ contributes also to UV and cisplatin-induced PCNA monoubiquitination (Terai et al., 2010). This monoubiquination occur on the same lysine residue K164 as RAD18-related PCNA monoubiquitination, and is antagonized by the action of the Ubiquitin-Specific Protease 1 (USP1). CRL4$^{\text{Cdt2}}$-dependent PCNA monoubiquitination is proposed to regulate PCNA-dependent TLS, associated with replication stress (Terai et al., 2010).
In addition to RAD18 and CRL4\textsuperscript{Cdt2}, RNF8 has been reported to be able to monoubiquitinate PCNA in the presence of UbcH5c, and polyubiquitinating PCNA in presence of UBC13/Uev1a. Both reactions are the same as the ones performed by RAD18-RAD6 and RAD5-UBC13 respectively. Upon RNF8 depletion, UV-dependent PCNA monoubiquitination is reduced, revealing a possible role of RNF8 in the DNA damage tolerance pathway (Zhang et al., 2008).

D) Additional levels of TLS regulation

In vertebrates, REV1 harbours a conserved Polymerase-Binding Domain (PBD) that mediates interactions with the other TLS polymerases Pol η, Pol ι, Pol κ, and the regulatory subunit of Pol ζ, Rev7 (Guo et al., 2003; Murakumo et al., 2001; Tissier et al., 2004). In this respect, REV1 could serve as a scaffold for translesion polymerases (Friedberg et al., 2005). In DT40 chicken cells, REV1 PBD domain is necessary for efficient bypass of [6-4] thymidine dimers photoproducts by Pol ζ and mediates the resistance to UV and cisplatin (Ross et al., 2005; Szuts et al., 2008). Deletion of the REV1 PBD domain in DT40 cells gives more frameshift deletion mutations (slippage) at [6-4]T=T sites, suggesting that REV1 regulates the frame fidelity of Polζ (Szuts et al., 2008).

REV1 may constitute a parallel pathway to PCNA\textsuperscript{monoUb} for TLS regulation, since REV1 deletion is not epistatic to defective PCNA ubiquitination (PCNA\textsuperscript{K164R} mutant) (Ross et al., 2005; Szuts et al., 2008). It has been shown in DT40 cells that TLS at stalled replication forks requires REV1 UBM and PDB domains, whereas PCNA\textsuperscript{monoUb} is surprisingly not essential for progression through the damage (Edmunds et al., 2008). However, PCNA\textsuperscript{monoUb} is necessary for filling postreplicative gaps. This highlights different functions of REV1 and PCNA\textsuperscript{monoUb} in DNA damage bypass regulation.

An additional level of TLS regulation is the posttranslational modifications of the Y-family DNA polymerases. In mammalian cells, ATR mediates Pol η phosphorylation in the Ser601 residue (Gohler et al., 2011). Despite its localization into nuclear replication factories after UV irradiation, the non-phosphorylatable mutant Pol η\textsuperscript{Ser601A} rescues only partially the UV-sensitivity of XP-V cells (Gohler et al., 2011). The E3 RING ubiquitin ligase PIRH2 monoubiquitinates Pol η at four different lysine residues in its C-terminal (Bienko et al., 2010). This monoubiquitination was proposed as a negative regulation of Pol η function through
masking its UBZ (Bienko et al., 2010; Jung et al., 2011). In *S. cerevisiae*, REV1 is phosphorylated by the ATR orthologue Mec1 (Pages et al., 2009; Sabbioneda et al., 2007).

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**Figure 4. 1 Translesion synthesis model** *(Lehmann et al., 2007)*

(1) The replication machinery including PCNA and replicative polymerase pol δ encounters a lesion (a CPD dimer in this case) and get stalled. (2) Rad18-RAD6 ubiquitin ligase complex is recruited at the stalled fork where it monoubiquitinates PCNA. The deubiquitinase USP1 that catalyses the opposite reaction is cleaved. For sake of clarity, only one PCNA site is shown to be ubiquitinated, although the three monomers of the PCNA homotrimer could be monoubiquitinated. (3) This increases the affinity for Y-family polymerases, in this case pol η, which carries out the bypass of the lesion thanks to a more open catalytic site than the replicative polymerase. (4) After bypassing the lesion pol η is replaced by the replicative polymerase pol δ that will continue DNA replication in a more accurate and efficient way.
4.1.4. The post-replication repair hypothesis

Exposing cells to UV light generates lesions that block the progression of replication forks. These lesions are either removed by nucleotide excision repair (NER) or tolerated by translesion DNA synthesis. Actually, two distinct models for TLS have emerged: active bypass at the blocked replication forks or post-replicative gap-filling mechanism (Edmunds et al., 2008; Lehmann and Fuchs, 2006). According to the first model, the TLS polymerase replace the stalled replicative polymerase and inserts few nucleotides opposite the lesion. Then, the replicative polymerase resumes DNA replication following the lesion bypass.

The post-replication repair hypothesis suggest that the stalled replication forks are reinitiated after the damage using for example the newly discovered primase-polymerase PrimPol (Mouron et al., 2013). This enzyme is a member of the Archaeo-Eukaryotic Primase (AEP) superfamily (Iyer et al., 2005). The AEP superfamily is defined by the DNA-dependent RNA polymerase Primase small subunit 1 (Prim1 or PriS) (Bianchi et al., 2013; Garcia-Gomez et al., 2013; Iyer et al., 2005). PrimPol is conserved in a broad range of unicellular and multicellular eukaryotes, including animals, plants, and protists (Iyer et al, 2005 #3017). However, it is absent in Drosophila and Caenorhabditis elegans. PrimPol can prime DNA synthesis using template pyrimidines (Bianchi et al., 2013; Garcia-Gomez et al., 2013), and primers synthesized by PrimPol can be extended by replicative DNA polymerases. This enzyme possesses also a DNA polymerase activity, capable of extending DNA/RNA chains, including its own primers. PrimPol has a relatively low fidelity, synthetizing around four nucleotides when bound to DNA (Keen et al., 2014), similar to low-fidelity Y-family polymerases. The zinc finger domain of PrimPol plays an important role in its catalytic activities. Mutation of zinc chelating residues suppresses primase activity in vitro (Keen et al., 2014; Mouron et al., 2013; Wan et al., 2013), and decreases the processivity of PrimPol.

This primase/polymerase is also a competent translesion DNA polymerase that can bypass different kinds of DNA lesions, such as 8-oxo-G, abasic sites, CPDs, and 6-4 photoproducts (Bianchi et al., 2013; Garcia-Gomez et al., 2013; Keen et al., 2014; Mouron et al., 2013). Additionally, it is required for the normal progression of replication forks during DNA replication, especially upon replication stress conditions (Bianchi et al., 2013; Mouron et al., 2013; Wan et al., 2013). Similarly, PrimPol is also important for the maintenance of the small circular mitochondrial genome (Garcia-Gomez et al., 2013).
Thanks to its primase activity, PrimPol can reprime stalled forks downstream the lesion. Fork repriming, generates single-stranded DNA (ssDNA) gaps opposite the lesions that will be subsequently filled by TLS polymerases (Lopes et al., 2006). In this scenario, TLS occurs in a temporally independent manner from DNA replication, and replication fork progression should not need TLS. In addition, if cells are TLS-deficient, ssDNA gaps are not filled inducing a G2-phase arrest through checkpoint activation. Consistent with this hypothesis, TLS-dependent DNA damage bypass after UV exposure can occur both in S-phase and G2-M-phases in synchronized mammalian cells (Diamant et al., 2012). However, upon DNA damage, PCNA monoubiquitination is observed mainly in S-phase in yeast (Daigaku et al., 2010; Davies et al., 2008), and XP-V cells that are defective in Polη, show replication defects after UV damage, suggesting a requirement for polymerase switch (Despras et al., 2010). Furthermore, another study suggested that there is a crosstalk between TLS polymerases and replicative polymerases. The authors showed that the Pol Delta Interacting Protein 38 (PDIP38) promotes the transient replacement of replicative polymerases by TLS polymerases at DNA damaged sites (Tissier et al., 2010). More recently, it has been shown using XP-V cells and XP-C cells (GG-NER defective) that both bypass and gap-filling are necessary for low-dose UV-induced DNA damage tolerance at replication forks (Quinet et al., 2014).

4.1.5. Implication of translesion polymerases in DNA repair

Although TLS is considered as a DNA damage tolerance pathway used by cells to prevent fork stalling, it seems to be more and more obvious that TLS can also be involved in DNA repair (Sale et al., 2012). Hereafter, some examples of TLS contribution to DNA repair mechanisms.

A) Pol η and pol ε are involved in DNA repair of oxidative damage

Oxidative stress generates oxidized bases, such as 8-oxoG. In eukaryotes, OGG1, a DNA N-glycosylase recognizes and removes oxidised purines from DNA (van der Kemp et al., 1996). In budding yeast, in the Ogg1 deletion mutant (ogg1Δ), inactivation of either RAD6, RAD18, Rad30polη or expressing a PCNA^K164R monoubiquitination defective mutant results in
more spontaneous mutagenesis, indicating that RAD6-RAD18-Rad30\textsubscript{pol}\textsubscript{75} and OGG1 contribute through two distinct pathways for error-free tolerance and repair of oxidative damage (de Padula et al., 2004; Haracska et al., 2000; van der Kemp et al., 2009b). Similarly, mismatch repair proteins MSH2 and MSH6 play a role in the same error-free pathway as Rad30\textsubscript{pol}\textsubscript{75} and RAD18 (de Padula et al., 2004; Haracska et al., 2000), implying that Pol\textsubscript{\eta} may act in MMR in \textit{S. cerevisiae}.

When an 8-oxoG is generated, it pairs with a C and is generally removed by OGG1 during base excision repair (van der Kemp et al., 1996). Nonetheless, 8-oxoG is not detected by the proofreading activity of the replicative polymerases (Hsu et al., 2004), which mis-incorporate an adenine in the opposite strand giving an A:8-oxoG mismatch (Shibutani et al., 1991). In this case, cells use a translesion DNA polymerase that replicates in an error-free manner the 8-oxoG in order to recover the genetic information and avoid the propagation of mutations. Then, the mismatched A is excised by the MMR pathway, and replace by a C using a translesion polymerase. In humans, \textit{in vitro} data suggest that both Pol \texteta and Pol \textital are involved in this pathway, while in budding yeast, which lacks Pol \textital, Rad30\textsubscript{pol}\textsubscript{75} may be the only polymerase that does this task (Haracska et al., 2000; Vaisman and Woodgate, 2001; Zhang et al., 2001; Zhang et al., 2000).

In mammalian cells, Both GFP-Pol \texteta and GFP-Pol \textital co-localise with 8-oxoG when the cells are irradiated with a laser, in a manner dependent on Pol\texteta UBZ and PIP box domains and Pol \textital first UBM domain (Petta et al., 2008; Zlatanou et al., 2011). The E3 ubiquitin ligase RAD18 and OGG1 are also found in this DNA repair factories (Lan et al., 2004; Nakajima et al., 2006).

In addition, PCNA was shown to be monoubiquinated outside of S-phase, particularly in quiescent and G1 cells upon UV or H\textsubscript{2}O\textsubscript{2} treatment (Ogi et al., 2010; Zlatanou et al., 2011). This suggest that, apart from its role in MMR and BER (Moldovan et al., 2007), PCNA\textsubscript{monoUb} can recruit TLS polymerases to assist DNA repair. The fact that PCNA\textsubscript{monoUb} in G1 cells depends on MMR proteins MSH2 and MSH6 further confirm the link between DNA repair and TLS (Zlatanou et al., 2011). Supposedly, the mismatch is first cleaved generating ssDNA coated by RPA that activate the checkpoint and induce the recruitment of RAD18-RAD6 ubiquitin ligase complex that ubiquitinates PCNA. At this stage, the gap is filled by the replicative polymerase Pol \textdelta and the TLS polymerase Pol \texteta catalyses the error-free bypass of 8-oxoG (de Padula et al. 2004; Zlatanou et al. 2011).
In human cells, both Pol η and Pol ι mutations exacerbate H$_2$O$_2$ sensitivity, indicating that Pol ι could be also implicated in MMR (Petta et al., 2008; Zlatanou et al., 2011). However, the kinetic of Pol ι chromatin recruitment following oxidative damage is slower than of Pol η, suggesting different roles in oxidative repair (Petta et al., 2008; Zlatanou et al., 2011). There are many evidences that point out the role of Pol ι in BER. The first one is that Pol ι can complement Polβ-deficient cell extracts, and has a dRP lyase activity for cleaving the phosphodiester bonds (Bebenek et al., 2001; Prasad et al., 2003). Also, a physical interaction between Pol ι and the scaffold protein XRCC1 was identified (Petta et al., 2008). Finally, BER activity is lower in Pol ι-deficient cell extracts (Petta et al., 2008).

B) Implication of Pol κ in Nucleotide Excision Repair

Several reports suggest a possible role for the Y-family DNA polymerase Pol κ in NER (Lehmann, 2011). Unlike XP-V cells that do not display NER defects (Cleaver, 1972), Pol κ-deficient cells are less efficient for removing [6-4]PP from DNA (Ogi and Lehmann, 2006). Depending on RAD18 and NER proteins, Polκ can localise within DNA repair factories together with Pol δ outside of S-phase (Ogi et al., 2010; Tsanov et al., 2014), suggesting that it could be recruited through interaction with the PCNA$^{\text{monUb}}$ observed in G1-phase (Ogi et al., 2010). However, following UV-irradiation PCNA$^{\text{monUb}}$ signal in G1-cells is very weak and is independent from NER factors (Ogi et al., 2010). Pol δ and Pol κ are involved in the same repair synthesis mechanism downstream of the excision step (Ogi and Lehmann, 2006). In quiescent cells, where the dNTPs concentrations are low, Pol κ which displays a higher affinity for dNTPs, may have a predominant role in this pathway over Pol δ (Ogi et al., 2010).

4.2. Template switch: error-free damage tolerance pathway

Template Switch (TS) is an alternative error-free pathway to tolerate DNA damage. It allows the cells to avoid the damage rather than bypassing it. During Damage Avoidance (DA), cells switch to the sister chromatid and use it as a template to recover the genetic information from it (Ulrich and Walden, 2010). PCNA polyubiquitination constitute the trigger for TS pathway (Figure 4. 2). Through interacting with RAD18, RAD5 E3 ubiquitin ligase recruits the
MMS2-UBC13 E2 heterodimer complex to chromatin, thus extending the ubiquitin moiety on PCNA\textsuperscript{monUb} and forming a polyubiquitin chain on Lys63 (Hoege et al., 2002; Parker and Ulrich, 2009; Stelter and Ulrich, 2003; Ulrich and Jentsch, 2000). In this reaction, PCNA has to be first monoubiquitinated at lysine 164 by RAD6-RAD18. In budding yeast, PCNA polyubiquitination on Lys63 was identified to be essential for UV-damage tolerance (Spence et al., 1995). Furthermore, budding yeast mutants in \textit{RAD5}, \textit{MMS2}, and \textit{UBC13} genes show an increased UV-mutagenesis independently of RAD30\textsuperscript{PolIII}, confirming the existence of a non-mutagenic RAD6 branch (Lawrence, 1994). Using a lesion-specific plasmid system that can discriminate between TLS and TS, it has been shown in yeast and in mammalian cells that DNA damage tolerance could occur through a non TLS-pathway dependent on the complementary strand (Adar et al., 2009; Baynton et al., 1998; Zhang and Lawrence, 2005). In humans, two homologues of RAD5, involved in PCNA polyubiquitination and required for the maintenance of genome stability, have been identified: SHPRH and HLTF (Motegi et al., 2008; Motegi et al., 2006; Unk et al., 2008; Unk et al., 2006).
The schema shows DNA damage tolerance pathway (DDT). (A) High fidelity replicative polymerases progression is blocked by lesions (yellow Square), resulting in stalled replication forks. These lesions are bypassed by DNA damage tolerance mechanism that replicates past damaged DNA using low-fidelity DNA polymerases (translesion synthesis) or the undamaged sister chromatid as a template (template switching). The key regulator of DDT pathway is the modification of PCNA. (B) PCNA monoubiquitination at K164 by RAD18-RAD6 E3-ligase promotes TLS pathway. Some TLS polymerases are more accurate than others depending on the type of lesion, for instance pol η is capable of bypassing CPDs with a relatively high fidelity. Following lesion bypass Usp1 deubiquitinates PCNA, thereby facilitating loading of the replicative polymerase to resume DNA synthesis. (C) Template switching is triggered by PCNA polyubiquitination on K164, mediated by RAD5 (human homologue, SHPRH or HLTF). This pathway is mediated by structural rearrangement of the replication fork either by recombination or fork reversal.
4.3. Fork restart

Replication have to restart DNA synthesis after resolving replication stress. Many mechanisms are involved in replication fork restart. Among these mechanisms, firing of dormant origins due to a recruitment of an excess of the helicase MCM2-7 during late mitosis and early G1-phase. It has been shown in *Xenopus laevis* that only a fraction of these origins are fired during a normal S-phase due to checkpoint activity (Woodward et al., 2006). In the presence of replicative stress more origins are fired in order to rescue replication forks. Consistent with this, partial knock-down of MCMs proteins in *C. elegans* (Woodward et al., 2006) or human cells (Ge et al., 2007), has no effect on normal replication but induces lethality and suppresses the use of dormant origins upon replication stress.

A mean to restart the fork is repriming behind the lesion, leaving ssDNA gaps that will be repaired during post-replication repair on both leading and lagging strands using translesion synthesis and recombination (see above) (Elvers et al., 2011; Lopes et al., 2006). The third major mechanism to restart DNA replication is fork regression and reversal. During this pathway, the newly synthesized leading- and lagging strands are unwound. Then, the parental strands anneal back after the block in order to establish dsDNA around the obstacle and make the lesion exposed to repair factors. At this point, the newly synthesized unwound strands anneal and form a reversed fork structure, called a "chicken foot structure" where typical replication fork (three-way junction) is converted into a four-way junction (Figure 4. 3) (Neelsen and Lopes, 2015). The formation of these structures was observed by extensive electron microscopic analyses coupled with 2D gel electrophoresis (Berti et al., 2013; Cotta-Ramusino et al., 2005; Follonier et al., 2013; Neelsen et al., 2013). Several proteins are involved in fork reversal, PARP is one of those (Ray Chaudhuri et al.). Defects in these factors, induce replication problems, genomic instability, and difficulties to resolve fork blocking, confirming their physiological role for fork restart (Atkinson and McGlynn, 2009).

Fork reversal is a complicated mechanism, not yet completely understood. Recent work has begun to elucidate some of the many cellular factors required for fork remodelling *in vivo*. When replication forks are uncoupled, ssDNA stretches accumulate and the recombinase RAD51 partially replaces RPA and converts these forks into reversed forks, especially following topoisomerase inhibition, nucleotide depletion, and in the presence of ICLs (Zellweger et al., 2015). Therefore, RAD51 loading at the extended ssDNA regions can prime
fork reversal, promoting the re-annealing of parental strands (Figure 4.3a). Interestingly, in low nucleotide availability condition, replication fork reversal partially depends on the human F-Box DNA Helicase protein 1 (FBH1) (Fugger et al., 2015). FBH1 presumably initiates the unwinding of the lagging strand (Masuda-Ozawa et al., 2013) (Figure 4.3a). In addition to RAD51 and FBH1, several other factors belonging to the Fanconi anaemia or homologous recombination pathways such as the breast cancer susceptibility proteins BRCA1 and BRCA2, the RAD51 paralogues, and FANCD2 were shown to participate in different steps of replication fork remodelling (Kim and D'Andrea, 2012) (Figure 4.3a).

Moreover, the SMARCAL1/HARP protein is a DNA translocase that can re-anneal ssDNA bubbles coated by RPA and is probably involved in the parental strands re-annealing during regression (Blastyak et al., 2007; Yusufzai and Kadonaga, 2008). SLX4 nuclease in complex with SLX1 was recently shown to participate to the cleavage of replication forks regressed by SMARCAL1/HARP (Couch et al., 2013). This complex can process fork structures and Holliday junctions (Fekairi et al., 2009; Svendsen et al., 2009). The FancM helicase is also capable of unwinding both fork structures and double Holliday-structures and catalyses fork regression in vitro (Gari et al., 2008).

Once reversed forks are formed, ssDNA regions on the regressed arm are expanded by nucleolytic degradation and recruit more RAD51, promoting invasion of the re-annealed homologue template strands and thus fork restarts in recombination-mediated manner (Figure 3.10a). Other mechanisms are involved in the restart or reversed forks. For instance, the RECQ1 ATP-dependent DNA helicase is able to bind reversed forks and restart them by branch migration (Figure 4.3b). In this pathway, RECQ1 is only activated when PARP is inactivated by replication stress and DNA repair signalling (Berti et al., 2013; Zellweger et al., 2015). Another pathway is mediated by the combined action of nucleolytic degradation and unwinding of the regressed arm using the ATP-dependent helicase–nuclease (DNA2) and the Werner syndrome helicase. Then, the recombination-dependent restart occurs (Thangavel et al., 2015). Another pathway is mediated by the combined action of nucleolytic degradation and unwinding of the regressed arm using the ATP-dependent helicase–nuclease (DNA2) and the Werner syndrome helicase. Then, the recombination-dependent restart occurs (Thangavel et al., 2015).
Figure 4. 3 Fork reversal as a mechanism for fork restart (Neelsen and Lopes, 2015)

(A) The involvement of homologous recombination factors in replication fork remodelling. Upon replication stress and fork uncoupling, RPA coating ssDNA is partially replaced by RAD51. Controlled resection of newly synthesized strands assists RAD51 loading and is mediated probably by the Fanconi anaemia (FA) and homologous recombination (HR) factors. RAD51 mediates homology search and strand invasion promoting the reannealing of parental strands, and priming fork reversal, which is assisted by F-box-containing DNA helicase 1 (FBH1)-mediated displacement of the growing lagging strand. Once reversed forks are formed, ssDNA regions on the regressed arm may also recruit RAD51, and promote homology-driven invasion of the reannealed template strands and thus result in recombination-mediated fork restart. (B) Restart mechanisms of reversed forks. The DNA helicase RECQ1 binds reversed forks and promotes branch migration to restart the fork. PARP transiently inhibit RECQ1 which is only reactivated (RECQ1*) when DNA repair and replication stress release allow local PARP inactivation. RECQ1 binding inhibits another pathway involved in reversed fork restart, consisting in the unwinding and the nucleolytic degradation of the regressed arm by the nuclease activities of WRN helicase and DNA2 helicase/nuclease. The resected regressed arm may promote the recruitment of branch migration factors or homologous recombination-dependent restart.
5. RAD18: a key regulator of cell response to DNA damage

The E3 ubiquitin ligase RAD18 together with its partner RAD6 is a key regulator of the global cell response to DNA damage as it is involved in many pathways, including DNA damage signalling, DNA damage tolerance, and DNA damage repair. Hereafter, I will provide structural insights into the RAD18-RAD6 complex and detail the involvement of this complex in dealing with DNA damage as well as the regulation mechanisms governing its functions.

5.1. Structural insight into RAD18-RAD6 complex

The human RAD18 cDNA was cloned by screening a human placenta cDNA library using a human EST clone that encodes a peptide with homology to the N-terminus of S. cerevisiae RAD18 and N. crassa UVS2 protein (Tateishi et al., 2000). The isolated hRAD18 gene encodes a protein of 495-amino acids and an estimated molecular weight of 56 kDa displaying an altered electrophoretic mobility since the protein is detected as an 80 kDa protein by western blot. Human RAD18 shares 20% sequence identity and 42% similarity with yeast RAD18, and both of them have a conserved ring-finger motif and a zinc-finger motif in the N-terminal domain (Figure 5. 1) (Tateishi et al., 2000). Using Fluorescence In Situ Hybridization (FISH) method and PCR mapping by using a radiation hybrid panel, the RAD18 gene has been mapped to a single locus on chromosome 3p24-25 (Tateishi et al., 2000). RAD18 localizes mainly in the nucleus.

RAD18 is a multidomain E3 ubiquitin ligase (Figure 5. 1) that has been reported to dimerize in vivo and in vitro (Masuda et al., 2012; Miyase et al., 2005; Notenboom et al., 2007; Ulrich and Jentsch, 2000). Dimerization occurs via an N-terminal region (Hedglin and Benkovic, 2015). Genetic data have previously shown RAD18 dimerization in yeast (Ulrich and Jentsch, 2000) and human cells (Miyase et al., 2005). In yeast, the RAD18-RAD18 interaction was mapped to the region comprising residues 83-248 that contains the Zinc Finger (ZnF) domain. Mutation in the human RAD18 ZnF domain (C207F) disrupts its dimerization. Therefore, the ZnF domain was suggested to be the dimerization domain (Miyase et al., 2005). More recent in vitro data do not support this conclusion since regions containing the ZnF domain (199–366) or isolated ZnF do not dimerize (Notenboom et al., 2007). Probably other domains and/or post-translational modifications are necessary for this dimerization. Consistent
with this possibility, it has been shown that RAD18 ubiquitination favours the dimer formation and strengthens the interaction between two RAD18 molecules to form a "RAD18•Ub–RAD18 (Zeman et al., 2014). The $\text{RAD18}^{C_{207}F}$ mutant has been shown to ubiquitinate PCNA in vivo and in vitro following UV damage (Miyase et al., 2005; Notenboom et al., 2007), and $\text{RAD18}$-null cells complemented with $\text{RAD18}^{C_{207}F}$ acquire resistance to DNA damaging agents, including UV, mitomycin C, MMS, and cisplatin (Miyase et al., 2005; Tateishi et al., 2000). Interestingly, cells expressing $\text{RAD18}^{C_{207}F}$ are sensitive to ionizing radiation, at least in G1-phase (Watanabe et al., 2009). This could be explained by the fact that the ZnF domain of RAD18 is required for its binding to ubiquitinated proteins at double-strand break sites (Huang et al., 2009; Notenboom et al., 2007). In addition, this mutant in the RAD18 ZnF domain is not ubiquitinated in cells (Miyase et al., 2005), but capable of auto-ubiquitination in vitro (Notenboom et al., 2007). The function of the ZnF remains unclear, in addition to its role in RAD18 dimerization, other reports suggest its possible contribution in DNA binding, ubiquitin binding, and controlling the cellular location of RAD18 ((Bailly et al., 1994; Jones et al., 1988; Miyase et al., 2005; Tateishi et al., 2000; Ulrich and Jentsch, 2000).

The RAD18 protein binds to the human homologs of the yeast RAD6 protein (UBE2A and UBE2B) through a conserved RING finger motif and a RAD6-Binding domain (R6B). The RAD6-binding domain is located at the C-terminus of RAD18 and interacts with the noncovalent ubiquitin interaction site on RAD6 (Bailly et al., 1997a; Ulrich and Jentsch, 2000). $\text{RAD18}^{A_{340-395}}$ deletion mutant loses its interaction with RAD6 in vitro, although localization to damage is not affected (Watanabe et al., 2004). At its N-terminal part, RAD18 contains a Ring domain, common in E3 ubiquitin ligases, which also helps RAD6 binding, independently of the R6B domain (Tateishi et al., 2000). Mutations in this region increase the sensitivity to DNA-damaging agents. Gel-filtrations experiments coupled to multi-angle static light-scattering show that RAD18 forms with RAD6 is a dimer of heterodimers where two RAD18 molecules are bound together through their zinc finger domain , and each of them is bound to RAD6 through the RING finger and RAD6 binding domains (Notenboom et al., 2007).

In addition, RAD18 also contains other domains that bind PCNA, RPA, or DNA (the SAP domain). RAD18 does not contain a classical PIP box, however, the N-terminal region of RAD18 contain a PCNA-binding motif that acts independently of the other domains in this region (Notenboom et al., 2007). Moreover, the exact binding site of RAD18 on PCNA remains unknown. Since overexpression of proteins characterised by high affinity to PCNA front side,
such as p15<sup>PAF</sup> and p21, does not impair PCNA monoubiquitination (Soria et al., 2006; Toledo et al., 2013), it was suggested that RAD18 may not require access to the front side of PCNA for monoubiquitination. RAD18 binds also to ssDNA at stalled replication forks through its SAP domain, following DNA damage (Bailly et al., 1994; Nakajima et al., 2006; Notenboom et al., 2007). In the actual model, both the SAP and RPA-binding domains participate to the recruitment of RAD18 to RPA-coated ssDNA generated at uncoupled replication forks (Davies et al., 2008; Huttner and Ulrich, 2008; Notenboom et al., 2007).

RAD18 also contains ATPase domain which function is currently unknown. Disruption of this domain does not affect the DNA binding of RAD18 or the RAD6/RAD18 complex and is dispensable for the RAD18-RAD6 interaction (Bailly et al., 1997a; Bailly et al., 1997b; Jones et al., 1988). Note that, except the zing finger domain (Rizzo et al., 2014), the ring finger domain (Huang et al., 2011), and the RAD6 binding domain (Hibbert et al., 2011), the crystal structure of full length RAD18 is not yet resolved.
Figure 5. Schematic representation of the RAD18 protein interactions (Notenboom et al., 2007)

The RAD18 ZnF binds to ubiquitin, while the SAP domain is a DNA binding domain. RAD6 interacts with the Ring domain (catalytic domain) and between residues 340 and 395 towards the C-terminus (RAD6 BD). Pol η binding domain is located between residues 401 and 445 and the PCNA-interacting region is contained within residues 16–366 (shaded in brown). Lysine residues K161, K261, K309 and K318 represents the four sites of RAD18 autoubiquitination, which are conserved between mouse and human RAD18.
5.2. Recruitment of RAD18 to DNA damage sites

According to the polymerase switch model, translesion polymerases replace the replicative polymerases, and get access to chromatin at sites of DNA damage during the S-phase in order to allow the release of stalled forks (Friedberg et al., 2005). Therefore, PCNA monoubiquitination through RAD18-RAD6 must be tightly coordinated with the arrests of replication forks.

When the replicative polymerases are blocked by bulky DNA lesions, the helicase continues to unwind the DNA double-helix, giving long stretches of single-stranded DNA (ssDNA) that is coated and stabilized by the ssDNA bonding protein RPA (Byun et al., 2005). Serval lines of evidence suggest that the RAD6-RAD18 complex may be recruited to stalled replication forks through interaction with this RPA-coated ssDNA. The first one is that RAD18 binds ssDNA through a DNA-interacting SAP domain that is necessary for recruitment of RAD18 to replication factories upon UV-irradiation (Byun et al., 2005; Nakajima et al., 2006; Notenboom et al., 2007). Also, in vitro PCNA\textsuperscript{monoUb} by RAD18-RAD6 complex requires the loading of the PCNA clamp onto ssDNA (Garg and Burgers, 2005; Haracska et al., 2006). In addition, aphidicolin-induced replication fork uncoupling in \textit{Xenopus} egg extracts leads to PCNA\textsuperscript{monoUb} (Chang et al., 2006). Interestingly, upon aphidicolin treatment, inhibition of replication fork uncoupling by blocking the helicase co-factor CDC45 using specific antibodies, prevents PCNA\textsuperscript{monoUb} (Chang et al., 2006). Finally, RPA interacts directly with RAD18 \textit{in vitro} in budding yeast through an RPA interacting domain, and this interaction is required for PCNA\textsuperscript{monoUb} after MMS-induced DNA damage (Davies et al., 2008), whereas RAD18 chromatin binding is independent of RPA in \textit{Xenopus} egg extracts (Recolin and Maiorano unpublished results).

Additionally, the MRN complex protein NBS1 has been shown to bind RAD18 after UV irradiation and mediate its recruitment to sites of DNA damage (Yanagihara et al., 2011). NBS1 knock-down strongly reduces PCNA\textsuperscript{monoUb} and disrupts Pol \textita{η} foci formation, giving an exacerbated UV sensitivity and elevated mutation rate (Yanagihara et al., 2011). The NBS1 binding domain was mapped on the C-terminal region of RAD18, and shares structural and functional similarities with the RAD6 binding domain. RAD18 homodimers can interact simultaneously with both NBS1 and RAD6. Therefore, NBS1 plays a role in translesion DNA synthesis in addition to its role in DSBs repair (Figure 5. 2) (Yanagihara et al., 2011).
Figure 5. 2 NBS1 role in translesion synthesis and homologous recombination (Yanagihara et al., 2011)

(A) NBS1 binds to E3 ubiquitin ligase RAD18 and targets it to site of UV lesions to mediate PCNA monoubiquitination and TLS-dependent lesion bypass. NBS1 binding domain on RAD18 shares structural similarity with RAD6 binding domain. (B) NBS1 plays also a role in DSBs repair within the MRN complex (see the text for more details).

Upon UV-induced DNA damage, RAD18 binds to DNA, but how RAD18 is targeted specifically to PCNA at stalled forks is poorly understood as RAD18 lacks PCNA-binding motifs. Few years ago, Spartan was identified as a scaffold for recruiting RAD18 to PCNA as it binds and bridges both RAD18 and PCNA (Centore et al., 2012). Consistent with this, DNA damage-induced PCNA monoUb was partially reduced in Spartan-depleted cells (Centore et al., 2012). However, several other publications have suggested different roles for Spartan in DNA damage signalling such as protecting the ubiquitin residue on PCNA or recruiting the ubiquitin-selective chaperone p97 (Davis et al., 2012; Juhasz et al., 2012; Machida et al., 2012; Mosbech et al., 2012).
In mammalian cells, RAD18 forms a complex with Polη (Figure 5. 3) (Day et al., 2010b; Watanabe et al., 2004), and this interaction is necessary for DNA damage tolerance (Barkley et al., 2012), as it targets RAD18 to PCNA and facilitates efficient PCNA monoUb. The S-phase checkpoint controls the RAD18–Pol η complex formation through CDC7 and CHK1 kinases (Day et al., 2010b; Watanabe et al., 2004). Surprisingly, Pol η stimulation of PCNAMonoUb is completely dissociable from its DNA polymerase activity. This may explain why catalytically inactive Pol η can partially rescue the DNA damage-sensitivity of XPV cells and induce the recruitment of other error-prone TLS polymerases (Polκ and Polτ) after UV irradiation (Ito et al., 2012b; Pavlov et al., 2001).

Figure 5. 3 RAD18 simplified interactome

The diagram shows the main proteins that interacts with RAD18, and which play a role in the global cell response to DNA damage. In green background, proteins involved in translesion DNA synthesis. Proteins involved in homologous recombination are depicted in purple background. Yellow background,
Recently, it has been shown that, upon DNA damage, c-Jun N-terminal Kinase (JNK) phosphorylates RAD18 specifically at S409 within the Polη binding domain, and in a checkpoint dependent manner. Interestingly, RAD18$^{S409}$ phosphorylation promotes association with Polη (Barkley et al., 2012). Furthermore, the serine-threonine kinase CDC7 together with its S-phase regulator ASK (DBF4) phosphorylates a cluster of serine residues within the Polη binding domain of RAD18, and positively regulates Pol $\eta$–RAD18 interaction in an ATR-CHK1-mediated checkpoint dependent manner (Day et al., 2010b; Vaziri and Masai, 2010). Upon replication fork block, CHK1 kinase inactivates the Anaphase-Promoting Complex/Cyclosome APC/C (Cdh1) through degradation of Cdh1, Thus APC/C (Cdh1) substrates are stabilized, including CDC7-ASK (DBF4) that phosphorylates RAD18 (Figure 5.4). Moreover, the ASK (DBF4) subunit interacts with the N-terminal region of RAD18 through its motif-C, a conserved C2H2-type zinc finger domain (Figure 5.4) (Yamada et al., 2013a). This interaction is necessary for RAD18 chromatin binding and subsequent Polη foci formation (Yamada et al., 2013a).
5.3. RAD18-RAD6 complex not only catalyses PCNA monoubiquination, but plays a major role in DNA repair and DNA damage signalling

PCNA is not the only substrate of RAD18. Early genetic screens of *Saccharomyces cerevisiae* mutants had suggested that RAD18 may be involved in DNA repair (Bailly et al., 1997b). Dysfunctions of the human RAD18 gene results in defective post-replication repair and exacerbated sensitivity to various DNA damaging agents, including UV, MMS, and MMC (Tateishi et al., 2000).

Several lines of recent evidence implicate RAD18 in ICL repair (Raschle et al., 2015) and FA pathway activation (Geng et al., 2010; Palle and Vaziri, 2011; Song et al., 2010; Williams et al., 2011). It has been reported that FANCD2 monoubiquitination is reduced in RAD18-deficient cells upon induction of ICLs (Williams et al., 2010), bulky adducts (Song et al., 2010) or treatment of cells with Topoisomerase I inhibitors (Palle and Vaziri, 2011), while it is stimulated by RAD18 overexpression. However the purified RAD6-RAD18 complex fails to monoubiquitinate FANCD2 in vitro suggesting the requirement of additional proteins (Song et al., 2010). Consistent with this possibility, Geng and colleagues suggested that the role of RAD18 in FA pathway is indirect. By ubiquitinating PCNA on Lys164, RAD18 promotes the recruitment of FANCL (E3 ubiquitin ligase) to chromatin, inducing FANCL-mediated FANCD2 and FANCI monoubiquitination (Geng et al., 2010). Moreover, FANCD2 monoubiquitination by RAD18 in response to ICL and bulky DNA lesions, depends also upon PCNA mono-ubiquitination and TLS polymerases binding (Geng et al., 2010; Song et al., 2010), whereas DNA Topoisomerase I inhibition induce a RAD18-mediated FANCD2 ubiquitination independently from TLS and PCNA\textsuperscript{monoUb}, but requires the E3 ubiquitin ligase activity of RAD18 (Palle and Vaziri, 2011). Furthermore, FANCD2 and RAD18 combined depletion does
not have additive effects on Topoisomerase I inhibition (Palle and Vaziri, 2011) or ICL sensitivity (Williams et al., 2011), indicating RAD18 and FANCD2 are epistatic for the FA pathway. In sum, these observations suggest that activation of the FA pathway by RAD18 is indirect and may depends upon activation of TLS.

Recently, RAD18 has also been shown to bind polyubiquitin chains on histone H2AX generated by RNF8 and RNF168. Then, RAD18 physically interacts with SLF1 and SLF2 (SMC5-SMC6 complex Localization Factor protein 1 and 2 respectively), linking RAD18 to the SMC5/6 complex that holds sister chromatids together in the vicinity of DNA crosslinks, thereby promoting the HR phase of ICL repair (Figure 5.5) (Raschle et al., 2015). SLF1 or SLF2 knockdown does not affect either FANCD2 or RAD18-dependent PCNA monoubiquitination in response to DNA damage (Raschle et al., 2015). By doing so, RAD18 seems to play a role in the different steps of ICL repair (Figure 5.7). Unexpectedly, FANCD2 monoubiquitination is still observed and the FA pathway is fully functional in RAD18−/− cells derived from knockout mice (Yang et al., 2016). The authors explained these observations by the fact that the previous in vitro studies were conducted using cancer-derived cultured cell lines that most of the time aberrantly overexpress RAD18, and FANCD2 monoubiquitination could be a neomorphic role of RAD18 in RAD18-overspressing cells. Moreover, RAD18 mutant mice and Fanc-deficient mice are phenotypically distinct, indicating a separation between RAD18 and FA pathways in vivo (Yang et al., 2016). This is consistent with the fact that no RAD18-deficient FA patients have been yet reported.

It has been shown also that RAD18 ubiquitinates the RFC component RFC2 in vitro and in cells (Tomida et al., 2008). RFC2 ubiquitination following MMS-treatment in decreased in RAD18−/− cells compared with matched RAD18+/+ HCT116 cells, suggesting that RFC2 ubiquitination is partially dependent on RAD18 (Figure 5.6) (Tomida et al., 2008).
Upon DNA damage, RNF8 and RNF168 E3 ubiquitin ligases polyubiquitinate H2AX. RAD18 recognizes and binds the polyubiquitin chains on histone H2AX. Then, RAD18 physically interacts with SLF1 and SLF2, promoting the recruitment of the SMC5/6 complex that holds sister chromatids together in the vicinity of DNA crosslinks, thereby promoting the HR phase of ICL repair.

RAD18 localizes both in the cytoplasm and in the nucleus. RAD18 nuclear translocation is increased in response to replication stress, upon dNTP depletion or double-strand DNA breaks (Masuyama et al., 2005). When DSBs are generated at unresolved stalled replication forks, RAD18 enhances the polymerization of the RAD51 recombinase (Kobayashi et al., 2015), and facilitates homologous recombination at the sites of breakage, thus reducing the toxic effect of non-homologous end joining at DSBs (Kobayashi et al., 2015; Saberi et al., 2007). Deletion of non-homologous end joining factor Ku70, restores the tolerance of $RAD18^{-/-}$ cells to camptothecin (topoisomerase I inhibitor) and olaparib (PARP inhibitor), two drugs that induce DSBs which are repaired exclusively by HR (Kobayashi et al., 2015). In response to CPT-induced DSBs, RAD18 is essential for proper FANCD2, BRCA2, and RAD51 foci formation. Knocking-down RAD18, reduces the protein levels of BRCA2 and RAD51, and affects FANCD2 ubiquitination and cell viability (Tripathi et al., 2016), indicating that RAD18 may function upstream these three proteins in the homologous recombination repair pathway. Immunoprecipitation experiments show that RAD18 physically interact with both FANCD2 and RAD51 (Tripathi et al., 2016). In *S. cerevisiae*, RAD18 is necessary for the formation of X-shaped Sister Chromatid Junctions (SCJs) at damaged replication forks (Branzei et al., 2008). This process involves the ubiquitin-conjugating enzymes MMS2 and UBC13 that mediate
PCNA polyubiquitination. The damage bypass through SCJs requires PCNA SUMOylation through the SUMO-conjugating enzyme Ubc9, and the recombination factor RAD51 (Branzei et al., 2008).

**Figure 5. 6 RAD18 known substrates**

RAD18 ubiquitinates a limited number of known substrates. Among them, PCNA was the first identified (Hoege et al., 2002; Kannouche et al., 2004), controlling TLS polymerases recruitment to damaged chromatin. Recently RAD18 has been shown to ubiquinate the Fanconi Anemia proteins FANCD2 (Song et al., 2010; Williams et al, 2011; Palle et al., 2011) and FANCI (Geng et al., 2010). 53BP1 is also ubiquitinated by RAD18 during non-homologous end joining (Watanabe et al., 2009). In yeast, the RAD6-RAD18 complex monoubiquitinates the Rad17 protein, promoting the recruitment of the 9-1-1 complex and efficient RAD53 phosphorylation (Fu et al., 2008). However, these observations were not confirmed by another independent study (Davies et al., 2010). RAD18 ubiquitinates also the RFC component RFC2 in vitro and in cells (Tomida J et al., JBC, 2008).

Unlike Polη, RAD18 rapidly relocalizes throughout the nucleus following X-ray irradiation. When recruited to ionizing radiation (IR)-induced sites of DSBs, RAD18 forms foci that co-localize with 53BP1, phosphorylated ATM, BRCA1, NBS1, and γ-H2AX (Huang et al.,...
RAD18 is capable of monoubiquitinating the KBD domain of 53BP1 at lysine 1268 in vitro (Figure 5.6), then it associates with 53BP1 and targets it to DSB sites only during G1-phase, promoting non-homologous end joining (Watanabe et al., 2009). When 53BP1 is mutated at the ubiquitinated lysine 1268, it loses the ability to bind efficiently the chromatin at the vicinity of DSBs. In RAD18-null cells, IR-induced nuclear Foci (IRIF), including BRCA1, MRE11/NBS1/RAD50 and 53BP1 proteins (Paull et al., 2000; Schultz et al., 2000) are disrupted and their viability after X-ray or UV irradiation is reduced (Huang et al., 2009; Watanabe et al., 2009). Furthermore, it was demonstrated that RAD18 is involved in Single-Strand Break Repair (SSBR) during S-phase independently from PCNA monoubiquitination and Polη chromatin binding, and HCT116 cells that are RAD18-deficient are sensitive to both X-ray irradiation and the topoisomerase I inhibitor camptothecin (CPT) capable of inducing single-strand breaks (Shiomi et al., 2007).

Interestingly, RAD18 knockout mouse embryonic stem cells display normal growth rates, but show hypersensitivity to DNA-damaging agents and defective post-replication repair (Tateishi et al., 2003). The mutation rate of knockout cells was comparable to wild type cells. However, spontaneous Sister Chromatid Exchange (SCE) was twice more frequent in the knockout cells than in normal cells. This suggest that RAD18 prevents illegitimate recombination and thus contributes to the maintenance of genomic stability through post-replication repair (Tateishi et al., 2003).

In addition to its role in DNA damage tolerance and DNA damage repair, RAD18 has been also involved in checkpoint signalling. Upon γ-radiation-induced DNA damage, RAD18 interacts with ubiquitinated chromatin factors, including the histone H2A through its zinc-finger domain and facilitates recruitment of the RAD9 component of the 9-1-1 checkpoint complex, to DNA double strand breaks, but does not contribute to downstream activation of the checkpoint kinases CHK1 and CHK2 (Inagaki et al., 2011). In yeast, the RAD6-RAD18 complex induces a DNA damage-dependent monoubiquitination of the Rad17 protein at the Lysine 197 (Figure 5.6), promoting the recruitment of the 9-1-1 complex and efficient RAD53 phosphorylation (Fu et al., 2008). However, these observations were not confirmed by another independent study (Davies et al., 2010).

Recently, monoubiquitinated PCNA has been shown to interact with the ATM cofactor ATMIN via WRN-Interacting Protein 1 (WRNIP1) (Kanu et al., 2016). RAD18 through ubiquitinating PCNA, ATMIN and WRNIP1 was shown to be required in ATM-dependent...
phosphorylation of Kap1, as well as p53 phosphorylation at serine 15 (Kanu et al., 2016). 53BP1 foci formation is also dependent on RAD18, after replication stress induced by aphidicolin, but not upon ionizing radiation-induced damage (Kanu et al., 2016). Consistent with this interpretation, RAD18 depletion in human cells reduces the chromatin recruitment of the DNA damage signalling factors ATM, γH2AX, as well as 53BP1 foci at the G2/M phase, and attenuates the activation of the G2/M checkpoint upon exposure to IR (Sasatani et al., 2015).

**Figure 5. 7 RAD18 role in interstrand crosslink (ICL) repair pathway** (Haynes et al., 2015)

RAD6-RAD18 ubiquitin ligase complex coordinates the activities of Fanconi anemia (FA) pathway, homologous recombination (HR), and translesion synthesis (TLS) during interstrand DNA crosslink (ICL) repair. (A) Evidence suggests that RAD6-RAD18 can mediate FANCD2 monoubiquitination and activation of FA network in cancer cell lines. In addition to its role in HR, monoubiquitinated FANCD2 also partakes in TLS polymerase Pol η recruitment. (B) RAD6-RAD18 complex also activates the error-prone translesion synthesis, component of DNA damage tolerance, by inducing PCNA monoubiquitination thus promoting the bypass of the DNA adducts. Besides their role in TLS, PCNA and Pol η participate to HR by regulating extension of D-loop structures. (C) RAD18 is also involved in HR through interacting with RAD51. The error-free arm of DNA damage tolerance (template switching) is activated by PCNA polyubiquitination, where RAD6 polyubiquitin chains are transferred to PCNA via HLFTE3 ligase.
5.4. Regulation of RAD18 function: USP1 and USP7

Given its pivotal role in governing the fine balance between replication fidelity and genome stability, RAD18 levels and activity must be tightly regulated to preserve genomic integrity. Very little is actually known about how RAD18 levels and activity are regulated during the cell cycle or after DNA damage.

Because RAD18-dependent PCNA\textsuperscript{monoUb} activates error-prone TLS polymerases, RAD18 activity should be strictly regulated in order to avoid undesired mutagenesis. This reaction is counteracted by the DUB Ubiquitin Specific Protease 1 (USP1) that removes the ubiquitin residue from the TLS polymerases scaffold, PCNA, preventing deregulated error-prone translesion DNA synthesis (Huang et al., 2006a). When cells are exposed to UV irradiation, USP1 is inactivated through an autocleavage reaction, thus enabling PCNA monoubiquitination and subsequent TLS activation (Huang et al., 2006a). It has been reported that USP1 depletion induces PCNA monoubiquitination (Simpson et al., 2006). Furthermore, ELG1 protein, Component of the RFC-like ELG1-RFC complex which appears to have a role in DNA replication, specifically directs USP1-UAF1 (USP1-Associated Factor 1) complex for PCNA deubiquitination (Lee et al., 2010).

Recently, USP7 was identified as another deubiquitinating enzyme of monoubiquitinated PCNA (Kashiwaba et al., 2015). Reducing USP1 or USP7 levels in human cells under UV- or H\textsubscript{2}O\textsubscript{2}-induced damage, has an additive effect on increasing PCNA monoubiquitination. Cell-cycle-synchronization experiments suggest that USP7 may deubiquitinate PCNA throughout interphase, while USP1 acts during the S-phase (Figure 5. 8) (Kashiwaba et al., 2015). In addition, USP1 suppression increases UV-induced mutagenesis, whereas USP7 suppression induces mutagenesis after H\textsubscript{2}O\textsubscript{2} treatment (Figure 5. 8). These findings suggest that USP1 deubiquitinates PCNA in a DNA replication-dependent manner and suppresses TLS mutagenesis, when USP7 suppresses the mutagenesis during DNA repair (Kashiwaba et al., 2015).
Figure 5.8 The role of USP1 and USP7 in regulating RAD18-mediated PCNA monoubiquitination (Kashiwaba S et al., 2015)

PCNA monoubiquitination by RAD6-RAD18 activates error-prone TLS DNA synthesis, promoting lesion bypass and mutagenesis. Both USP7 and USP1 deubiquitinate PCNA thus suppressing TLS-related mutagenesis. USP1 suppresses UV-induced mutations during S-phase, whereas USP7 suppresses specifically \( \text{H}_2\text{O}_2 \)-induced mutagenesis in interphase.

Very recently, USP7 was identified as a critical regulator of RAD18 protein levels (Zlatanou et al., 2016). USP7 binds directly to RAD18 via a consensus USP7-binding motif, and disassembles the polyubiquitin chains on RAD18 both \textit{in vitro} and \textit{in vivo}, preventing its proteasomal degradation (Zlatanou et al., 2016). In USP7-depleted cells, RAD18 is destabilized and UV-induced PCNA monoubiquitination and Pol \( \eta \) chromatin binding are disrupted at stalled replication forks with reduced DNA damage tolerance (Zlatanou et al., 2016).

RAD18 function is also regulated through its auto-monoubiquitination. When the cells are treated with MMS or \( \text{H}_2\text{O}_2 \), RAD18 is deubiquitinated and activated (Zeman et al., 2014). The ubiquitinated form of RAD18 loses its capacity to interact with the Helicase-Like Transcription Factor (HLTF) or SNF2 Histone linker Plant homeodomain RING Helicase (SHPRH), two downstream E3 ligases essential for error-free DNA damage bypass. Instead, monoubiquitinated RAD18 interacts with the zinc finger domain of another nonubiquitinated RAD18, forming a dimer RAD18\( \text{Ub} \)-RAD18 that may inhibit RAD18 function in \textit{trans}. 121
Additionally, ubiquitination prevents RAD18 from forming foci at sites of DNA damage, thus reducing PCNA monoubiquitination (Zeman et al., 2014).

Moreover, the RAD18 gene was shown to be under the control of the transcription factor E2F3 (Varanasi et al., 2012). E2F3 directly associates with the RAD18 promoter and controls its activation. When E2F3 is knockdown, PCNA_{monoUb} and RAD18 levels are strongly reduced. Conversely, RAD18 ectopic expression is sufficient to rescue the PCNA_{monoUb} defect in E2F3 knockdown cells (Varanasi et al., 2012).

In addition to these regulation pathways, recently the micro RNA miR-145 was found to target directly RAD18 and reduce its levels. Interestingly, targeting RAD18 by miR-145 enhanced DNA damage in ColtRectal Cancer (CRC) cells after 5-Fluoro Uracil (5-FU) treatment (Liu et al., 2015).

6. Oncogene-induced replication stress in cancer progression and recurrence

Cancer is a major health issue responsible for around 25% of deaths in developed countries (Jemal et al., 2011). It is defined as uncontrolled and abnormal cell proliferation. Even though cancers are very heterogeneous, they display shared properties referred as “Cancer Hallmarks”. This term was originally introduced sixteen years ago by Hanahan & Weinberg (Hanahan and Weinberg, 2000). Since that time, the number of cancer hallmarks significantly increased, including independency from proliferative signalling and unlimited replicative potential, resistance to growth suppressors, escape from apoptosis, tissue invasion and metastasis, capacity to induce angiogenesis, deregulated cellular metabolism, genomic instability, escape to immune response, and tumour-induced inflammation (Hanahan and Weinberg, 2000, 2011; Luo et al., 2009). It is obvious that some of these hallmarks are more relevant for certain cancer-types development than others. The most frequent mutations found in human cancers are linked to cancer hallmarks (Figure 6. 1). Mutations in oncogenes and tumour suppressors that regulate cell growth can explain the sustained proliferation of cancer cells, whereas the capacity to escape from apoptosis can be explained by the accumulation of mutations in checkpoint genes such as the TP53, ATM, ATR, or MDM2 (Macheret and Halazonetis, 2015). These two hallmarks, in addition to genomic instability, could be related to
the replication stress. In the oncogene-induced replication stress model, mutations in the genes driving cell proliferation (oncogenes and tumour suppressors) induce DNA replication stress and accumulation of DSBs, which, consecutively, leads to genomic instability and selects precancerous lesions for escape from apoptosis (Figure 6.2) (Macheret and Halazonetis, 2015). For these reasons, DNA replication stress can be considered as a hallmark of cancer cells.

**Figure 6.1 The most frequent genetic mutation found in human cancers are linked to cancer hallmarks (Macheret and Halazonetis, 2015)**

The figure represents the most frequently targeted genes by single nucleotide substitution (SNS), amplification (Ampl), and deletion (Del) in cancer cells. The mutations of these genes are related to cancer hallmarks. The Chromatin modifiers could not be directly linked to a specific cancer hallmark. Oncogenes are written in green letters, tumor suppressors in red letters, and NOTCH1 in blue letters.

Oncogenes are genes that when deregulated drive cancer development (Bishop, 1987; Land et al., 1983). These genes share two main properties: capacity to transform cells when mutated or overexpressed and being mutated in human cancers. When activated, these cancer-driver genes induce DNA DSBs and replication stress in human precancerous lesions and cancers. The vast majority of oncogenes stimulate the activity of G1 and S-phases CDKs and deregulate the cell cycle (Hartwell and Kastan, 1994; Ortega et al., 2002). By doing so,
oncogenes may induce DNA replication stress in human precancerous lesions leading to the formation of DSBs (Bartkova et al., 2005; Bartkova et al., 2006; Di Micco et al., 2006; Gorgoulis et al., 2005).

Expressing oncogenes in non-transformed primary cells also induces DNA replication stress. These accumulate stretches of ssDNA and DSBs specifically in S-phase. Additionally, they display premature termination of DNA replication forks and Loss Of Heterozygosity (LOH) targeting preferentially the common fragile sites (Bartkova et al., 2006; Di Micco et al., 2006).

Oncogene activation leads to genomic instability in human precancerous lesions through several mechanisms. Telomeres erosion and shortening is one such a mechanism, which can give rise to genomic instability before telomerase expression is induced (Maser and DePinho, 2002). Telomere erosion is common in human precancerous lesions (Hansel et al., 2006), however sometimes genomic instability at common fragile can occur before changes telomere shortening become detectable (Gorgoulis et al., 2005).

Mutations in caretaker genes that preserve genomic integrity generate also genomic instability. This is the case for the genes involved in cell cycle checkpoints and DNA repair (Hartwell and Kastan, 1994). For instance, mutations in DNA MMR genes cause Microsatellite INstability (MIN), which is a type of genomic instability (Lengauer et al., 1998). Surprisingly, p53-null mice do not present genomic instability (Lane, 1992). Another possibility is that oncogene-induced DNA damage, rather than inactivating caretaker genes, may induce DNA double strand breaks that can lead afterwards to Chromosomal INstability (CIN).

Genomic instability seems to be essential for cancer development as it allows the normal cell to accumulate all the mutations necessary to be transformed to a cancer cell (Beckman and Loeb, 2006). However, the role of genomic instability in tumour progression remains an elusive concept.

6.1. DNA damage response as a barrier to malignant transformation that precancerous cells can sometimes override

When oncogenes are activated, they generate DNA damage as a consequence of unscheduled stimulation of cell proliferation in differentiated cells. In normal conditions, the
DNA damage response acts to repair the damage, and if it is unrepairable, an apoptosis program is activated, involving the genome guardian p53 (Figure 6.2). Different lines of evidence point to p53 as a key tumorigenesis barrier in precancerous lesions. First, apoptosis and senescence are both p53-dependent mechanisms. Also, in human cancers, precancerous lesions evolution and the escape from tumorigenesis barrier is very often correlated with p53 mutations, and it has been shown in p53-deficient mice that the transition from precancerous lesions to cancer is more rapid (Bartkova et al., 2005; Campisi, 2005; Gorgoulis et al., 2005; Kastan and Bartek, 2004). In the oncogene-induced DNA damage model, p53 is activated in precancerous lesions by the DNA damage checkpoint pathway, however p53 can be also activated through the Alternative Reading Frame (ARF) tumour suppressor protein pathway (Sherr et al., 2005). Several studies using cultured cells and mouse models confirm a role of ARF in oncogene-induced apoptosis and senescence (Sherr et al., 2005). Interestingly, knocking-out either ARF or ATM in lymphoma mouse model accelerates lymphomagenesis. This suggests that both ARF and the DDR pathways possess a tumour suppressor potential, however whether ARF activates p53 remains unclear. Analysis of human cancers biopsies suggest that in cancer cells p53 is mostly activated by the DNA damage checkpoint (Bartkova et al., 2006). During the DNA damage response, DSBs activate p53 mainly through the kinases ATM and CHK2 (Kastan and Bartek, 2004). ATM is also one of the most mutated gene in human cancers (Greenman et al., 2007). Furthermore, heterozygous mutations in CHK2 gene predispose germline cells to cancer and causes similar phenotypes as p53 germline mutations (Bell et al., 1999). Moreover, in breast and lung carcinomas as well as in certain melanomas, expression of 53BP1 and MDC1, two DDR proteins acting upstream of CHK2 and p53, is strongly reduced (V. G. Gorgoulis et al., Nature, 2005).

In cultured primary cells, overexpressing oncogenes induces apoptosis and/or senescence, but ATM inhibition suppresses this response (Bartkova et al., 2006; Di Micco et al., 2006). Also, oncogene-induced apoptosis in mouse tumour models is dependent on the DDR pathway, as inhibiting CHK2, ATM, or TIP60 DDR proteins promotes tumour progression (Bartkova et al., 2006; Di Micco et al., 2006). In addition, ATM- or CHK2-deficient mice, or mice with a p53 that cannot be phosphorylated by ATM and CHK2 develop more tumours (Westphal et al., 1997).

Nevertheless, p53 remains the most frequently mutated DNA damage checkpoint gene in human cancer. This is probably due to the fact that p53 can easily be inactivated by a single
amino acid mutations within the p53 DNA binding domain because of its very low melting temperature. In addition, these mutations generate frequently dominant-negative forms of p53. Unlike ATM or CHK2 mutations, cancer cells mutated in p53 possess an efficient G2/M DNA damage checkpoint and DSBs repair that contribute to cell survival and tumour progression (Kastan and Bartek, 2004).

![Figure 6. 2 Model for oncogene-induced replication stress and how the DNA damage response acts as a barrier to malignant transformation (Halazonetis et al., 2008)](image)

When oncogenes are activated in normal cells, they induce an aberrant cell proliferation, giving rise to precancerous lesions. As a consequence of rapid proliferation and unscheduled DNA replication, cells accumulate replication stress and DSBs that are normally sensed by cell cycle checkpoints which activate repair pathways or apoptosis/senescence via p53, halting the progression of the precancerous lesions. When checkpoint proteins are mutated, this barrier is not anymore functional, and precancerous lesions can be transformed to cancer.
6.2. Cancer stem cells: a niche of cells responsible for resistance and recurrence of tumours

It is nowadays assumed that within the tumour, malignant cells co-exist and interact with non-malignant reactive tissue. This results in tumours composed of normal cells and heterogeneous cancer cell populations (Marusyk et al., 2012). Among the cancer cell population, there is a niche of cells gathering the properties of cancer cells and stem cells at the same time, called “Cancer Stem Cells” (CSCs). These cells have the unique ability to give rise to all cell types found in a particular cancer. CSCs are tumorigenic and this is ensured through the two stem cell processes: self-renewal and differentiation into different cell types. Moreover, CSCs are suspected to be responsible for the recurrence of tumours as they can persist in a tissue and resist to the therapy, to cause subsequently relapse and metastasis. That is why CSCs field is one of the hottest topics in current cancer research (Medema, 2013), and there is an intense research focus on cancer stem cells to identify new specific therapies that target this population (Atlasi et al., 2014).

The cancer stem cell model was first evoked in the mid-1990s, when John Dick, a stem cell biologist from the University of Toronto had isolated in leukaemia patients blood rare cells that were capable of giving rise to a new leukemia when injected into mice and seemed to play a major role in cancer progression (Bonnet and Dick, 1997; Lapidot et al., 1994; Larochelle et al., 1996). Nevertheless, the CSCs theory has been one of the most controversial topic during the last ten years in current biomedical research. In fact, many different nomenclatures were used to refer to these cells such as stem cell-like cancer cells, tumour-initiating cells or tumour-propagating cells (Wang et al., 2015), and sometimes CSCs hypothesis is used rather than CSCs theory (Venere et al., 2013). Actually, the main controversy over the CSC theory concerns the origin and frequency of these cells as well as their specific phenotypes and functions (Lathia, 2013).

The main argument in favour of the CSCs theory is the experimental evidence that there is a hierarchy governing the organisation of a cancer within a tissue similar to a normal tissue, with rare undifferentiated CSCs on the top of the pyramid responsible for maintaining the whole tumour cell population (Ito et al., 2012a). CSCs share several proprieties with normal stem cells (Figure 6. 3) (Antoniou et al., 2013). First, they are capable of self-renewal, meaning that CSCs can renew themselves indefinitely and stay a lifetime as undifferentiated cells. In addition to
self-renewal, asymmetric division is another feature of CSCs allowing them to generate differentiated daughter cells which constitute the bulk of the tumour. Unlike CSCs, these daughter cells have limited or no proliferation capacities, meaning that these daughter cells have a negligible contribution to the long-term sustenance of the tumour (Clevers, 2011). Relying on their unique self-renewal and unlimited replication properties, only CSCs are able to initiate tumours (Tirino et al., 2013). Interestingly, CSCs are incredibly resistant to DNA-targeting chemicals and ionizing radiations. This is probably due to higher DNA repair capacities (Skvortsov et al., 2015), and the fact that they grow more slowly than other malignant cells (Kaiser, 2015). In addition, CSCs possess active drug efflux system (Chen et al., 2013; Lou and Dean, 2007) and increased resistance to reactive oxygen species (Wang et al., 2015).

**Figure 6. 3 Current cancer stem cell theory and therapy targeting** (Wang et al., 2015)

Cancer stem cells are responsible for maintaining the whole tumour bulk thanks to their self-renewal and unlimited replication capacities. Under certain microenvironment conditions, normal cancer cells can convert to cancer stem cells, demonstrating the plasticity of a cancers. During tumour progression, different cancer stem cell clones coexist (depicted in different colours. (A) A cancer stem cells arise from mutations in normal stem cells, progenitor cells and/or differentiated cells. (B) The generated cancer stem cell divides asymmetrically and gives a daughter cancer stem cells and differentiated bulk cancer cells that can be mutated subsequently. A new cancer stem cell can be created from mutated bulk cancer cell or another cancer stem cell. (C) Cancer stem cells represent different clones that coexist and
are responsible for tumour heterogeneity. (D) After a conventional chemotherapy, most of the bulk cancer cells are killed, however chemo-resistant cancer stem cells are largely maintained, leading to tumour relapse. (E) Targeting specifically the cancer stem cells leads to gradual and slow tumour regression, leaving time for new cancer stem cells to eventually be generated from mutated bulk cancer cells and cause tumour relapse. (F) Killing both cancer stem cells and the bulk cancer cells at the same time may lead to tumour eradication.

In the current model, CSCs arise mainly from transformed normal stem cells (Pardal et al., 2003). This is due to the fact that the majority of cancers develop in epithelial tissue in which only stem cells spend enough time in the body to accumulate the mutations necessary for developing into cancer (Figure 6.1). However, more recent studies show the high plasticity of CSCs, and that they can come from a progenitor or even differentiated normal cancer through mutation, epigenetic modifications, or both (Gupta et al., 2011; Nguyen et al., 2012; Woll et al., 2014; Zomer et al., 2013). An example of this plasticity is provided by inducing CSCs in human colon cancer cells by simply expressing a set of defined factors including OCT3/4, SOX2 and KLF4 (Oshima et al., 2014). This experiment is very reminiscent of the ability of differentiated cells to be reprogrammed into embryonic stem cells by overexpression of a defined set of similar transcription factors to generate induced Pluripotent Stem Cells (iPSCs) (Takahashi and Yamanaka, 2006), which may suggest a link between cancer development and a return to an undifferentiated state, characteristic of early stages of embryonic development. Interestingly, such a link was previously proposed for the development of aggressive human tumours (Ben-Porath et al., 2008).

Cancer stem cells represent a rare proportion of the total cells in a tumour and they are the only one having the capacity to initiate tumours. For instance, in Acute Myeloid Leukaemia (AML) only 0.0001% of total cells can initiate tumours (Bonnet and Dick, 1997; Quintana et al., 2008; Schatton et al., 2008). However, other findings suggested that the stem cell-like cancer cells could reach a proportion of 25% in a tumour (Kelly et al., 2007; Krivtsov et al., 2006), challenging the classical CSCs theory.

Actually, the detection of cancer stem cells in a sample relies on three methods, including cell surface marker expression assay, sphere formation assay, and in vivo tumour initiating assay coupled with limited dilution assay (Figure 6.4). In the in vivo tumour initiating assay, cancer cells are sorted based on surface markers, then xenografted into immune-deficient mice (Tsuyada and Wang, 2013). This assay is considered as the “gold standard” to define
human CSCs, but it presents some intrinsic limitations (Woll et al., 2014). For example, the microenvironment depends on the mouse tissues into which human cancer cells are transplanted and is usually different from the original environment. This could explain, in addition to tumour heterogeneity, the differences in CSCs frequency among studies in which different animal or cancer cell models had been used.

**Figure 6. 4 Features of cancer stem cells** (Lathia et al., 2015)

Cancer stem cells (CSCs) are defined by functional characteristics that include sustained self-renewal, persistent proliferation, and the capacity of initiating tumours upon secondary transplantation, which is considered as the functional CSC assay. CSCs also share common features with somatic stem cells, including low frequency within a tissue (or tumour in this case), stem cell marker expression (examples relevant to GBM and the brain are shown), and the ability to generate multiple lineages of progeny.

Among different cancer types, Glioblastoma (GBM) is one of the most refractory to therapy, characterized by its self-renewing population, the tumorigenic cancer stem cells (CSCs) that contribute to tumour initiation, recurrence, metastasis and resistance to therapeutic. Glioblastoma is classified by the World Health Organization (WHO) as a grade IV glioma. It
is the most prevalent and lethal primary intrinsic brain tumour (Stupp et al., 2009). The brain gives rise to tumours with defined cellular organisation, suggesting the existence of hierarchy in this cancer (Reya et al., 2001). Atop of this hierarchy there are stem cells, which are rare self-renewing cells, with some them quiescent, and the others highly proliferative (Barker et al., 2010). Several groups demonstrated the existence of this self-renewing, tumorigenic cells within that gliomas and other primary brain tumours (Galli et al., 2004; Hemmati et al., 2003; Ignatova et al., 2002; Singh et al., 2003).

This tumour displays a highly infiltrative phenotype in the surrounding brain tissue but rarely metastasizes to other organs. GBM therapy consists mainly on removing the tumour mass using surgical resection followed by concurrent radiation therapy combined to temozolomide (TMZ; a DNA alkylating agent) and subsequent additional adjuvant TMZ therapy. Additional therapies to fight GBM progression are being made using targeted immunotherapies (e.g., bevacizumab) (Lathia et al., 2015). Unfortunately, conventional treatment never lead to complete cure of the cancer, but rather offers patients a better quality of life with an additional survival time.

GBM is one of the most genomically characterized cancer types, allowing the classification of this tumours into several groups depending on transcription profiles: proneural, neural, classical, and mesenchymal, genetic: Isocitrate DeHydrogenase 1 (IDH1) mutations, and epigenetics: CpG Island Methylator Phenotype (CIMP) (Olar and Aldape, 2014; Weller et al., 2009). This markers and others, such as Methyl Guanine MethylTransferase (MGMT) promoter methylation and the deletion of chromosomes 1p and 19q in oligodendrogiomas, might be used as prognosis indicators. For instance, mutations in IDH1 gene, is generally correlated with long-term survival (Lathia et al., 2015).

Most actual glioma CSCs markers are similar to those from normal stem cells, despite the controversy concerning the linkage between glioma CSCs and normal stem cells. Among this marker, transcription factors and structural proteins essential for Neural Stem and Progenitor Cells (NSPC), including OLIG2 (Ligon et al., 2007), NANOG (Ben-Porath et al., 2008; Suva et al., 2014), SOX2 (Hemmati et al., 2003), NESTIN (Tunici et al., 2004), MYC (Kim et al., 2010), MUSASHI1 (Hemmati et al., 2003), BMI1 (Hemmati et al., 2003), and Inhibitor of Differentiation protein 1 (ID1) (Anido et al., 2010). More recently, it was suggested that enhanced aldehyde dehydrogenase (ALDH) activity is a hallmark of cancer stem cells, measurable by the aldefluor assay (Ginestier et al., 2007). However, the cell surface markers
are the most useful for enriching CSCs from Non-Stem Tumour Cells (NSTCs) using traditional sorting methods such as flow cytometry. Many potential cell surface markers have been proposed, such as CD133 (Hemmati et al., 2003), CD44 (Liu et al., 2006), CD15 (Son et al., 2009), integrin α6 (Lathia et al., 2010), L1CAM (Bao et al., 2008), and A2B5 (Ogden et al., 2008). These surface markers mediate the interaction of the cells with their microenvironment. CD133 (also called Prominin-1) was the first proposed marker. This glycoprotein is expressed on the surface of neural stem cells and enriched in cells with high self-renewal, proliferation and differentiation rates (Singh et al., 2003). Although CD133 continues to be the most commonly used cell surface marker, it is controversial as it gives false-negatives for identifying CSCs in some tumours (Beier et al., 2007). Moreover, CD133 expression is cell cycle regulated, which implies that potentially slow-cycling CSCs lack CD133 expression during G0/G1 cell cycle phase are not detected (Sun et al., 2009). Additionally, the acetylated CD133 (AC133) surface epitope is related to stem cells and decreases with differentiation, whereas the expression of Prominin-1 mRNA is not a stemness marker (Kemper et al., 2010).

Other markers had emerged, such as integrin α6 that have been suggested for CSCs and NSTCs segregation (Lathia et al., 2010). More recently, CD44 and CD15 have been proposed as potential associated markers with specific subgroups of GBM (Bhat et al., 2013). However, all these markers must be used with caution, because of the multiplicity of stem cells population within a given tissue type and the inherent adaptability of cancer cells.

In addition to surface marker, other methods have been described to enrich for glioma CSCs, such as the abilities to form neurospheres in serum-free medium or efflux of fluorescent dyes based on the hypothesis that stem cells contain drug efflux transporters (Goodell et al., 1996; Kondo et al., 2004; Yu et al., 2008). Neurosphere culture selects for cells that can grow in stem cell medium, however this method fails to recapitulate the heterogeneity of the original tumour in vivo (Lee et al. 2006 (Lathia et al., 2011; Lee et al., 2006) (Venere et al., 2011), a characteristic that freshly isolated CSCs using marker expression maintain (Singh et al., 2004).

During brain development, several regulatory pathways orchestrate the tissue growth. Notch and Nuclear Factor κB (NF-κB) signalling together with the ephrins and Bone Morphogenetic Proteins (BMPs) pathways guide the fate of NSPCs (Lathia et al., 2015). CSCs use these developmental programs in order to maintain their undifferentiated state, thus increasing their maintenance and survival. The most common pathways activated in CSCs include Notch, BMP, NF-κB, and Wnt signalling (Day et al., 2013; Li et al., 2009; Lubanska et
al., 2014; Rheinbay et al., 2013; Yan et al., 2014b). This pathways are activated through niche factors and help cancer stem cells and progenitor to maintain tumour growth. The niche factors include microenvironmental and metabolic factors in addition to a combination of genetic and epigenetic alterations (Figure 6.5).

The Notch pathway plays an important role during neural development, sustaining NSPC populations and inhibiting neuronal differentiation. In GBM, Notch pathway is aberrantly activated stimulating a stem-like state of astrocytes with increased proliferation (Jeon et al., 2008). Inhibiting the γ-secretase, an enzyme that activate the Notch pathway, induce the depletion of CSCs, highlighting the dependence of glioma CSCs on Notch signalling (Fan et al., 2010; Fan et al., 2006). Recently, it has been shown that Notch1, one of the four notch pathway receptor in mammals, is responsible for angiogenic properties and Pericyte-Like cell differentiation of glioblastoma stem cells (Guichet et al., 2015).

BMPs signalling induce the differentiation of NSPC toward an astroglial lineage, this is why it has been proposed as a possible differentiation therapy for GBM (Piccirillo et al., 2006). However, despite its expression in primary GBM tissue, glioma CSCs are highly resistant to the differentiation effects of BMPs because they express a foetal BMP receptor that does not recognize the protein (Lee et al., 2008) and additionally secrete BMP antagonists, especially Gremlin1 (Yan et al., 2014a).

Wnt signalling is also highly active in CSCs and is essential for maintaining the stem cell phenotype. Modulation of Wnt/β-catenin signalling through PLAGL2 which is highly amplified in gliomas, suppress CSCs differentiation (Zheng et al., 2010). Chromatin modifications mapping in CSCs shows widespread activation of Wnt pathway genes through loss of Polycomb-mediated repression. The CSCs chromatin state is thought to be dependent on ASCL1 transcription factor, which activates Wnt signalling (Rheinbay et al., 2013).

Many growth factor signalling pathways have been shown to contribute to GBM function and maintenance. For example, PDGFRβ signalling contributes to CSCs self-renewal, invasion and tumour growth through activating downstream proteins (Kim et al., 2012b). EGFR signalling activates AKT, and induces ID3, IL-6, and IL-8, contributing to CSCs maintenance. In addition to its role in stress responses, Transforming Growth Factor β (TGF-β) stimulates CSCs self-renewal, and helps for maintaining the stemness of these cells through inducing the expression of SOX2 and SOX4 (Ikushima et al., 2009).
Figure 6.5 Intrinsic and extrinsic regulators of cancer stem cells state (Lathia et al., 2015)

The schema shows Cell-dependent (intrinsic) and environment-dependent (extrinsic) factors that regulate the CSC state. The main intrinsic regulators include genetic, epigenetic, and metabolic regulation, while extrinsic regulators include interaction with the microenvironment, including niche factors and the immune system.
Chapter II
Results
II. Results

1. "PIP degron proteins, substrates of CRL4\textsuperscript{Cdh2}, and not PIP boxes, interfere with DNA polymerase \( \eta \) and \( \kappa \) focus formation on UV damage"

1.1. Article introduction

The most accepted model for the recruitment of TLS polymerases onto DNA lesions is the polymerase switch model. In this process, the RAD6-RAD18 complex induces PCNA\textsuperscript{monoUb}, thus increasing the affinity of TLS polymerases for chromatin-bound PCNA (Lehmann et al., 2007). Y family TLS polymerases (Pol \( \eta \), Pol \( \kappa \), Pol \( \iota \), and REV1) interact with PCNA\textsuperscript{monoUb} through their evolutionary conserved UBDs (Bienko et al., 2005), and form microscopically visible replication factories (Guo et al., 2006; Kannouche and Stary, 2003; Kannouche et al., 2004; Ogi et al., 2005; Tissier et al., 2004). These polymerases also harbour another domain, called “the PIP box motif”, contributing to their interaction with PCNA hydrophobic pockets. The role of this motif in regulating TLS polymerases recruitment was not extensively studied as its interaction with PCNA does not dependent on reversible posttranslational modifications. However, the hydrophobic pockets of PCNA interact with many proteins that compete out for binding during DNA replication and repair (Moldovan et al., 2007). On another end, the homotrimeric structure of this sliding clamp limits the number of proteins that can bind at a given time (Gulbis et al., 1996; Krishna et al., 1994). This implies that competition between different PCNA partners may constitute a potential mechanism for TLS regulation. It is possible that other mechanisms than PCNA\textsuperscript{monoUb} may contribute to TLS activation through displacing other PCNA partners. This hypothesis is interesting and has not yet been thoroughly explored. Consistent with this, it was reported that the CDKs inhibitor p21 can compete out GFP-Pol \( \eta \) from forming TLS foci, in a PIP box-dependent manner (Soria et al., 2008). The p21 protein contains a specialized PIP box motif, known as “PIP degron” that targets p21 for proteasomal degradation upon binding to PCNA. In this work, the authors proposed that p21 proteasomal degradation after DNA damage may regulate the access of Pol \( \eta \) to replication and/or repair factories. This study shed light on the possibility that degradation of another PIP degron-containing protein, CDT1 (Arias and Walter,
after UV damage, may be important to regulate the access of Pol η to PCNA. DNA damage-dependent degradation of CDT1 was previously reported to constitute a novel checkpoint that inhibits licensing in the presence of DNA damage. However evidence supporting this conclusion is weak. In particular, in this work the authors did not explore the possibility that slowdown of DNA synthesis upon DNA damage-dependent CDT1 degradation may be an indirect effect of the ATR/ATM checkpoint (Higa et al., 2003). CDT1, like p21 and few other partners of PCNA, contain a specialised PIP degron motif that confers both high-affinity PCNA binding and proteasomal degradation following CRL4Cd2-mediated polyubiquitination.

1.2. Project objectives

The first objective of this work was to determine the role of CDT1 degradation upon UV damage. Since CDT1 contains a PIP degron, we wanted to test whether it can compete out GFP-Pol η foci formation, in a PIP box-dependent manner, and if CDT1 degradation via the E3 ubiquitin ligase CRL4Cd2 is required for localisation of Pol η in subnuclear foci. Another question was if CRL4Cd2 pathway targets specifically proteins harbouring a PIP degron motif which confers a high affinity for PCNA binding rather than all PCNA partners. This possibility also implies that the CRL4Cd2 degradation pathway may have a new function in regulating PCNA-Pol η interactions. Moreover, it would be interesting to know whether a short PIP box peptide is sufficient to block the access of Pol η to replication factories, and what is the minimal motif necessary for this activity. Finally, we wanted to determine whether CDT1 may also compete out other Y-family polymerases for forming replication factories, and for this reason we have decided to analyse the recruitment of another TLS polymerase, Pol κ.
1.3. The article: "PIP degron proteins, substrates of CRL4Cdt2, and not PIP boxes, interfere with DNA polymerase η and κ focus formation on UV damage"

PIP degron proteins, substrates of CRL4\textsuperscript{Cdt2}, and not PIP boxes, interfere with DNA polymerase η and κ focus formation on UV damage
PIP degron proteins, substrates of CRL4Cdt2, and not PIP boxes, interfere with DNA polymerase η and κ focus formation on UV damage

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ABSTRACT

Proliferating cell nuclear antigen (PCNA) is a well-known scaffold for many DNA replication and repair proteins, but how the switch between partners is regulated is currently unclear. Interaction with PCNA occurs via a domain known as a PCNA-Interacting Protein motif (PIP box). More recently, an additional specialized PIP box has been described, the “PIP degron”, that targets PCNA-interacting proteins for proteasomal degradation via the E3 ubiquitin ligase CRL4Cdt2. Here we provide evidence that CRL4Cdt2-dependent degradation of PIP degron proteins plays a role in the switch of PCNA partners during the DNA damage response by facilitating accumulation of translesion synthesis DNA polymerases into nuclear foci. We show that expression of a nondegradable PIP degron (Cdt1) impairs both Pol η and Pol κ focus formation on ultraviolet irradiation and reduces cell viability, while canonical PIP box-containing proteins have no effect. Furthermore, we identify PIP degron-containing peptides from several substrates of CRL4Cdt2 as efficient inhibitors of Pol η foci formation. By site-directed mutagenesis we show that inhibition depends on a conserved threonine residue that confers high affinity for PCNA-binding. Altogether these findings reveal an important regulatory role for the CRL4Cdt2 pathway in the switch of PCNA partners on DNA damage.

INTRODUCTION

Proliferating cell nuclear antigen (PCNA), a processivity factor for replicative DNA polymerases, acts as a docking molecular platform for many factors, and orchestrates several aspects of DNA metabolism such as DNA replication and repair (1). Its homotrimeric ring-shaped structure (2,3) could in theory provide an interaction surface for up to three partners at a time, although binding can be mutually exclusive (4). Binding occurs through a small and highly adaptable PCNA-Interacting Protein motif (PIP box) that tethers partners to a hydrophobic pocket on PCNA (1). To ensure stable interaction, some factors, like the cyclin-dependent kinase inhibitor p21, have evolved a strong binding affinity (5), thus efficiently competing out other factors for binding (5,6).

Other PCNA partners, such as members of the Y-family of translesion synthesis DNA polymerases (TLS pols) that carry out DNA lesions bypass (7), also require an ubiquitin-binding motif that tethers them to an ubiquitin group covalently attached to PCNA (8). Monoubiquitylation of PCNA that occurs on DNA damage, increases the affinity of TLS pol η for PCNA (9–11) and may constitute a mechanism to switch from replicative to TLS pols at stalled replication forks (12). Pol η is recruited at sites of ultraviolet (UV) damage on chromatin to bypass the major UV-induced DNA lesion, the thymine-thymine cyclobutane pyrimidine dimer photoproduct (13,14), and can be visualized by expression of cGFP-tagged Pol η in cells (15). In addition, emerging evidence implicates Y-family TLS pols also in DNA repair (16) outside the S-phase of the cell cycle (17,18). For instance, Pol κ is recruited to UV-damage sites to carry out nucleotide
excision repair (NER) (19) in the G1-phase or in quiescent cells (18).

Some PCNA partners are targeted for proteasomal degradation on interaction (20) via polyubiquitination by the E3 ubiquitin ligase Cullin 4-RING Ligase (CRL4)-Ddb1-Cdt2 (CRL4<sup>CD2</sup>). In this reaction, PCNA provides a molecular platform where CRL4<sup>CD2</sup> and the substrate meet (21). Recently, it was discovered that a ‘degron’ module, hereafter called PIP degron, that lies within the PIP box and adjacent amino acids, is essential for degradation (22). Compared with a canonical PIP box (of signature Q/N-x-x-Ψ-x-x-φ-θ, where Ψ is a hydrophobic residue, mostly M, L, V or I, and φ and θ are aromatic amino acids such as F or Y), a PIP degron contains both a TD motif and a basic amino acid four residues downstream, of signature ’Q/N-x-x-Ψ-T-D-φ-θ-x-x-x-R/K’ (22,23). Despite intensive investigations, the biological role of this degradation pathway is not completely understood, in particular on DNA damage (20). In metazoans, CRL4<sup>CD2</sup> substrates include replication licensing factor Cdt1 (24,25), p21 and the histone methyltransferase Set8 (26,27). Cdt1 catalyzes loading of the Mcm2-7 helicase at replication origins (33,34) and PCNA-triggered Cdt1 degradation in S-phase prevents re-polymerization and preserves genome stability (24,35-40).

Interestingly, Cdt1 is rapidly polyubiquitinated after DNA damage (within minutes) via the CRL4<sup>CD2</sup> pathway (25,41-43) much faster than during a normal S-phase (28,44-46) by both chromatin-bound PCNA and the SFC<sup>Skp2</sup> ubiquitin ligase (36,38). Pol η degradation after DNA damage via the CRL4<sup>CD2</sup> pathway in Caenorhabditis elegans, was proposed as a mechanism to inhibit TLS at completion (47). All these studies implicate CRL4<sup>CD2</sup> in regulating the interaction of PCNA with specific DNA repair and lesion-bypass factors after DNA damage.

We made the hypothesis that CRL4<sup>CD2</sup> may clear PCNA from PIP degron-containing partners to improve accessibility to repair factors. By using the Cdt1 PIP degron, as a tool to test this hypothesis, we provide evidence that PCNA-triggered degradation of Cdt1 is required for efficient cGFP-TLS-Pol η and -Pol K focus formation on UV damage. By extending this assay to other PIP boxes, we found that this is a specific feature of PIP degrons of Cdt1, p21 and Set8 and not of canonical PIP boxes, like Fen1 or Pl5(PAF). These results support a model for PCNA partners switch triggered by DNA damage, orchestrated by CRL4<sup>CD2</sup>.

**MATERIALS AND METHODS**

**Plasmids**

Human pEGFP-Pol η (15), pEGFP-Pol κ (48) and pcDNA3-HA-p21<sup>WAF1</sup> (49) were as described. cDNA clones of human Cdt1, Fen1 and mouse Cullin 4A were purchased from IMAGENES. pcDNA3-Cdt1-HA, pcDNA3-HA-Fen1 and pcDNA3-Cullin 4A-Myc<sub>e</sub> were generated by polymerase chain reaction (PCR) and cloning into pcDNA3-HA C-terminal, pcDNA3-HA N-terminal and pcDNA3-Myc<sub>e</sub>, respectively, as previously described (50). The deletion mutants Cdt1ΔPIP (Δ aa 1-14) and dCullin4A (Δ aa 252-759) were generated by PCR mutagenesis and cloned into pcDNA3-HA C-terminal and pcDNA3-Myc<sub>e</sub> respectively. To generate Cdt1-PIP<sup>Fen1</sup> chimera, a PpuMI restriction site was created at the 5' of Cdt1ΔPIP-HA by introducing a silent mutation by PCR. A duplex of annealed oligonucleotides encoding the PIP box of Fen1 with its C-terminal flanking region was inserted into the HindIII-PpuMI sites to produce pcDNA3-Cdt1-PIP<sup>Fen1</sup>-HA. The same strategy was used to generate Cdt1 mutPIP and Cdt1 R<sup>44A</sup>. The myc-PIP box constructs were generated by a similar approach. Annealed oligonucleotides encoding the SV40 large T antigen NLS were inserted into the Neo<sup>e</sup>-EcoRI sites of pcSV3+MT (51). A second duplex encoding a PIP box with its C-terminal flanking region was subsequently inserted into the EcoRI-Kdel sites. C-terminally HA-tagged Cdt1 variants were also subcloned into pBluescript prokaryotic vector (52). All constructs were verified by DNA sequencing.

**Cell culture, infection, transfection and electroporation**

NIH-3T3, U2OS and Platinum-E (Cell Biolabs) cells were grown in Dulbecco’s modified eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics. For infection, viral particles were generated by transfecting Platinum-E eucaryotic packaging cell line with retroviral expression vector (pBabe-puro) encoding Cdt1 variants using homemade PEI reagent. The viral supernatant was diluted (10-1000-fold) in normal growth medium to obtain low Cdt1 expression levels. Forty-eight hours after infection, cells were selected in puromycin (2.5 μg/ml)-containing medium. Selected populations were expanded and promptly used for experiments. Cells were transfected with Lipofectamine (Invitrogen). To achieve low Cdt1 expression levels, cells were transfected with pcDNA3-Cdt1-HA and empty vector at 1:20 ratio. For high expression levels, a ratio of Cdt1: empty vector of 3:1 was used. Before electroporation, NIH-3T3 cells were incubated in RPMI-1640 medium for 30 min. After trypsinization, 1 × 10<sup>5</sup> cells/ml were resuspended in RPMI-1640 medium and 4 × 10<sup>5</sup> cells were mixed with 30 μg of plasmid DNA, and exposed to a single voltage pulse (300 V, 500 μF). Gene-Pulser, Bio-Rad) at room temperature. Electroporated cells were allowed to recover for 5 min in the medium before replating.

**Irradiation**

In all experiments, UV-C irradiation at 254 nm was performed with microprocessor-controlled croslinker (BIO-LINK<sup>®</sup>) at a dose of 20 J/m<sup>2</sup> unless stated otherwise.

**Cell viability experiments**

Cells were plated at 1.0 × 10<sup>5</sup> per well in 12-well plates and UV irradiated. Forty-eight hours after irradiation, cell viability was determined using the CellTiter-Glo<sup>®</sup> Luminescent Cell Viability assay (Promega).
Antibodies

ORC2 antibody was from Marcel Mächali (IGH, Montpellier). Pol η, Mmc2 (AbCam), PCNA and β-actin (Sigma), HA (Y-11, Santa Cruz Biototechnology), Myc9B11, PR-Set/Sct8, P-p53 (Ser15), P-H2AX (Ser139; Cell Signalling) and Cdt1 (Millipore).

Immunofluorescence and microscopy

Cells were grown on coverslips before co-transfection. Four hours after UV-C irradiation, cells were fixed with 3.2% paraformaldehyde for 15 min at room temperature, washed three times with phosphate buffered saline (PBS) and permeabilized with 0.2% Triton X-100 for 5 min on ice. To detect Cdt1-HA variants or myc-PIP box peptides, cells were blocked with PBS + 3% bovine serum albumin (BSA) for 30 min and incubated with anti-HA or anti-myc primary antibodies, respectively, for 1 h at room temperature. Cells were washed twice with PBS + 3% BSA and incubated with Texas Red-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch) for 1 h at room temperature. After washing twice with PBS + 3% BSA, cells were incubated with ProlongGold DAPI (Invitrogen), eGFP-Po1 η or eGFP-Pol κ foci were analyzed with Leica DMi6000 epifluorescence microscope (RIO imaging facility). Images were acquired using a CoolSnap HQ CCD camera (Photometrics) and metatmosphere software (Molecular Devices).

Foci formation assay

Cells were co-transfected with eGFP-Pol η or eGFP-Pol κ and Cdt1 variants, p21, Fen1 or myc-PIP box constructs and incubated for 24 h before UV-C irradiation. Four hours after irradiation, cells were fixed, washed three times with PBS and mounted with Prolong Gold DAPI (Invitrogen). The percentage of eGFP-Pol η or eGFP-Pol κ expressing cells displaying eGFP-Pol η or eGFP-Pol κ foci was determined by scoring at least 200 nuclei for each condition. Nuclei containing <30 foci were scored as negatives. Means and standard deviation (error bars) of three independent experiments are shown.

Cell lysis and immunoprecipitation experiments

Co-immunoprecipitations were performed as described (50). Briefly, cells were rinsed once in PBS and incubated with ice-cold lysis buffer (50 mM Tris- HCl, pH 7.4, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 1% Triton X-100 and protease inhibitors) for 30 min on ice before scraping. Equivalent amounts of protein were incubated for 4 h at 4°C with HA-coupled protein A agarose beads (Roche). After extensive washing with lysis buffer, bound proteins were eluted in Laemmli buffer. Alternatively, after cell lysis, whole cell extracts were clarified by centrifugation at 16 000g for 10 min at 4°C. Protein concentration of the clarified lysates was estimated using BCA method (Pierce).

Chromatin isolation

Chromatin-enriched and soluble fractions were prepared using CSK-extraction procedure. Briefly, cell pellets were lysed in CSK buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM EDTA, 1 mM MgCl2, 0.5 mM DTT, 1 mM ATP, 0.2% Triton X-100 and protease inhibitors) for 10 min on ice. After centrifugation at 800g for 3 min at 4°C, the supernatant (Triton-soluble fraction) was recovered. The pellet (Triton-insoluble fraction) was resuspended in CSK buffer and incubated for 10 min on ice. After centrifugation, the pellet (chromatin-enriched fraction) was lysed in Laemmli Buffer.

UV-induced cell death assay

This assay was performed as previously described (53). Briefly, cells were electroporated with Cdt1 variants or myc-PIP box constructs and incubated for 24 h before 10 J/m2 UV-C irradiation. Twenty-four hours after irradiation, cells were harvested, washed twice in PBS and fixed in ice-cold 70% ethanol at −20°C overnight. Thawed cells were washed twice in PBS and incubated with 50 μg/ml RNase A at 37°C for 1 h. DNA was stained with propidium iodide (25 μg/ml). Cells were analyzed with a FACScalibur flow cytometer using CellQuestPro software. The percentage of cells displaying a DNA content lower than 2C was assessed.

RESULTS

CRL4<sup>CD2</sup>-mediated proteolysis facilitates UV-induced eGFP Pol η and Pol κ focus formation

On irradiation of cells with UV light, Pol η is recruited to chromatin by interaction with PCNA and accumulates in discrete, microscopically visible foci that co-localize with sites of UV damage, visualized on expression of Pol η fused to enhanced green fluorescent protein (eGFP-Pol η). Importantly, eGFP-Pol η restores the UV sensitivity of cells mutated in the Pol η gene (XP-V mutant) and therefore constitutes a useful marker of translational DNA synthesis in vivo (15).

We sought to determine whether failure to degrade CRL4<sup>CD2</sup> substrates on DNA damage might impact on eGFP-Pol η foci formation. We first treated cells with the proteasome inhibitor MG132 to stabilize all CRL4<sup>CD2</sup> substrates after UV damage (25,27), and observed a strong decrease in the number of cells with eGFP-Pol η foci (Supplementary Figure S1A). Next, we disrupted the CRL4<sup>CD2</sup> pathway by expressing a dominant negative mutant of Cullin 4A (54), a scaffold for CRL4<sup>CD2</sup> complexes (Supplementary Figure S1B-E), and obtained a similar result. These results show that ubiquitin-dependent proteasomal degradation is a prerequisite for eGFP-Pol η focus formation, thus implicating the CRL4<sup>CD2</sup> pathway in this regulation.

Next, we determined to which extent partial inhibition of CRL4<sup>CD2</sup>-dependent proteolysis affects eGFP-Pol η focus formation compared with inhibition of Cullin 4A. For this purpose, we generated two nondegradable mutants of the CRL4<sup>CD2</sup> substrate Cdt1, described in Figure 1A. The first one (R+4A mutant) binds PCNA but cannot be degraded (22), since it is mutated in the PIP degron by alanine substitution (A) of the basic
Figure 1. The Cdt1 \( \text{R}^{+4A} \) mutant affects UV-induced eGFP-Pol \( \eta \) and \( \kappa \) focus formation. (A) Sequence comparison between Cdt1 WT PIP box, the degron mutant R+4A and the PIP box mutant mutPIP. The residues essential for interaction with PCNA are shown in red (or light gray), and the residues that define the PIP degron are shown in blue (or dark gray). \( \Phi = \) Val, Leu, Ile, Met; \( \theta = \) Tyr, Phe. (B) The basic residue (R) four amino acids downstream (+4) of the Cdt1 PIP box is required for UV-induced Cdt1 degradation. NIH-3T3 cells were transduced with pBabe-puro retroviral vector encoding either HA-tagged Cdt1 WT, Cdt1 \( \text{R}^{+4A} \), Cdt1 mutPIP or empty vector (EV) and selected with puromycin for 2 days. Cell lysates were prepared 4 h after mock (–) or UV (+) irradiation and analyzed by western blot to detect both endogenous (End) and ectopically expressed human Cdt1 (Ect), that is smaller in size than the mouse orthologue. (C and D) NIH-3T3 cells transduced as in (B) were subsequently transduced with eGFP-Pol \( \eta \) or eGFP-Pol \( \kappa \) (panel D) and
residue (R) essential for CRL4^3H2-dependent degradation, four amino acids (~4) downstream of the PIP box. The second is mutated in the PIP box (mut PIP) and therefore cannot interact with PCNA (21). NIH-3T3 cells were transduced with retroviral vectors encoding HA-tagged Cdt1 variants expressed to similar levels than endogenous Cdt1 to perform the experiments described below (Figure 1B). Consistent with previous reports (22,23), Cdt1^{R4A} remains stable on UV damage, as the Cdt1^{mutPIP} variant, whereas Cdt1 wild-type (WT) and endogenous Cdt1 are efficiently degraded (Figure 1B). Importantly, eGFP-Pol η foci formation was also reduced in cells expressing the Cdt1^{R4A} mutant compared with cells expressing Cdt1^{WT} (Figure 1C), while Cdt1^{mutPIP} did not have a significant effect.

We also analyzed formation of eGFP-Pol ι foci, another PIP box-containing TLS polymerase also implicated in NER in the G1-phase of the cell cycle. We observed that on UV damage, eGFP-Pol ι focus formation occurred mainly in cells that stained negative for Cyclin A (Figure 1E, compare black bars with gray bars, and Supplementary Figure S2A) in line with previous observations (19,55). Importantly, we observed inhibition of eGFP-Pol ι focus formation by the Cdt1^{R4A} mutant (Figure 1D and E, R+4A), unveiling the DNA damage-dependent contribution of CRL4^3H2 in G1-phase, when the S-phase CRL4^3H2 pathway is off (20). Importantly, the percent of Cyclin A-negative cells (~60% of the total cell population), did not change in cells expressing the different Cdt1 variants (Supplementary Figure S2B), consistent with the cell cycle distribution of NIH-3T3 cells (Figure 2G). These results are consistent with a previous report showing that UV-dependent Pol ι focus formation is excluded from S-phase cells (18). Of note, expression of nondegradable Cdt1 (R^{4A} mutant) at endogenous levels inhibits both eGFP-Pol η and -Pol ι focus formation, to a lower extent compared with treatment with the proteasome inhibitor MG132, or to Cullin 4A inhibition. These data further support the role of Cdt1 in the chromatin fraction specifically on UV irradiation (Figure 2E, lanes 7–8), and its recruitment is impaired on expression of Cdt1^{WT}, and not of Cdt1^{mutPIP} (lanes 9–10). Finally, expression of Cdt1 mutated in the key residues essential for PCNA interaction (Cdt1^{mutPIP}) did not affect eGFP-Pol η foci formation (Supplementary Figure S4C). Taken together, these results show that Cdt1^{mutPIP} loses the potential to inhibit recruitment of Pol η to chromatin and to impair Pol η foci formation on UV irradiation. We also verified that high Cdt1^{WT} expression did not impair UV-induced PCNA monoubiquitylation (Figure 2E), essential for Pol η recruitment to damage sites, nor induce global cell cycle changes (Figure 2G). These observations rule out the possibility that Cdt1^{WT} overexpression may affect Pol η focus formation indirectly via interference with PCNA monoubiquitylation, or via an indirect cell cycle effect.

**Figure 1. Continued**

Irradiated as in (B). Control cells were transduced with empty vector. Four hours after irradiation, the distribution of eGFP-Pol η or -Pol ι was examined by fluorescence after fixation. Scale bar: 10 μm. (Right panel) The percentage of eGFP-Pol η-expressing cells in which Pol η was localized in nuclear foci was assessed. Means and standard deviation of three independent experiments are shown. **P < 0.0001. (E) The percentage of eGFP-Pol η-expressing cells in which Pol ι was localized in nuclear foci (black bars) that also stained negative for Cyclin A (gray bars) was assessed. Means and standard deviation of three independent experiments are shown. **P < 0.0001. (F) Cell viability of cells transduced as in (B) with Cdt1^{WT}, Cdt1^{mutPIP} or empty vector. **P < 0.01 (n = 3). NS, nonsignificant.
Figure 2. Overexpression of Cdt1 impairs eGFP-Pol η focus formation after UV irradiation, in a PIP box-dependent fashion. (A) Western blot of NIH-3T3 cells transiently transfected with pcDNA3[Eμ promoter] vector encoding Cdt1-HA WT as indicated in ‘Materials and Methods’ section. Cells were UV irradiated (−) or mock irradiated (++) 24 h after transfection. Four hours later, cells were harvested and processed for immunoblotting with antibodies to HA, or β-actin. (B) NIH-3T3 cells were co-transfected with eGFP-Pol η and either 8eDNA3 vector encoding Cdt1-HA WT, Cdt1-HA ΔPIP or empty vector and UV irradiated as in (A). Four hours after irradiation, cells were fixed and stained with anti-HA antibody. The distribution of eGFP-Pol η was detected by GFP fluorescence (green), and the staining of Cdt1-HA (red) obtained by indirect immunofluorescence. Scale bar: 10 μm. (C) Quantification of the data shown in (B). Means and standard deviation of three independent experiments are shown. (D) Western blot of NIH-3T3 cells co-transfected with eGFP-Pol η and the indicated constructs and irradiated as in (B). The sequence of Cdt1ΔPIP is shown (dotted line represents deleted residues). The residues essential for interaction with PCNA are shown in red (or light gray). (E) Western blot showing PCNA-mediated recruitment of Pol η to chromatin after UV irradiation. UV-induced binding of Pol η to chromatin. U2OS cells were transfected with the indicated plasmids. Cells were detergent-extracted 4 h after mock (−) or UV (++) irradiation. The distributions of Pol η, (continued)
and also show that PCNA is still competent to be posttranslationally modified.

In mammalian cells, expression of Cdt1 at high levels promotes DNA re-replication only in certain cell lines (57,58). Consistent with this observation, we observed that expression of Cdt1 at high levels induces re-replication in human U2OS cells, and not in mouse NIH-3T3 cells (Supplementary Figure S4D); however, interference with UV-induced eGFP-Pol η foci was observed in both cell lines (Figure 2B and C and Supplementary Figure S4B), suggesting that the ability of Cdt1 to interfere with Pol η focus formation is independent from its function in DNA replication.

The Cdt1 PIP box is sufficient to impair Pol η foci formation and to induce UV-dependent cell death

To determine whether the PIP box of Cdt1 on its own affects Pol η focus formation, we generated and expressed only the Cdt1<sup>WT</sup> PIP box, or a mutant that cannot interact with PCNA (Cdt1<sup>MUT</sup>), fused to the c-myc epitope and the SV40 nuclear localization signal to facilitate nuclear retention (Cdt1-myc-PIP box, Figure 3A and B). We verified by immunoprecipitation that Cdt1-myc-PIP box<sup>WT</sup> and not Cdt1-myc-PIP box<sup>MUT</sup> interacts specifically with PCNA (Figure 3C). Strikingly, we observed that expression of Cdt1-myc-PIP box<sup>WT</sup> impaired UV-induced Pol η focus formation, while the mutant Cdt1-myc-PIP box<sup>MUT</sup> that cannot interact with PCNA showed no inhibition (Figure 3D and E), similar to what observed with full-length Cdt1. Similarly, Cdt1-myc-PIP box<sup>WT</sup> severely impaired formation of UV-induced Pol κ focus, while Cdt1-myc-PIP box<sup>MUT</sup> showed no inhibition (Supplementary Figure S3C). Notably, both full-length Cdt1 and Cdt1-myc-PIP box<sup>WT</sup> impaired UV-induced Pol κ focus formation more efficiently than Pol η foci (compare Supplementary Figure S3B with Figure 2C, and Supplementary Figure S3C with Figure 3E), consistent with the lower PCNA-binding affinity of the Pol κ PIP box compared with that of Pol η (59).

Upon UV damage, Pol η is essential for cell viability (15). Hence, if expression of Cdt1 interferes with TLS (as well as other DNA repair pathways, see ‘Discussion’ section) it is expected that cell death should increase specifically only on UV irradiation. This was previously shown to be the case for p21 (60). To address this question, we quantified the formation of hypodiploid (sub-G1) cells after UV irradiation as previously described (53). Consistent with data shown in Figure 1F, we observed a 3-fold increase of UV-specific sub-G1 cells on expression of Cdt1<sup>WT</sup> and not Cdt1<sup>MUT</sup>P Hip (Figure 3F and Supplementary Figure S4E). In addition, expression of Cdt1-myc-PIP box<sup>WT</sup> also induced UV-dependent cell death to a similar level than full-length Cdt1 (FL, Figure 3F). Importantly, neither Cdt1<sup>MUT</sup> nor Cdt1-myc-PIP box induced cell death in the absence of UV damage, but specifically affected cell viability only on DNA damage, suggesting interference with TLS function and/or other repair pathways.

Collectively, these results demonstrate that the Cdt1 PIP box on its own is both required and sufficient to inhibit UV-induced Pol η foci and to induce UV-dependent cell death.

PIP degrons of Cdt1, p21 and Set8 are potent inhibitors of UV-induced Pol η foci

As we observe for Cdt1, it was previously shown that p21 overexpression can also interfere with UV-induced Pol η foci formation via its PIP box (60). This raises the question of whether this is a general property of all PIP box-containing proteins (61). To address this question, we compared the ability of Cdt1, p21 as well as another PIP box-containing protein, the flap endonuclease-1 Fen1, to impair formation of eGFP-Pol η foci after UV-induced damage. We observed that when expressed at similar levels (Figure 4A), only Cdt1 and p21 could interfere with formation of eGFP-Pol η foci, while Fen1 only had a marginal effect (Figure 4B), consistent with a previous report showing that the PCNA-binding affinity of p21 is 725-fold higher than that of Fen1 (5).

To extend our analysis to other PCNA-binding proteins, we used the same strategy described in Figure 3A, to create a series of myc-PIP box constructs (Figure 4C) belonging to two distinct categories, (i) substrates of CRL4<sup>Cdc2</sup> (PIP degrons) and (ii) canonical PIP boxes. These constructs were expressed at similar levels in NIH-3T3 cells (Figure 4D), and they all localized in the nucleus (panel E). Consistent with the weak inhibition of Pol η focus formation observed by expression of Fen1 (panel B), the expression of either myc-PIP box<sup>Fen1</sup> or myc-PIP box<sup>INPAP</sup> did not impair the assembly of Pol η foci following UV irradiation (panels E-F). In contrast, PIP degrons such as Cdt1, p21 and Set8 showed strong inhibitor activity (panel F). These results suggest that PIP degrons, rather than canonical PIP boxes, can efficiently compete for PCNA binding, although it cannot be excluded that exceptions to this rule may exist.

The conserved TD motif of PIP degrons is critical for interference with Pol η focus formation

To address whether interference with eGFP-Pol η focus formation is a specific feature of a PIP degron, we exchanged the PIP degron of Cdt1 with the canonical PIP box of Fen1 (Figure 5A) and tested the ability of the resulting chimeric protein Cdt1-PIP<sup>Fen1</sup> to interfere with Pol η accumulation into foci. Interestingly, we observed that the ability of Cdt1 to impair Pol η focus
formation after UV damage was strongly reduced when its PIP degron was exchanged with the PIP box of Fen1 (Figure 5B-C), and was comparable with that observed with Cdt1 lacking its PIP box (Cdt1<sup>ΔPIP</sup>), shown in Figure 2B-C. Importantly, Cdt1-PIP<sup>Em1</sup> mutant was expressed at a similar level as Cdt1<sup>WT</sup> and was chromatin bound (Figure 5D).

Next, we sought to identify the residues within the Cdt1 PIP degron, which confer its ability to impair UV-induced Pol η focus formation. PIP degrons differ
Figure 4. PIP degron peptides derived from CRL4<sup>Spec</sup> targets inhibit accumulation of Pol η into UV-damage foci. (A) NIH-3T3 cells transfected with pCDNA3 vector encoding either HA-tagged full-length Cdt1, p21 or Fen1 were analyzed by Western blot with antibodies specific for HA or β-actin. (B) NIH-3T3 cells were co-transfected with eGFP-Pol η and the indicated constructs. The percentage of eGFP-Pol η-expressing cells displaying Pol η foci was determined 4 h after mock (−) or UV (+) irradiation. Means and standard deviation of three independent experiments are shown. (C) Sequences used to generate myc-PIP box peptides derived from CRL4<sup>Spec</sup> substrates (PIP degrons) or stable PCNA partners (Canonical PIP boxes).
Figure 5. The Fen1 PIP box does not interfere with eGFP-Pol η focus formation. (A) Comparison of the sequence of the Cdt1 PIP box with that of Fen1 (left panel). Description of Cdt1-PIP<sup>Sen</sup> chimeric fusion (right panel). (B) The Cdt1-PIP<sup>Sen</sup> chimer does not interfere with UV-induced Pol η foci. NIH-3T3 cells were co-transfected with eGFP-Pol η and either the indicated constructs or empty vector. Four hours after mock (-) or UV irradiation (+), cells were fixed and immunostained with anti-HA antibody. The distribution of eGFP-Pol η (green) and the staining of Cdt1-HA (red) in the same cell are shown. Scale bar: 10 μm. (C) The percentage of eGFP-Pol η-expressing cells displaying Pol η foci was determined. Means and standard deviation of three independent experiments are shown. (D) Subcellular fractionation of NIH-3T3 cells transfected with the indicated Cdt1 variants or empty vector (ctrl). Four hours after mock (-) or UV irradiation, cells were lysed and fractionated into soluble (cytoplasmic) and insoluble (chromatin-bound) fractions. Extracts were analyzed by western blot with the indicated antibodies. Activation of the DNA damage checkpoint was monitored with an anti-phospho-specific H2AX antibody (γH2AX).

Figure 4. Continued (canonical PIP boxes). The residues essential for interaction with PCNA are shown in red (or light gray), residues that define the PIP degron are shown in blue (or dark gray). (D) Western blot of NIH-3T3 cells transfected with either the indicated myc-PIP box constructs, described in (C), or empty vector (ctrl.). Cell lysates were immunoblotted with antibodies specific to myc or β-actin. (E) and (F) Comparison of the potential of PIP degron- and canonical PIP box-containing peptides to inhibit Pol η focus formation. NIH-3T3 cells were co-transfected with eGFP-Pol η and the indicated myc-PIP box constructs and irradiated as in (B). Four hours later, cells were fixed and stained with anti-myc antibody and observed by fluorescence microscopy (E). The percentage of eGFP-Pol η-expressing cells in which Pol η accumulates in nuclear foci was assessed (F). The distribution of eGFP-Pol η (green) and the staining of myc-PIP box peptides (red) in the same cell are shown (F). Scale bar: 5 μm.
from canonical PIP boxes in that they contain two highly conserved motifs (Figure 6A), (i) a TD motif and (ii) adjacent basic residues required for CRL4\(^{Adp2}\)-dependent degradation (22,23,44). The TD motif confers high-affinity PCNA-binding (22,23) and is absent in the Cdt1-PIP\(^{Fem1}\) chimera (Figure 5A) as well as in the vast majority of PCNA-binding proteins (Figure 6B and Supplementary Figure S5A), suggesting that it may be important for interference with Pol \(\eta\) focus formation. To test this possibility, we individually disrupted either...
the TD motif of Cdt1 by substitution of the threonine residue by alanine (Cdt1<sub>T5A</sub>), or, as a control, we mutated the basic motif downstream of its PIP box by replacing all basic residues by alanine (Cdt1<sub>R3A</sub>), as pictured in Figure 6A. Immunoprecipitation experiments show that both Cdt1<sup>WT</sup> and Cdt1<sup>R3A</sup> efficiently interact with PCNA (Figure 6C, IP: HA, lanes 3 and 5), whereas the Cdt1<sup>T5A</sup> mutant has a much weaker PCNA-binding affinity (lane 4), confirming previous results (22,23). We observed that the Cdt1<sup>T5A</sup> mutant impaired the accumulation of Pol η into foci in a similar way as to Cdt1<sup>WT</sup> (Figure 6D and E), indicating that the basic (RRR) motif is not required for inhibition of Pol η focus formation. In contrast, the Cdt1<sup>R3A</sup> mutant only weakly affected Pol η focus formation, although it bound chromatin with similar efficiency as Cdt1<sup>WT</sup> (Supplementary Figure S5D, compare lanes 9–10 with lanes 13–14).

Collectively, these results show that a point mutation within Cdt1 PIP degron considerably reduces both its affinity for PCNA and the potential to impair Pol η focus formation. We conclude that the threonine residue within the Cdt1 PIP degron, which is conserved among PIP degrons, is a critical residue for interference with Pol η focus formation after UV irradiation.

**DISCUSSION**

In this work, we have provided evidence that activation of CRL4<sup>CDc2</sup> by DNA damage facilitates access of specific repair factors to PCNA, such as TLS Pol η and Pol κ, showing that CRL4<sup>CDc2</sup> plays an additional role in TLS DNA synthesis, independent of PCNA monoubiquitylation (62) involving efficient clearing of PIP degrons from PCNA on UV damage. This regulation may ensure that PIP degrons, which have a stronger affinity for PCNA than canonical PIP boxes, are efficiently removed because their presence may be deleterious for the cell at a given time. For example, it was shown that p21 interferes with TLS activity <i>in vivo</i> (63), as well as with TLS Pol η focus formation (60) via its PIP box. Moreover, removal of chromatin-bound Cdt1 in early S-phase constitutes one mechanism to limit replication to only one round per cell cycle. On DNA damage, Cdt1 is degraded within minutes (25,35), but the biological significance of this instant degradation has been so far elusive. Because Cdt1 has no further role in replication after MCM2-7 recruitment (64), its degradation may be important to avoid reinitiation at just fired origins, or alternatively, to facilitate TLS-dependent replication fork restart. Under our experimental conditions, we could not observe rereplication on stabilization of Cdt1 after UV damage, nor hyperactivation of the DNA damage checkpoint, a molecular sign of abnormal replication (58,65), suggesting that removal of Cdt1 after damage may have no roles in repressing reinitiation of DNA synthesis. In contrast, our data indicate that expression of the Cdt1 PIP degron on its own affects cell viability on UV damage, similar to full-length Cdt1, suggesting interference with TLS function, independently from Cdt1 function in DNA replication. There is evidence that replication fork restart at UV lesions requires Pol η (66), which may suggest that removal of Cdt1 from PCNA after UV damage in early S-phase may facilitate Pol η recruitment to reduce replication stress. This interpretation is also consistent with a previous report showing that Pol η is essential for cell viability on UV damage (15), and that disruption of its PCNA interaction region strongly affects cell viability (67). In support of this possibility, recent data show that proteolysis of chromatin-bound FBH1 helicase (another PIP degron) is important to maintain genomic stability by preventing homologous recombination after replication stress (68,69) and facilitate lesion bypass (70). Moreover, p21 degradation has been now shown to be important in replication bypass at forks stalled by UV lesions (71). Finally, recent data indicate that activation of CRL4<sup>CDc2</sup> after DNA damage may be also important to stall ongoing DNA synthesis by degradation of the p12 subunit of DNA polymerase δ (72).

The experimental data provided in our work suggest that regulated proteolysis of PCNA cofactors may represent a way to regulate specific interactions, an attractive model previously proposed as mechanism to increase PCNA accessibility (31,61,63), in addition to reversible posttranslational modifications of PCNA such as ubiquitylation and sumoylation (1).

**Possible role of PCNA-dependent degradation in DNA repair**

New emerging evidence (55,73–75) suggests that TLS also occurs outside S-phase (and this work). Pol κ functions in NER in G1 as well as in quiescent cells (18,19). A recent report implicates also Pol η in mismatch repair (MMR) and shows that PCNA monoubiquitylation in G1 after oxidative stress is dependent on the MMR machinery in mammalian cells (17). These studies suggest that TLS polymerases can also function in G1 in a way coupled to DNA repair. Our observations further suggest that activation of CRL4<sup>CDc2</sup> on DNA damage in G1 is important to remove PIP degrons proteins thus facilitating Pol κ recruitment. CRL4<sup>CDc2</sup>-mediated degradation of Cdt1 after DNA damage in G1 was recently shown to be dependent on the early incision steps of NER and subsequent PCNA loading (42,44,76), suggesting the existence of a time window when CRL4<sup>CDc2</sup> substrates compete out TLS polymerases for binding to PCNA during the early steps of DNA repair. This possibility is supported by our data obtained with Cdt1<sup>R3A</sup> mutant or a dominant negative mutant of Cullin 4A. Activation of CRL4<sup>CDc2</sup>-mediated proteolysis may be a general mechanism that cells use to facilitate the interplay of specific repair factors on chromatin-bound PCNA after DNA damage, and be relevant to DNA repair by facilitating partners switch.

**Mechanism of interference of CRL4<sup>CDc2</sup> substrates with TLS polymerases**

A detailed analysis of CRL4<sup>CDc2</sup> substrates has shown that a conserved TD motif confers high-affinity binding to PCNA (22), consistent with our findings that the threonine residue (T5) within the PIP degron of Cdt1 is required for both strong binding to PCNA and inhibition
of Pol η focus formation. Canonical PIP boxes such as Fen1 lack the TD motif (Figure 5A). The p21 PIP box binds tightly to PCNA, with higher affinity than the PIP box of Fen1 (5), and we have shown that Cdt1 fails to impair Pol η focus formation when its PIP degron is replaced with the canonical PIP box of Fen1. Finally, introduction of a TD motif into canonical PIP boxes enhances binding to PCNA (22,23). Interestingly, the presence of a TD motif within the PIP box of PCNA-binding proteins is rare and it seems to be specifically present in CRL4<sup>cdc20</sup> substrates (Supplementary Figure S5A). Y-family DNA polymerase also have noncanonical PIP boxes (Supplementary Figure S5A) resulting in suboptimal PCNA-binding affinity (59). Consistent with this observation, both the PIP box and the ubiquitin-binding motifs are required for efficient Pol η accumulation to sites of UV damage (67). Hence, we propose that CRL4<sup>cdc20</sup> substrates interfere with recruitment of PCNA partners bearing noncanonical PIP boxes through an affinity-driven competition dependent on a highly conserved TD motif. At the molecular level, this model implies that PIP degrons should be able to mask the binding site for Pol η on PCNA (hydrophobic pocket). This hypothesis is supported by radiographic co-crystal structures (59,77) showing that Pol η PIP box motif interacts with PCNA on the same site bound by p21 (Supplementary Figure S5B).

In conclusion, the results presented in this work highlight the importance to remove PIP degrons from PCNA to facilitate TLS and probably other DNA repair mechanisms, such as NER and/or HR (70). Given their strong affinity for PCNA, failure to remove such factors may compromise DNA repair efficiency. Cdt1 is an oncogene (78) overexpressed in several human cancers and cancer cell lines (79,80). It will be interesting to determine if TLS function or DNA repair is affected in cancer cells overexpressing Cdt1, or other degrons, and if this may alter the sensitivity of cancer cells to specific chemotherapies.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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**Conflict of interest statement.** None declared.

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PIP degron proteins, substrates of CRL4\(^{\text{Cd2}}\), and not PIP boxes interfere with DNA polymerase \(\eta\) and \(\kappa\) focus formation upon UV damage

Nikolay TSANOV, Chames KERMI, Philippe COULOMBE, Siem VAN DER LAAN, Dana HODROJ and Domenico MAIORANO

SUPPLEMENTARY MATERIALS AND METHODS

**FACS analysis**
Cells were co-transfected with pcDNA3-Cdt1-HA and pEGFP in the ratio of 10:1. Three days post-transfection, cells were harvested and fixed in 1% paraformaldehyde for 15 min at room temperature. After washing twice in PBS, cells were fixed in ice-cold 70% ethanol at -20\(^\circ\)C overnight. Thawed cells were washed twice in PBS and incubated with 50 \(\mu\)g/mL RNase A at 37\(^\circ\)C for 1h. DNA was stained with propidium iodide (25 \(\mu\)g/mL). Cells were analyzed with a FACScalibur flow cytometer using CellQuestPro software. Transfected cells were gated on the GFP signal.

**Cyclin A staining**
Cells expressing either Cdt1\(^{\text{WT}}\) or the Cdt1\(^{\text{R+4A}}\) mutant were transfected with either eGFP-Pol \(\eta\) or Pol \(\kappa\)-expressing plasmids. Twenty-four hours post-transfection cells were UV-irradiated. Following fixation cells were stained with an antibody specific for Cyclin A (sc-751, Santa Cruz Biotechnology) and analyzed by indirect immunofluorescence.

**siRNA**
The expression of Polk in NIH3T3 cells expressing the Cdt1\(^{\text{R+4A}}\) mutant was achieved as previously described (1). Total cell lysates were prepared 72 hours post-RNAi treatment and analyzed by western blot. The viability of cells was determined as described in Materials and Methods using the CellTiter-Glo® Luminescent Cell Viability assay (Promega).
SUPPLEMENTARY FIGURES LEGENDS

Supplementary Figure S1. Effects of CRL4Cdh2 inhibition on the relocalization of Pol η into nuclear foci after UV-induced DNA damage.

(A) U2OS cells transfected with eGFP-pol eta were pretreated with the proteasomal inhibitor MG132 for 1 hour prior to mock (-) or UV-irradiation (+). Control cells were treated with DMSO. After irradiation, cells were incubated for 4 hours in the presence of MG132 or DMSO, and the percentage of eGFP-Pol η-expressing cells displaying nuclear foci was assessed after fixation. Means and standard deviation of three independent experiments are shown.

(B) Schematic representation of mouse Cullin 4A wild-type (WT) and dominant-negative (dn) mutant constructs.

(C) UV-induced degradation of Cdt1 is inhibited by the dnCul4A mutant. NIH3T3 cells were transfected with the indicated constructs. After 24h, cells were mock (-) or UV- irradiated, and 4h later processed for immunoblotting with antibodies to myc, HA and β-actin.

(D) dnCul4A impairs the assembly of UV-induced Pol η foci. NIH3T3 cells were co-transfected with eGFP-Pol η and either dnCul4A-myc, or WT Cul4A-myc, or empty vector. Twenty-four hours post-transfection, cells were irradiated as in (A). Four hours later, the distribution of eGFP-Pol η in the nucleus was analyzed after fixation. Scale bar : 10μm.

(E) The percentage of eGFP- Pol η-expressing cells in which pol eta was localized in nuclear foci (shown in panel D) was assessed. Means and standard deviation of three independent experiments are shown.

Supplementary Figure S2. Cell cycle-dependent localization of eGFP-Pol κ.

(A) NIH-3T3 cells expressing either Cdt1WT or the Cdt1R14A mutant of the experiment shown in Figure 1 were analyzed for eGFP-Pol κ nuclear foci formation and Cyclin A staining by immunofluorescence after fixation. Insets show selected cells (indicated by a white arrow) to appreciate formation of eGFP-Pol κ foci in Cyclin A-negative cells. Scale bar: 10 μM.

(B) The percentage of Cyclin A positive and negative cells in the total population of cells expressing either the empty vector, Cdt1 wild-typeWT, the Cdt1R14A or the Cdt1M131P mutants and transfected with eGFP-Pol κ, was assessed after fixation. Means and standard deviation of three independent experiments are shown.
(C) Western blot of NIH3T3 cells expressing the Cdt1<sup>R4A</sup> mutant described in Figure 1 and treated with either a control siRNA (luciferase, Luc) or Pol κ-specific siRNA.

(D) Viability curves of NIH3T3 cells expressing the Cdt1<sup>R4A</sup> mutant treated with either a control siRNA (luciferase, Luc) or Pol κ-specific siRNA. NS, non-significant (n=3).

Supplementary Figure S3. Cdt1 interferes with UV-induced eGFP-Pol κ focus formation in a PIP box-dependent manner.

(A) NIH-3T3 cells were co-transfected with eGFP-Pol κ and either Cdt1<sup>WT</sup>, Cdt1<sup>PIP</sup>, or empty vector. Four hours after mock (-) or UV-irradiation (+), cells were fixed and stained with anti-HA. Scale bar: 10 μm.

(B) Quantification of the data shown in (A). Means and standard deviation of three independent experiments are shown.

(C) NIH-3T3 cells were co-transfected with eGFP-Pol κ and either myc-PIP box<sup>Cdt1WT</sup>, myc-PIP box<sup>Cdt1Mut</sup>, or empty vector and irradiated as in (B). Four hours later, the percentage of eGFP-Pol κ-expressing cells in which Pol κ was localized in nuclear foci was assessed. Means and standard deviation of three independent experiments are shown.

(D) Y-family DNA polymerases have non-canonical PIP boxes. Comparison of the PIP box sequences of Y-family DNA polymerases with that of the canonical PIP box (consensus).

Supplementary Figure S4. Cdt1 interference with eGFP-Pol η foci formation is independent of its function in DNA replication.

(A) Subcellular fractionation of NIH-3T3 cells transfected with wild-type Cdt1 (WT) or a Cdt1 mutant that lacks the PCNA-interaction motif (APIP), or empty vector (ctrl.). Cells were mock (-) or UV-irradiated (+) 24 hours post transfection and 4h later were lysed and fractionated into soluble (cytoplasmic fraction) and insoluble (chromatin-bound) fraction as described in Materials and methods. Extracts were analyzed by western blot with the indicated antibodies. Activation of the DNA damage checkpoint was monitored by western blot with an anti-phospho-specific p53 antibody.

(B) Expression of Cdt1 at high levels in human U2OS cells inhibits pol η foci formation. U2OS cells co-transfected with eGFP-Pol η and either Cdt1<sup>WT</sup> or empty vector were mock (-) or UV-irradiated (+). The percentage of eGFP-Pol η-expressing cells in which pol η was localized in nuclear foci was assessed. Means and standard deviation of three independent experiments are shown.
(C) A functional PCNA-Interaction motif (PIP box) is required for the inhibition of pol eta focus formation by Cdt1. NIH3T3 cells were co-transfected with eGFP-Pol η and the indicated constructs. After 24 hours, cells were irradiated as in (A) and the percentage of cells displaying eGFP- Pol η foci was assessed. Means and standard deviation of three independent experiments are shown.

(D) Overexpression of Cdt1 induces DNA re-replication in U2OS cells. Cells were transfected with the indicated Cdt1 constructs or empty vector (Vector). After 3 days, cells were fixed and stained with propidium iodide, and processed for FACS analysis. The extent of re-replication is indicated by the percent (%) of cells displaying DNA content higher than 4C.

(E) Cdt1 increases UV-induced cell death in a PIP box dependent fashion. NIH-3T3 cells were electroporated with either pcDNA3 vector encoding Cdt1WT, or Cdt1mutPIP, or empty vector. Twenty-four hours post-transfection, cells were mock- or UV-irradiated with 10 J/m². Cells were fixed and stained with propidium iodide 24 hours after irradiation, and processed for FACS analysis. The percentage of sub G1 cells was assessed.

Supplementary Figure S5. Conservation of the TD motif within PCNA-interacting proteins.

(A) Presence of the TD motif within PCNA-interacting motifs. Comparison of the sequences of the PIP box motifs of thirty known partners of PCNA in humans. The PIP boxes have been organized in several categories: Canonical PIP boxes (consensus), Non-canonical PIP boxes (differ from consensus), and PIP degrons (substrates of CRL4CDK2). The residues essential for optimal PCNA-interaction are shown in red and the residues that define the PIP degron are shown in blue. The proteins that contain a TD motif within their PIP box are indicated.

Ψ = Val, Leu, Ile, Met; θ = Tyr, Phe.

(B) Pol eta binds PCNA through a non-canonical PIP box onto the same hydrophobic pocket as p21. The images show the structures of PCNA-p21 complex (a) (2) and PCNA-pol eta complex (b) (3). The hydrophobic pocket onto PCNA was colored (orange: Pro 234, Pro 129, yellow: Tyr 250, light green: Leu 47, dark green: Ile 128). Pol eta binds PCNA through a non-canonical PIP box that lacks the glutamine residue (Q1), resulting in lower PCNA-binding affinity (3). The positions of the TD motif and the R+4 residue in the structure of p21-PCNA are shown. The images were generated using PDB accession numbers 1AXC (a) and 2ZVK (b) and Swiss-PDB Viewer (4). The sequences of the PIP box motifs are indicated under the images.
SUPPLEMENTARY REFERENCES


Supplementary Figure S2

A

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- eGFP-Pol κ
- Cyclin A
- DAPI

B

eGFP-Pol κ-transfected cells

C

siLuc siPolκ

Polκ  β-actin

D

Cell viability (% of mock)

- cdt1 R+4A si luc.
- cdt1 R+4A si polk

UV dose (J/m²)
A

Empty Vector  Cdt1-HA

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B

% of cells with eGFP-Polh foci

Empty    Empty   WT   ΔPIP

C

% of cells with eGFP-Polh foci

Empty    Empty   WT   ΔPIP

D

Canonical PIP box

12345678  QXXVXX00

Y-family of DNA polymerases

polη: -MQTLLESFFKPLT
polε: -KHTLDIFPPK
polδ: -KGLIDIFTL-

Rev 1: PIP box absent
A

PCNA-interacting motifs

Canonical PIP box

QxxYX00

12345678

Fen1  QGRLDFF
p15(PAF) QKIGEFFF
p66(pol δ) QVSITGFF
LigI  QRHMSF
MSH3  QAVLSRF
APE2  QRNLKSYF
XPG  QRLEDSFF
MYB  QVQVDNF
UNG2  QKTLYSQL
RECC5  QNLIRHFF
BLM  QTRVKDF
MDM2  QMIVLTYF

Canonical PIP boxes

Non-canonical PIP boxes

Polymerase

pol η  MQTLESFF
pol κ  KHTLDIFF
pol λ  KGLIDXYI
PARP1  QBLIKMIF
WRN  QRKLLRD
DNMT1  QTITSHF
Rfc1  MDRIKFF
pol β  QLQKHF
pol λ  SVPVLLEF
ING1b  QHLVNYV
WSTF  QDEIAEDY
NCL1  QRLHSTAF
p57  GPLISBFF

B

p21 PIP box peptide

(a)

pol η PIP box peptide

(b)

M4  Y8
T5  F7
Q1  R+4
D6
F6
T+4

PIP box sequence:

Q1-XX-M4-T5-D6-F7-Y8-XX-X-R+4

M1-XX-L4-E5-S4-F3-F8-XX-X-X-T+4
2. "RAD18 Is a Maternal Limiting Factor Silencing the UV-Dependent DNA Damage Checkpoint in Xenopus Embryos"

2.1. Article introduction

The cell cycle of early embryos is very rapid and contracted, and the S-phase checkpoint that delays cell division upon DNA damage (Anderson et al., 1997; Hensey and Gautier, 1997) or unreplicated DNA (Dasso and Newport, 1990; Kimelman et al., 1987) is inefficient. This may represent an adaptation to ensure rapid proliferation. Checkpoint activation is observed near the MBT concomitant to the instauration of gap-phases (Clute and Masui, 1997; Newport and Kirschner, 1982a, b). The molecular mechanisms responsible for checkpoint suppression in early embryos are currently very poorly understood. Previous studies using *Xenopus in vitro* egg extracts (Dasso and Newport, 1990) and in live embryos (Conn et al., 2004; Kappas et al., 2000) have shown that checkpoint activation depends upon the nucleus-to cytoplasmic (N/C) ratio, and not upon transcription nor translation, suggesting titration of maternal limiting factors whose identity is currently unknown. More recently, genetic data in *C. elegans* (Holway et al., 2006; Ohkumo et al., 2006) have implicated a Y-family TLS polymerase (Pol η) in this regulation, since early embryos mutated in this enzyme delay the cell cycle after exposure to DNA damaging agents. However, whether this function is conserved in vertebrates and the molecular mechanism underlying this regulation is completely unknown. TLS polymerases are specialized in the replication of damaged DNA although they are error-prone due to the presence of a larger catalytic site than replicative DNA polymerases (Sale et al., 2012). Y-family TLS polymerases are recruited to damage sites through interaction with monoubiquitinated PCNA (PCNA\textsubscript{monoUb}) catalysed by the RAD6-RAD18 ubiquitin ligase, while the USP1 ubiquitin hydrolase catalyse the opposite reaction (Ulrich and Takahashi, 2013).

2.2. Project objectives

The main objective of my thesis was to understand the molecular basis of checkpoint silencing in early embryos. For this, we have taken advantage of *cell-free* extracts derived from
activated Xenopus eggs. This *in vitro* system faithfully reproduces the developmentally-regulated activation of the DNA damage checkpoint observed *in vivo* (Anderson et al., 1997; Conn et al., 2004; Kappas et al., 2000; Newport and Dasso, 1989). This is achieved by increasing the concentration of Xenopus sperm nuclei into a fixed volume of egg extract, thus mimicking the increase in N/C ratio in the early embryo close to MBT (over 400 nuclei/μl of egg extract) (Dasso and Newport, 1990). Biochemical characterization of chromatin assembled in Xenopus egg extracts at low nuclear-to-cytoplasmic (N/C) ratio provided us with evidence that checkpoint repression in Xenopus eggs is a consequence of strong inhibition of replication fork uncoupling mediated by constitutive PCNA$_{\text{monoUb}}$, and subsequent TLS Pol η chromatin binding, irrespective of DNA damage. As RAD6-RAD18 is the ubiquitin ligase responsible for PCNA$_{\text{monoUb}}$, we checked whether it could be the maternally supplied limiting factor responsible for checkpoint silencing in early embryos. Another important point was to check if this regulation is reversible and can be reactivated in Xenopus egg extracts at high N/C ratio. Moreover, it would be interesting to know how RAD18 and PCNA$_{\text{monoUb}}$ are developmentally regulated in dividing Xenopus embryos. The following step was to investigate whether this embryonic-like checkpoint silencing could be reactivated in somatic mammalian cells upon overexpressing RAD18 by the analysis of GFP-Pol η replication factories formation, replication fork uncoupling (RPA foci), and checkpoint silencing.

From the abovementioned data it is predictable that cells overexpressing RAD18 may display resistance to certain DNA damaging agents, in particular to those that are dealt with by translesion DNA synthesis, such as DNA adducts generated by UV lesions or cisplatin, as well as bases alkylation. One of our objectives was to measure the sensitivity of cells expressing high levels of wild-type RAD18 or of specific RAD18 mutants (C28F and C207F), exposed to DNA damaging agents by performing survival assays.

Since overexpressing RAD18 in cells could be responsible for overriding the DDR activation which considered as a barrier to malignant transformation (Halazonetis et al., 2008), we have explored the possibility that RAD18 may be overexpressed in cancers. In other terms, it is possible that forced expression of RAD18 may phenocopy mutations in checkpoint genes and as such promotes proliferation and resistance of cells exposed to DNA damage or cells experiencing replication stress generated by activation of an oncogene.
2.3. The article: "RAD18 Is a Maternal Limiting Factor Silencing the UV-Dependent DNA Damage Checkpoint in Xenopus Embryos"

RAD18 Is a Maternal Limiting Factor Silencing the UV-Dependent DNA Damage Checkpoint in Xenopus Embryos
RAD18 Is a Maternal Limiting Factor Silencing the UV-Dependent DNA Damage Checkpoint in *Xenopus* Embryos

**Graphical Abstract**

**Highlights**
- DNA damage tolerance protein RAD18 is a checkpoint-limiting factor in *Xenopus* embryos
- At low N/C ratios, RAD18 suppresses the checkpoint and induces resistance to DNA damage
- There is constitutive PCNA$^{mLib}$ and DNA POL$\Pi$ recruitment to chromatin at a low N/C ratio
- RAD18 is overexpressed in glioblastoma cancer stem cells

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**In Brief**
The DNA damage checkpoint is silent in the rapidly dividing cells of early *Xenopus* embryos. Kermi et al. find that high levels of the maternally provided DNA-damage tolerance protein RAD18 suppress checkpoint activity in early embryos. As cells divide, DNA abundance increases relative to RAD18, allowing checkpoint activation at the midblastula transition.

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RAD18 Is a Maternal Limiting Factor Silencing the UV-Dependent DNA Damage Checkpoint in Xenopus Embryos

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SUMMARY

In early embryos, the DNA damage checkpoint is silent until the midblastula transition (MBT) because of maternal limiting factors of unknown identity. Here we identify the RAD18 ubiquitin ligase as one such factor in Xenopus. We show, in vitro and in vivo, that inactivation of RAD18 function leads to DNA damage-dependent checkpoint activation, monitored by CHK1 phosphorylation. Moreover, we show that the abundance of both RAD18 and PCNA monoubiquitylated (mUb) are developmentally regulated. Increased DNA abundance limits the availability of RAD18 close to the MBT, thereby reducing PCNA mUb and inducing checkpoint derepression. Furthermore, we show that this embryonic-like regulation can be reactivated in somatic mammalian cells by ectopic RAD18 expression, therefore conferring resistance to DNA damage. Finally, we find high RAD18 expression in cancer stem cells highly resistant to DNA damage. Together, these data propose RAD18 as a critical embryonic checkpoint-inhibiting factor and suggest that RAD18 deregulation may have unexpected oncogenic potential.

INTRODUCTION

Early embryonic cleavages are rapid, consisting of alternating S and M phases with virtually absent Gap phases (Graham and Morgan, 1966). In this contracted cell cycle, the S phase checkpoint delaying cell division upon DNA damage (Anderson et al., 1997; Henssey and Gautier, 1997) or unreplicated DNA (Dasso and Newport, 1990; Kimelman et al., 1987) is inefficient and may represent an adaptation to ensure rapid proliferation. The molecular mechanisms responsible for checkpoint inhibition in early embryos are poorly understood. Previous studies in Xenopus (Conn et al., 2004; Dasso and Newport, 1990; Kappas et al., 2000) have shown that checkpoint activation depends on the nuclear-to-cytoplasmic (N/C) ratio because of the absence of cell growth and not on transcription or translation, suggesting titration of maternal limiting factors of unknown identity. Genetic data in C. elegans (Holway et al., 2006; Ohkumo et al., 2006) have implicated a translesion DNA polymerase (TLS POL1) specialized in the replication of damaged DNA (see Sale et al., 2012, for a review). POL1 is recruited to DNA damage upon binding to PCNA monoubiquitylated by the RAD6 (E2)-RAD18 (E3) ubiquitin ligase complex, whereas the USP1 ubiquitin hydrolase catalyzes the opposite reaction (see Ulrich and Takahashi, 2013, for a review). In S phase, checkpoint activation relies upon replication fork uncoupling generated by DNA damage, such as UV irradiation. Excess single-stranded DNA (sssDNA), generated in this process by the action of the helicase, is the primary substrate initiating ATR-dependent checkpoint signaling (Byun et al., 2005). Here we provide evidence that checkpoint repression in Xenopus eggs is a consequence of replication fork uncoupling inhibition mediated by RAD18, a critical factor for PCNA monoubiquitylated (PCNA mUb). We also show that this regulation is reversible and can be reactivated by increasing RAD18 abundance, resulting in resistance to DNA-damaging agents that is relevant to cancer recurrence.

RESULTS AND DISCUSSION

Constitutive TLS POL1 Binding to Chromatin at a Low N/C Ratio

To understand the molecular grounds of embryonic checkpoint silencing, we used cell-free extracts derived from activated Xenopus eggs. This in vitro system faithfully reproduces the developmentally regulated activation of the DNA damage checkpoint observed in vivo (Anderson et al., 1997; Conn et al., 2004; Kappas et al., 2000; Newport and Dasso, 1989). This is achieved...
by adding a sufficient amount of sperm nuclei into a fixed volume of egg cytoplasm, reaching a critical N/C ratio that triggers checkpoint activation (400 nuclei/μl; Dasso and Newport, 1990).

Figures S1A–S1C show that UV-irradiated sperm nuclei, added at a low N/C ratio into egg extracts naturally synchronized in very early S phase, fail to delay both DNA synthesis and mitotic entry compared with a high N/C ratio. Consistent with previous observations in vivo (Conn et al., 2004; Kappas et al., 2000), we did not observe CHK1 phosphorylation at a low N/C ratio (Figure 1A, top, lane 2), whereas this occurred normally at a high N/C ratio as expected (Kumagai et al., 1998). Inhibition of CHK1 phosphorylation was also observed using a fixed amount of damaged sperm nuclei while increasing the extract volume (Figure S1D), therefore strengthening the conclusion that checkpoint activation is sensitive to the N/C ratio and not to the total amount of DNA damage.

Using the ssDNA binding protein RPA (Recolin et al., 2012; Walter and Newport, 2003) as a readout for replication fork uncoupling, we observed that RPA greatly accumulated onto chromatin in S phase at a high N/C ratio upon UV irradiation, as expected, (Figure 1A; bottom, lane 4), whereas RPA accumulation was strongly reduced at a low N/C ratio (lens 2), suggesting inefficient replication fork uncoupling. This is consistent with previous observations in C. elegans embryos (Halway et al., 2006; Ohkuro et al., 2006) as well as with a reduced production of ssDNA in human embryonic stem cells (Desmarais et al., 2012). In addition, UV-dependent accumulation of the ATR-interacting protein (ATRIP), recruited by RPA and required for checkpoint signaling, was also strongly abolished, whereas it was recruited normally at a high N/C ratio. At a low N/C ratio, ATR was bound to chromatin and showed modest accumulation upon UV irradiation, similar to ATRIP. Efficient replication fork uncoupling was observed at a low N/C ratio by blocking DNA synthesis with aphidicolin, an inhibitor of replicative DNA polymerases (Figure S1E), suggesting that the uncoupling defect is specific to UV damage. Interestingly, at a low N/C ratio, we
observed that POLγ is chromatin-associated with or without DNA damage (Figure 1A, bottom, lanes 1 and 2). In contrast, at a high N/C ratio, POLγ was recruited only after UV irradiation (Figure 1A, lanes 3 and 4). We also verified the presence of replicative polymerases on chromatin at a low N/C ratio (Figure S1F) and observed that POLγ abundance is similar to that of POLε (Figure S1G). Strikingly, at a low N/C ratio, PCNA<sup>MMTS</sup> was observed on chromatin irrespective of DNA damage (Figure 1A, lanes 1 and 2), whereas, at a high N/C ratio, PCNA<sup>MMTS</sup> was present mainly upon UV irradiation (bottom, lane 4), as reported previously (Chang et al., 2006). Damage-independent POLγ recruitment at a low N/C ratio was reduced by addition of Geminin, an inhibitor of replication fork formation (Figure S1H), suggesting replication fork dependency for binding. Because TLS POLγ replicates past UV lesions, constitutive POLγ binding may avoid fork stalling by UV lesions, therefore preventing replication fork uncoupling and ssDNA formation. In turn, this leads to failure to recruit checkpoint factors (ATRIP), and precludes checkpoint activation. Constitutive TLS in early embryos may be important to tolerate not only external damage but also endogenous replication stress induced by a high concentration of nucleotides. This possibility is in line with evidence suggesting that ribonucleotide triphosphate (NTP) incorporation causes replication stress and induces PCNA<sup>MMTS</sup> in yeast and that TLS activity is required for replication resumption (Lazzaro et al., 2012).

RAD6-RAD18 and Not POLγ Is Titrated from Egg Cytosol at a High N/C Ratio

Maternally supplied inhibitor(s), present in limited amounts in egg cytoplasm and titrated progressively on chromatin during embryonic cleavages, may be responsible for checkpoint silencing (Conn et al., 2004; Dasso and Newport, 1990; Kappas et al., 2000). The data shown in Figure 1A and previous data on C. elegans (Holway et al., 2006; Okumura et al., 2008) implicate TLS components. We analyzed the abundance of several TLS factors remaining in the cytoplasm after incubation with sperm nuclei at a low or high N/C ratio and observed that USP1, POLγ, PCNA, RPA, CHK1, and ATR levels did not change (Figure 1B, Figure S1J), suggesting that they are in excess over the DNA. By raising specific antibodies (Figure S2A), we observed that RAD6 and RAD18 were depleted from the extract and less abundant on chromatin at a high N/C ratio (Figure 1B). Reduced RAD18 and RAD6 chromatin binding at a high N/C ratio correlates with both reduced PCNA<sup>MMTS</sup> and POLγ chromatin binding in the absence of UV damage, whereas USP1 binding did not change significantly, suggesting that RAD18 is more limiting near the midblastula transition (MBT). To investigate whether this is due to titration or destabilization, we analyzed RAD18 binding to chromatin at increasing N/C ratios, with or without the proteasome inhibitor MG132 (Figure S2B, top), and observed a gradual decline in both conditions, suggesting titration, although MG132 increased RAD18 abundance at a high N/C ratio. Moreover, a kinetic analysis of chromatin binding in vitro shows that RAD18 is absent from chromatin at the end of S phase (Figure S2B, bottom), whereas, in the presence of MG132, its abundance is increased, suggesting destabilization after replication. Together, these results suggest that both titration and destabilization limit the availability of RAD18 at a high N/C ratio.

In mammalian cells, RAD18 is recruited to chromatin upon DNA damage by physical interactions with the DBF4 subunit of the CDC7 protein kinase (Yamada et al., 2013). Interestingly, immunoprecipitation experiments show complex formation between RAD18 and the Xenopus DBF4-related protein DRF1 at a low N/C ratio in the absence of damage (Figure 1C). This complex was virtually undetectable at a high N/C ratio, even when an excess of RAD18 immunoprecipitates, compared with a low N/C ratio, was analyzed (Figure 1C, right). This observation suggests that, at a low N/C ratio, high RAD18 abundance promotes DNA damage-independent complex formation with DRF1 to constitutively target RAD18 to replication forks.

RAD18 and PCNA<sup>MMTS</sup> Are Developmentally Regulated

Analysis of RAD18 and PCNA<sup>MMTS</sup> abundance in embryos at different stages of development shows that RAD18 decreases during embryogenesis, starting from stage 4, and drops to very low levels at stage 6.5 (pre-MBT; Figure 1D). RAD18 decline is paralleled by a correspondent decrease in PCNA<sup>MMTS</sup>. Similar to what was observed in vitro (Figure S2B), injection of MG132 into embryos, at a dose that does not interfere with the timing of MBT onset (Brandt et al., 2011), did not affect the decline of RAD18 levels prior to stage 6, although it significantly increased RAD18 abundance at stage 6.5 (Figure 1E). This result suggests that, in vivo, both titration and destabilization limit RAD18 abundance near the MBT. In contrast, DRF1 did not show significant changes up to stage 7, similar to what has been reported previously (Collart et al., 2013; Takahashi and Walter, 2003), suggesting that RAD18 is more limiting than DRF1. This possibility is supported by the observation that DRF1 is not depleted from egg cytoplasm at a high N/C ratio (Figure S1I) and is consistent with DRF1 titration at a higher N/C ratio (around 3,000 nuclei/μl; Collart et al., 2013), whereas onset of the DNA damage checkpoint occurs at 400 nuclei/μl (Conn et al., 2004; Dasso and Newport, 1990; Kappas et al., 2000). Therefore, reduced RAD18 abundance and not DRF1 is likely responsible for DNA damage-dependent checkpoint activation, although it cannot be excluded that the titration of RAD18 stabilizing factor(s) may also contribute. Quantification of RAD18 shows that its concentration in Xenopus eggs is relatively low (~0.25 ng/embryo, 3.5 μM; Figure S2C), over 1,000 times less than PCNA. Genetic evidence in C. elegans proposes POLγ as a repressor of the checkpoint (Holway et al., 2006; Okumura et al., 2006; Roenink et al., 2012). Although we found that, in Xenopus, POLγ is implicated, it is not limiting because it is not quantitatively depleted at a high N/C ratio. We speculate that, at a high N/C ratio, a reduced abundance of RAD18 may be counteracted by USP1, resulting in reduced PCNA<sup>MMTS</sup>.

RAD6-RAD18 Inhibits the UV-Dependent DNA Damage Checkpoint In Vivo at a Low N/C Ratio

We next removed RAD18 from egg extracts using specific antibodies. As expected (Bally et al., 1994), RAD18 depletion also partially removed RAD6 (Figure 2A, lane 2; Figure S2D) but not POLγ, RPA, or PCNA. RAD18 depletion drastically reduced PCNA<sup>MMTS</sup> upon UV irradiation at a low N/C ratio, as well as POLγ chromatin binding, and, importantly, induced UV damage-dependent CHK1 phosphorylation (Figure 2B, lane 3). In
RAD18 depletion induces CHK1 phosphorylation at a low N/C ratio upon UV damage (Figure 2B), indicating that this phenotype is specific to a low N/C ratio and, likely, that it is not due to the accumulation of unpaired DNA because neither RAD6 nor RAD18 are required for nucleotide excision repair (Hishida et al., 2005). Moreover, pre- and post-MBT embryonic extracts appear to have a similar DNA repair capacity (Anderson et al., 1997). Furthermore, the N/C ratio, and not the total amount of damaged DNA, is critical for checkpoint activation (Conn et al., 2004), ruling out differences in DNA repair rates.

Reconstitution of RAD18-depleted extracts at a low N/C ratio (Figure S2F) with a recombinant His$_6$-RAD6-RAD18 complex (Figure 2C) inhibited UV damage-dependent CHK1 phosphorylation (Figure 2D, lane 5), excluding the implication of co-depleted proteins. Furthermore, this complex, and not recombinant RAD6, rescued defective PCNA$^{mbt}$ in RAD18-depleted extracts, demonstrating that it is functional (Figure S2G). Also, CHK1 phosphorylation was induced by UV damage when recombinant PCNA$^{mbt}$ mutant that cannot be monoubiquitylated, and not wild-type (WT) PCNA, was added to extracts at a low N/C ratio (Figure S2H). Of note, the recently discovered PRIMPOL (see Helledge, 2013, for a review), was not bound to chromatin at a low N/C ratio (Figure S2I), ruling out active UV lesion bypass or replication fork restart by this polymerase. Finally,
recombinant His6-RAD6-RAD18 repressed both RPA accumulation and CHK1 phosphorylation, normally observed at a high N/C ratio upon UV irradiation (Figure 2E; compare lanes 2 and 3), suggesting inhibition of replication fork uncoupling.

RAD18 Silences the UV-Dependent DNA Damage Checkpoint in Xenopus Embryos

To obtain evidence for checkpoint inhibition by RAD18 in vivo, we overexpressed either RAD18WT or catalytically inactive RAD18 (G28F mutant) by microinjection of the corresponding mRNA into embryos at the two-cell stage. Embryos injected with either water (mock) or RAD18WT mRNA developed normally and reached stage 6.5 with or without UV irradiation (Figure 2F). In contrast, UV-irradiated embryos injected with RAD18G28F were delayed in the embryonic cleavages from one cell cycle after UV irradiation (Figure S2I). Consistent with this phenotype, UV-irradiated embryos expressing the RAD18G28F mutant accumulated CHK1 phosphorylation, whereas expression of RAD18WT inhibited it (Figure 2G; compare lane 3 with lane 4). Importantly, no spontaneous CHK1 phosphorylation was observed in embryos injected with either RAD18 mRNAs (Figure S2K; -UV), showing that RAD18 is not implicated in DNA damage-independent developmental activation of CHK1, as reported for DFR1 (Collart et al., 2013). These data, together, show that RAD18 inhibition is sufficient to give to the embryo the competence to activate the DNA damage checkpoint. Because RAD18 is also implicated in double-strand break repair (Huang et al., 2009; Szöts et al., 2006; Watanabe et al., 2009), it might also contribute to silencing the checkpoint upon γ-irradiation, although we have not tested this possibility.

Reactivation of Embryonic-like Checkpoint Silencing in Mammalian Cells by RAD18 Upregulation

Next we analyzed the consequences of increasing RAD18 abundance in somatic mammalian cells. RAD18 overexpression did not induce significant cell-cycle changes (Figure S3A) and, consistent with previous reports (Bi et al., 2006; Davies et al., 2008), induced constitutive PCNA™ (Figure 3A). Importantly, overexpression of either RAD6 or RAD18 and RAD6 was not sufficient to induce constitutive PCNA™ to a level similar to that of RAD18 alone (Figure S3B). Of note, and unlike what was observed in Xenopus at a low N/C ratio (Figure 1A), the amount of PCNA™ observed in asynchronous cells expressing RAD18 increased after UV irradiation. Moreover, eGFP-POLγ nuclear foci formed, even in the absence of DNA damage, only upon RAD18 overexpression (Figures 3B and 3C). Most importantly, UV-dependent CHK1 phosphorylation was reduced significantly in asynchronous cells expressing RAD18 (Figure 3D; Figure S3D), suggesting that, in mammalian cells, high RAD18 abundance is sufficient to inhibit UV-dependent checkpoint activation, in line with a previous observation in yeast (Daigaku et al., 2010). A very similar result was obtained upon expression of PCNA™ fused to ubiquitin (Figures 3E and 3F) that mimics constitutive PCNA™ (Kanai et al., 2015). Moreover, expression of RAD18 lacking CDC7 phosphorylation sites (RAD18™™, also required for POLγ binding (Durando et al., 2013), did not induce constitutive eGFP-POLγ nuclear foci and acts as a dominant negative because it inhibits eGFP-POLγ focus formation after UV damage (Figure S3C), consistent with a previous report (Day et al., 2010). This mutant did not inhibit CHK1 phosphorylation compared with RAD18WT (Figure S3D), suggesting that checkpoint silencing depends on RAD18 phosphorylation by CDC7. Similarly, the TLS-deficient RAD18™™ mutant did not induce constitutive eGFP-POLγ nuclear foci (Figure S3E). Finally, and entirely consistent with results in Xenopus (Figure 1A), high RAD18 expression also strongly repressed RPA foci formed upon UV irradiation in mammalian cells (Figure 4A), suggesting inhibition of replication fork uncoupling. Together, these observations show that RAD18 overexpression is sufficient to induce constitutive PCNA™ independently of RAD6, although we could not formally prove it in Xenopus because we failed to express active recombinant RAD18 without RAD6. This suggests that, when overexpressed, RAD18 may either bypass the RAD6 requirement or RAD6 may use another UV-inducible intermediate. This latter possibility may explain why, in C. elegans, RAD6 mutations did not delay mitotic entry in early embryos upon DNA damage (Holway et al., 2006).

We next determined whether cells expressing RAD18 display increased resistance to DNA damage resulting from impaired checkpoint activation. To this end, we generated stable cell lines expressing ectopic RAD18 at a similar level as endogenous RAD18. Expression of RAD18WT significantly increased cell viability upon exposure to either UV irradiation or to the chemotherapeutic relevant drug cisplatin, whereas RAD18™™ did not (Figures 4B and 4C). Taken together, these results link RAD18 expression to checkpoint inactivation and resistance to DNA-damaging agents.

RAD18 Is Overexpressed in Cancer Stem Cells Highly Resistant to DNA Damage

Resistance to DNA-damaging agents is linked to cancer recurrence. We observed high RAD18 expression in a colon cancer-derived cell line resistant to oxaliplatin (HTC116) compared with the HCT8-sensitivity cancer cell line (Figure S4A) as well as in the highly DNA damage-resistant brain cancer glioblastoma (Figure 4D). Importantly, we observed high RAD18 expression specifically in glioblastoma cancer stem cells isolated from tumor biopsies (CD34+) and not in their differentiated counterparts that express RAD18 at similar levels than HeLa cells (Figure 4E). In contrast, expression of RAD6 and of other TLS-, checkpoint-, and proliferation-relevant proteins was not increased in glioblastoma (Figure S4B). This result is consistent with GEO profile data showing high RAD18 mRNA expression in glioblastoma cancer stem cells. Moreover, very recent data implicate RAD18 in the therapeutic resistance of colon cancer cells (Liu et al., 2015). Furthermore, RAD18 downregulation in the U87 glioblastoma cell line induced sensitivity to cisplatin (Figure 4F), whereas RAD18 re-expression induced a dramatically increased viability, suggesting acquired resistance. Because glioblastoma is resistant to cisplatin, this observation puts forward RAD18 as a target for sensitizing glioblastoma to cisplatin. Together, our findings suggest that increased RAD18 expression has a positive effect on proliferation upon DNA damage by shutting checkpoint activation, therefore confering resistance to DNA damage, and show high RAD18 expression specifically in cancer stem cells that are implicated in resistance to therapy.
In conclusion, this work suggests that constitutive PCNA$^{164R}$, driven by RAD18, is responsible for silencing the UV damage checkpoint in Xenopus embryos by inhibiting replication fork uncoupling, a critical determinant for checkpoint signaling. Genetic data in C. elegans (Holway et al., 2006; Ohkuno et al., 2006) and the presence of constitutive PCNA$^{164R}$ in early Drosophila embryos (E. Lo Furno, I. Busseau, and D. M., unpublished data) make likely that this regulation may be conserved in other organisms. Recent data suggest that the DNA damage checkpoint affects TLS through the regulation of a RAD18-CDC7-DBF4 complex (Yamada et al., 2013). Our observations show that RAD18 deregulation affects the DNA damage checkpoint, suggesting cross-talk between these two pathways. This may constitute an additional mechanism, aside from the mutator activity, linking TLS deregulation to cancer (Abertella et al., 2005). In this perspective, RAD18 deregulation might have a previously unrecognized oncogenic potential relevant to the therapeutic resistance of certain cancer subtypes, such as those of embryonic origin or those generated by dedifferentiation of somatic cells.

### EXPERIMENTAL PROCEDURES

#### Xenopus Egg Extract Preparation and Use

Experiments with Xenopus were performed in accordance with current institutional and national regulations approved by the Minister of Research under...
Figure 4. High RAD18 Expression Is Associated with Resistance to DNA Damage
(A) RAD18 expression inhibits UV-dependent RPA focus formation in mammalian cells. HEK293T cells transfected with the indicated expression vectors stained with DAPI to visualize DNA and RPA2 antibodies were visualized by fluorescence microscopy. Scale bar, 10 μm. Quantification of RPA2 foci from the experiment described in (A) is also shown. Means ± SD are shown (*P < 0.01, n = 3).
(B and C) Survival curves of asynchronous NIH 3T3 cells stably expressing either empty vector or low levels of RAD18wt or RAD18C91F mutant challenged by the indicated doses of UV-C or cisplatin (CisA). CisA normalized to non-irradiated cells (mock). Means ± SD are shown (**P < 0.01, n = 3).
(D) Expression of RAD18 mRNA in glioblastomas (CD133+, Glioma) compared with HeLa cells by RT-PCR. Means ± SD are shown (n = 3).
(E) Western blot of total cell extracts from glioblastoma biopsies (grade 4), differentiated counterparts (progenitors, CD133+), or HeLa cells. TOP: western blot of U87 glioblastoma cell extracts treated with control siRNA (siLuc) or a RAD18-specific siRNA (siRAD18) or co-transfected with RAD18 siRNA and a plasmid expressing RAD18wt (siRAD18 + RAD18). Bottom: survival curves of U87 glioblastoma cells treated as described for the top and challenged with the indicated doses of cisplatin compared with non-treated cells (mock). Means ± SD are shown (n = 3, see also Figure S4).

supervision of the Departmental Direction of Population Protection (DDPP).
Interphase and cycling Xenopus egg extracts were prepared and used as described previously (Murray, 1991; Racolins, 2012). UV irradiation of sperm chromatin and isolation of chromatin fractions was performed as described previously (Racolins, 2012).

Xenopus Embryos and Microinjection Experiments
Embryos were prepared by in vitro fertilization using standard procedures (Sivel et al., 2000). UV-irradiated at the two-cell stage, and microinjected with the indicated mRNAs. Total protein extracts were obtained by collecting staged embryos according to Nieuwkoop and Faber normal tables.

mRNA Synthesis
In vitro transcription was performed using the mMESSAGE mACHINE kit (Ambion). mRNA was ethanol-precipitated and dissolved in water ready for microinjection.

Cell Culture
Cells were cultured and maintained under standard conditions. For transient expression, HEK293T cells were transfected with calcium phosphate. Twenty-four hours post-transfection cells were mock- or UV-irradiated and collected at the indicated time points. Whole cell extracts were clarified by centrifugation.

Immunofluorescence Microscopy and Focus Formation Assay
Cells were grown on coverslips prior to co-transfection. Four hours after UV-C irradiation, cells were fixed with 3.2% paraformaldehyde for 15 min at room temperature and washed three times with PBS. After washing twice with PBS + 3% BSA, cells were mounted with ProlongGold DAPI (Invitrogen). eGFP-POLκ foci were analyzed with a Leica DM6000 epifluorescence microscope (Montpellier Réunion InterOrganisme facility). Images were acquired using a CoolSnap HQ charge-coupled device (CCD) camera (Photometrics) and MetaMorph software (Molecular Devices). The percentage of eGFP-POLκ-expressing cells displaying eGFP-POLκ foci was determined.
by scoring at least 200 nuclei for each condition. Nuclei containing less than 30 foci were scored as negatives.

**Generation of Stable NIH 3T3 Cells Expressing RAD18**

Cells were infected with viral particles generated by transfecting the Platinum-E ectopic packaging cell line (Cell Biolabs) with retroviral vectors (psLP-pure) encoding RAD18 variants (WT, C26F) using Lipofectamine (Invitrogen). The viral supernatant was collected to infect cells. Forty-eight hours post-infection, cells were selected in medium containing puromycin (2.5 μg/ml, Sigma). Selected populations were expanded and used promptly.

**Cell Viability Experiments**

Cells were plated at 1.0 × 10^5 cells/well in 12-well plates and UV-irradiated or exposed to the indicated concentrations of ADAM. At 48 hr post-irradiation, cell viability was determined using the CellTiter-Glo luminescent cell viability assay (Promega).

**siRNA**

U87 cells were co-transfected with small interfering RNA (siRNA) using JetPrime reagent (POLYplus). Twenty-four hours after transfection, cells were trypsinized and seeded in 12-well plates at a density of 10^5 cells/well. Twenty-four hours later, cells were treated with cisplatin (Sigma).

**Statistical Methods**

Unless stated otherwise, data are presented as mean values ± SD of three independent experiments. For the data shown in Figures 1E, 5F, and 4B and Figures S2B, S3E, and S4B, unpaired, two-tailed t-tests were performed. p values are represented as **(p < 0.001), *p < 0.01, p < 0.05.** All statistical analyses were calculated using GraphPad Prism 6 software. Significance was assumed when p < 0.05.

**ACCESSION NUMBERS**

The accession number for the Xenopus laevis Rad18 cDNA reported in this paper is GenBank: HF586915.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.06.002.

**AUTHOR CONTRIBUTIONS**


**ACKNOWLEDGMENTS**

We thank M. Michal for encouragement, J. Moreau for help with microscopy, M. Larrens for RAD6-RAD18 purification, P. Coulombe for help with stable cell lines, A. Elosua for technical assistance, K. Cimprich for XRAD18 cDNA, P. Kenmochi for EdFP-POly, C. Vaziri for RAD18a, C. Mashtarin for PCNA-ubiquitin, M. Akiyama (KPOLy), A. Kamapati and W. Dunphy (KORF1), and J. Huang (PRIMPIC) for antibodies, P.H. Nasheuer for the gift of recombinant POly, and J. Sale and J. Hutchins for critical reading of the manuscript. Grants supporting this work were from ARC (3165), FRM (DIEQ200712010548), Ligue contre le Cancer (to C.K.), and ANR (CHECKDEV).

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Supplemental Information
RAD18 Is a Maternal Limiting Factor Silencing the UV-Dependent DNA Damage Checkpoint in Xenopus Embryos
Chames Kermi, Susana Prieto, Siem van der Laan, Nikolay Tsanov, Bénédicte Recolin, Emmanuelle Uro-Coste, Marie-Bernadette Delisle, and Domenico Maiorano
SUPPLEMENTAL INFORMATION

Supplemental Figures legend.

Figure S1, related to Figure 1

(A) DNA synthesis, monitored by incorporation of a nucleotide precursor, of egg extracts supplemented with sperm nuclei at low N/C ratio in the presence (+) or absence (-) of UV-irradiated chromatin (300 J/m²).

(B) Cycling egg extracts were incubated at room temperature for the indicated times in the absence or presence (C) of cycloheximide and analysed by western blot to detect the phosphorylated forms of MCM4 (arrows), a CDK1 substrate (Hendrickson et al., 1996). UV irradiation did not induced mitotic delay, similar to the non-irradiated control (right panel). In contrast, extracts supplemented with sperm nuclei at high N/C ratio were strongly delayed in interphase (middle panel). No MCM4 phosphorylation was observed in the presence of cycloheximide at both low and high N/C ratio (C) as expected (Maiorano et al., 2004), due to inhibition of CYCLIN B synthesis, indicating failure to enter mitosis.

(D) Checkpoint suppression by decreasing the N/C ratio. Equal amounts of sperm nuclei (1000 nuclei/µl) UV-irradiated (lanes 1-4) or not (lane 5) were incubated at room temperature for sixty minutes with increasing volumes of egg extracts (dilution factors X3, X6, X9 respectively for lanes 2-4 compared to lane 1). Nuclei were then isolated and CHK1 phosphorylation analyzed by western blot with a specific antibody. CHK1 serves here as loading control.
(E) Aphidicolin induces replication fork uncoupling at low N/C ratio. Egg extracts were supplemented with sperm nuclei at low N/C ratio in the presence (+) or absence (-) of 100 μg/ml of aphidicolin. Chromatin fractions were isolated and analysed by western blot with the indicated antibodies.

(F) Association of replicative DNA polymerases α and δ catalytic subunits with chromatin at low N/C ratio. Western blot of chromatin fractions obtained at low N/C ratio and *Xenopus* egg extracts (LSS) with the indicated antibodies.

(G) Quantification of POL帮我 binding to chromatin at low N/C ratio compared to recombinant POL帮我 and POLα (Rec). Mol: number of molecules per replication unit.

(H) Chromatin binding of POL帮我 at low N/C ratio is inhibited by GEMININ. Chromatin binding was analysed as in (F) in the absence (-) or presence (+) of 100 nM of recombinant GEMININ. ORC1 was used in this experiment as a chromatin loading control.

(I) Comparison of abundance of the indicated proteins (as determined by western blot) remaining in the egg cytoplasm after incubation with sperm chromatin at low or high N/C ratio. Egg extracts were supplemented with either 100 (low N/C ratio) or 1000 (high N/C ratio) nuclei/μl and incubated at room temperature for ninety minutes.

**Figure S2, related to Figure 2**

(A) Western blot of *Xenopus* egg extracts probed with either pre-immune (PI), RAD18 or RAD6 anti-serum. kDa indicates molecular weight standards.

(B) Effect of MG132 on RAD18 stability in vitro. (Upper panel) Titration of RAD18 onto chromatin by increasing the N/C ratio (nuclei/μl of egg extract) in the absence (DMSO) or presence of MG132. Western blot of chromatin sample taken at 70
minutes after incubation at room temperature and isolated as described in Experimental Procedures. Lanes 1-6 and 7-12 were spliced together from the same blot. Quantification is shown. Means and standard deviation of two independent experiments are shown. (Lower panel) Dynamics of RAD18 chromatin binding as described above. Quantification is shown. Means and standard deviation of two independent experiments are shown.

(C) Quantification of RAD18 stored in the *Xenopus* egg. Western blot of samples of recombinant XRAD18 (lanes 1-7) and 1 µl of *Xenopus* egg extract (~ 25 µg of total proteins, corresponding to about 2 embryos assuming that one egg yields about 0.5 µl of extract) with the anti-RAD18 antibody. Ng indicates nanograms of recombinant RAD18. Western blot signals were quantified using the Image J software and expressed in the graph as relative optical density (ROD).

(D) Immunoprecipitation of RAD18 co-precipitates the RAD6 protein. Egg extracts were incubated with either mock or RAD18-specific antibodies for 1 hour at 4 °C and immunoprecipitates were analysed by western blot with the indicated antibodies.

(E) RAD18 depletion (∆RAD18), compared to control depletion (∆Mock), does not stimulate UV-dependent CHK1 phosphorylation at high N/C ratio. Analysis of CHK1 Thr344 phosphorylation at high N/C ratio of the experiment described in Figure 2D.

(F) Western blot of chromatin fractions isolated from *Xenopus* egg extracts incubated with sperm chromatin at low N/C ratio, after treatment with non-specific (∆Mock) or RAD18-specific (∆RAD18) antibodies.

(G) The Rad6-Rad18 recombinant complex rescues defective PCNAmutb of RAD18-depleted egg extracts. Egg extracts depleted of RAD18 (∆RAD18) were reconstituted (+) or not (-) with increasing amounts of a His6-RAD6-RAD18 recombinant (Rec)
complex (corresponding to + and ++ in the panel legend), or recombinant His<sub>6</sub>-RAD6, and sperm chromatin at high N/C ratio. PCNA<sup>UB</sup> was determined by western blot on chromatin fractions isolated after incubation at room temperature for 90 minutes.

**H** PCNA<sup>K164R</sup> induces CHK1<sup>5344</sup> phosphorylation upon UV irradiation in *Xenopus* egg extracts at low N/C ratio. Analysis of CHK1 phosphorylation in egg extracts supplemented with either wild-type (WT) PCNA or PCNA mutated in the lysine 164 residue (K164R) and sperm nuclei at low N/C ratio.

**I** PRIMPOL does not bind to chromatin at low N/C ratio in *Xenopus* egg extracts. Western blot of chromatin fractions isolated from *Xenopus* egg extracts incubated with sperm chromatin at high or low N/C ratio and analysed with either PCNA or PRIMPOL antibodies.

**J** Cell cycle duration of live embryos injected with the indicated mRNA at the 2-cell stage, UV-irradiated (+UV) or not (-UV) at stage 3. Data are represented as scatter dot plot. Time points and medians are represented. Fifteen individual embryos were followed through early divisions until 300 min after the first cleavage. Each time point corresponds to the cleavage of an individual cell from embryos.

**K** RAD18 overexpression suppresses UV-dependent CHK1 phosphorylation in *Xenopus* embryos. Western blot of embryos protein extracts (from Figure 2F; see Experimental Procedures) obtained from embryos injected with the RAD18 mRNA or water (H<sub>2</sub>O) and probed with the indicated antibodies.
Figure S3, related to Figure 3

(A) Cell cycle profile of cell overexpressing RAD18. FACS analysis of HEK293T cells expressing either the empty vector (EV) or RAD18. The percentage of cells in each cell cycle phase is shown. 2N and 4N indicate DNA content.

(B) RAD18 and not RAD6 stimulates PCNA^{mutB} in mammalian cells. Western blot of total cell extracts of HEK293T cells expressing the indicated constructs described in Figure 3B and analysed with PCNA antibodies.

(C, left panel) HEK293T cells were co-transfected with HsRAD18 wild-type or Rad18 missing the CDC7 phosphorylation sites (Δ401-445 mutant) and a vector expressing the eGFP-POL{\textsubscript{11}}. Twenty-four hours later cell were UV-irradiated and processed for immunofluorescence microscopy as described in Experimental Procedures. Scale bar: 10\,\mu m. (Right panel) Quantification of eGFP-POL{\textsubscript{11}} foci from the experiment described in panel C. Means and standard deviation of three independent experiments are shown.

(D) Determination of CHK1\^{S345} phosphorylation in HEK293T cells expressing empty vector, RAD18\textsuperscript{WT} or Rad18\textsuperscript{Δ401-445}, in the absence (-) or presence (+) of UV irradiation. Samples were analysed 240 minutes post-UV irradiation. The first four lanes were spliced together with the last two lanes from the same blot.

(E) HEK293T cells were co-transfected with empty vector, HsRAD18\textsuperscript{WT} or Rad18\textsuperscript{C29F} mutant and a vector expressing the eGFP-POL{\textsubscript{11}}. Twenty-four hours later cell were UV-irradiated and processed for immunofluorescence microscopy as described in Experimental Procedures. Scale bar: 10\,\mu m. Quantification shows means and standard deviation of three independent experiments (*\,p<0.01).
Figure S4, related to Figure 4

(A) Expression of RAD18 in HCT116 and HCT8 colorectal cancer cell lines respectively resistant or sensitive to oxaliplatin treatment (Balin-Gauthier et al., 2008). Asterisk indicates a non-specific cross-reacting polypeptide.

(B) Expression of DNA damage response proteins in glioblastoma. Western blot of total extracts obtained from glioblastoma biopsies (Glioblastoma, grade 4) or differentiated counterparts (progenitors, CD133-) or HeLa cells.
Supplemental Experimental Procedures

Cloning procedures and plasmids

A *X. tropicalis* RAD18 homologue (Genbank EST: AL881643) was identified in the databank by performing a BLAST search using human and mouse RAD18 proteins. Two oligonucleotides specific of XtrRAD18 were synthesized, XtrR18F (5′-GGAATTCCGTTCAAATGTATAATGCTCA-3′) and XtrR18R (5′-CTGAGCATTATACATTTTGAACGAATTCC-3′) containing a synthetic EcoRI restriction site (underlined), and used as primers in PCR reactions with 5′ or 3′ primers specific of a *X. laevis* ovary cDNA library made in lambda gt10 vector (Rebagliati et al., 1985). PCR products were blunt-end ligated to pRSET expression vector (Invitrogen) to generate XI{Rad18}^{Mor} or XI{Rad18}^{Cher}. Recombinant plasmids were sequenced on both strands. Full-length *X. laevis* RAD18 was kindly provided by K. Cimprich (Stanford University, USA). The sequence of the *X. laevis* RAD18 gene has been deposited to the EMBL genebank (accession number CCQ71719.2). The PCNA His{e}-K164R mutant was generated as previously described (Chang et al., 2006). The *Xenopus* RAD6 gene was obtained from NIBB *Xenopus* cDNA Resource (NIB, Japan). Full length XRAD6 cDNA was amplified by PCR using 5′ (5′-CCCCTCGAGTTAGGAATCATTTCAACTTGCTC-3′) and 3′ (5′-CCGGATCCATGTCACCACCC-3′)-specific primers containing a synthetic BamHI or XhoI restriction site (underlined). The PCR product was cloned into the pFastBacHtb vector (Pharmingen) digested with the same restriction enzymes to obtain the recombinant plasmid pFastBacHis{e}XRAD6. The cDNA was sequenced on both strands. *Xenopus* full-length RAD18 was cloned into the
pFastBac1 vector as the Xhol-BamHI DNA fragment. The recombinant plasmid pFastBacXRAD18 was sequenced on both strands. The HsRad18<sup>Δ401-445</sup> and Rad18<sup>Δ200F</sup> mutants were previously described (Huang et al., 2009; Watanabe et al., 2004). The plasmid expressing Pcnα(K164R)-Ubiquitin fusion was previously described (Kanao et al., 2015).

Expression of recombinant proteins
His<sub>6</sub>-XIRAD18<sup>Cter</sup> was expressed in the E. coli strain BL21 Star (DE3). Cells were grown at 37 °C over night and diluted 100-fold into fresh LB until OD<sub>600</sub> reaches 0.6. Then cells were left to shake at room temperature for 30 minutes and the expression of the recombinant protein was induced by addition of 0.5 mM IPTG. Cultures were left to shake for 3 hours at room temperature and harvested by centrifugation. The His<sub>6</sub>Pcnα<sup>K164R</sup> mutant was expressed and purified as previously described (Chang et al., 2006). A recombinant His<sub>6</sub>-RAD6-RAD18 complex was expressed in baculovirus-infected cells and purified to homogeneity on a Nickel column as previously described (Watanabe et al., 2004), followed by gel filtration. Recombinant GEMININ was expressed in bacteria and purified as previously described (Tada et al., 2001).

Antibodies
XIRAD18 antibodies were raised against His<sub>6</sub>-XIRad18<sup>Cter</sup> (amino acids 243-496) expressed and purified in bacteria by nickel affinity chromatography (Qiagen). Crude serum was also affinity-purified by affinity chromatography using the same antigen used to immunize rabbits coupled to Sepharose by standard procedures. RPA2 antibodies were previously described (Cuvier et al., 2006). XPOL<sub>11</sub> antibodies were
raised against full recombinant protein expressed in bacteria as previously described (Yagi et al., 2005). RAD6 antibodies were generated by injection of rabbit with recombinant, baculovirus-expressed *Xenopus* RAD6. The following antibodies were also used: human phospho-CHK1 (Ser\(^{345}\), Cell Signaling; 2341; recognizes S\(^{344}\) XCHK1); CHK1 (G-4, sc-8408, Santa Cruz biotechnology); H3 (ab1791, AbCam); PCNA (PC10, Sigma); MCM4 (Coue et al., 1998); MCM2 (ab4461, AbCam), β-tubulin (T3526, Sigma), USP1 (14346-1-AP, Proteintech); DRF1 (Yanow et al., 2003), PRIMPOL (Wan et al., 2013). ATRIP was a kind gift of K. Cimprich (Stanford University, USA). ATR antibodies were raised as previously described (Hekmat-Nejad et al., 2000). ORC1 antibody was a gift of M. Mêchali.

**Immunodepletion procedures**

RAD18 was removed from egg extracts by two rounds of depletion with affinity-purified RAD18 antibodies coupled to DynaBeads (Invitrogen). This procedure allows minimal dilution of the extracts during the depletion procedure avoiding spontaneous checkpoint activation likely due to dilution of the RAD6-RAD18 complex. Egg supernatants were thawed and supplemented with cycloheximide on ice and beads were added to the extract in a 50% ratio (V:V). For immunoprecipitations, extracts were diluted ten-fold in XB buffer supplemented with protease inhibitors and incubated with Rad18 antibody for 1 hour at 4°C. Immunocomplexes were collected with Protein A sepharose, washed in XB buffer and neutralized in Laemmli buffer.
RNA extraction, reverse transcription and quantitative real-time PCR

Total RNA was isolated with TRizol reagent (Invitrogen). Reverse transcription was carried out using random hexanucleotides (Sigma) and Superscript II First-Strand cDNA synthesis kit (Invitrogen). Quantitative PCR reactions were performed using Lightcycler SYBR Green I Master mix (Roche) on Lightcycler apparatus (Roche). All primers used were intron spanning and to ensure specificity melt-curve analysis were carried out at the end of all PCR reactions (primer sequences available upon request). The relative amount of target cDNA was obtained by normalisation using geometric averaging of an internal control gene (HPRT, Hypoxanthine-Guanine Phosphoribosyl Transferase).

Patients and tumour samples.

Tumour sample were obtained from patients diagnosed for type IV grade glioma (i.e. glioblastoma) and undergoing surgery at the neurosurgery department of the Rangueil Hospital (Toulouse, France). All subjects provided their informed written consent before their surgery and the protocol followed the declaration of Helsinki guidelines and was approved by local ethics committee.

Supplemental References


Chapter III
Discussion
III. Discussion

1. PIP degron proteins, substrates of CRL4\(^{Cdt2}\), and not PIP boxes, interfere with DNA polymerase \(\eta\) and \(\kappa\) focus formation on UV damage

During an unperturbed cell cycle, the E3 ubiquitin ligase CRL4\(^{Cdt2}\) plays an important role in preventing DNA re-replication thus preserving the genome stability by targeting for proteasomal degradation different cell cycle-regulated proteins, including the licensing factor CDT1, and the mammalian methyltransferase PR-Set7/Set8 (Arias and Walter, 2006; Ralph et al., 2006; Tardat et al., 2010; Zhong et al., 2003). Moreover, CRL4\(^{Cdt2}\) has also been implicated in the regulation of TLS independently from the ubiquitin-proteasome system, through catalysing PCNA\(^{monoUb}\) at Lys164 in mammalian cells (Terai et al., 2010). In this work, we have shown that, activation of the CRL4\(^{Cdt2}\) degradation pathway following DNA damage, facilitates the access of translesion DNA polymerases to PCNA, in a PCNA\(^{monoUb}\) -independent fashion. This function of CRL4\(^{Cdt2}\) implicates the degradation of substrates that bind PCNA with high affinity, including the licensing factor CDT1. CRL4\(^{Cdt2}\) clears out PIP degron-containing proteins from PCNA, thus enhancing the recruitment of the translesion polymerases Pol\(\eta\) and Pol\(\kappa\) to chromatin-bound PCNA.

1.1. CRL4\(^{Cdt2}\) regulates Pol \(\eta\) recruitment to DNA damage sites

Our results suggest that CDT1\(^{R+4A}\) competes out Pol \(\eta\) and Pol \(\kappa\) for PCNA binding. However the inhibitory effect is partial and not fully consistent with the complete inhibition observed when the PIP box of Pol \(\eta\) is mutated (Bienko et al., 2010). This could be explained by the fact that in this experiment the degradation of only a single CRL4\(^{Cdt2}\) substrate is impaired (CDT1), whereas when the function of CRL4\(^{Cdt2}\) is impaired all its substrates are stabilised, and therefore a stronger defect in formation of GFP-Pol \(\eta\) foci is observed. To fully test this hypothesis, it would be interesting to mutate other PIP degrons (such as p21 and Set8) and analyse the extent of TLS polymerases focus formation. In addition, CDT1\(^{R+4A}\) may not be very stable in S-phase, when GFP-Pol \(\eta\) foci mostly form. In fact, CDT1 is also targeted for
proteasomal degradation during S-phase by the E3 ubiquitin ligase SCF, independently from CRL4Cdt2 (Nishitani et al., 2006). To test this possibility, an additional mutation in the cyclin-binding motif of CDT1, responsible for its phosphorylation by CDK2-cyclin A, and subsequent SCF-mediated polyubiquitination and proteasomal degradation (Sugimoto et al., 2004), may increase the capacity of CDT1R4A to impede Pol η localisation to replication factories.

1.2. Possible role of CRL4Cdt2-dependent degradation in DNA repair

Our work also suggest that TLS may also occur outside of S-phase. This is in line with several emerging evidences (Daigaku et al., 2010; Diamant et al., 2012; Karras and Jentsch, 2011; Soria et al., 2009) suggesting that TLS polymerases are involved in DNA repair. For instance, Pol κ has been proposed to function in NER in G1 as well as in quiescent cells (Ogi and Lehmann, 2006; Ogi et al., 2010). Moreover, after oxidative stress in G1-phase, PCNA is monoubiquitinated and Pol η recruited in a MMR machinery-dependent manner (Zlatanou et al., 2011). These studies and the present work suggest that TLS polymerases can also function in G1 in a way coupled to DNA repair. Our data show that CRL4Cdt2 pathway is important for removing PIP degron proteins after UV damage, not only in S-phase but also in G1-phase, thus facilitating Polκ recruitment. Activation of CRL4Cdt2-mediated proteolysis may be a general mechanism that cells use to facilitate the interplay of specific repair factors on chromatin-bound PCNA after DNA damage, and be relevant to DNA repair by facilitating partners switch.

1.3. Hierarchical interactions with PCNA

Recruitment of PCNA partners is hierarchical due to important variations in the binding affinities of different PIP box motifs (Moldovan et al., 2007). Our work support the idea that the small variations in the sequence of a PIP box may have dramatic effects on its ability to compete out Pol η from replication factories. We have also shown that PIP degron bearing proteins have a higher potential to inhibit GFP-Pol η foci formation than canonical PIP boxes. For instance, we demonstrated that the T5 residue within its PIP degron confers high PCNA-binding affinity and high potential to compete out Polη from replication factories. The T5 is highly conserved in substrates of CRL4Cdt2 that compete out Pol η (Havens and Walter, 2011;
Moldovan et al., 2007). However, it will be interesting to mutate T5 also in p21 and PR-Set7/Set8 and to assess the effect on GFP-Pol η foci formation. This could explain the need to destroy PIP degron-containing proteins during S-phase and/or after DNA damage in order to increase the chances of Y-family polymerases to gain access to chromatin-bound PCNA.

Since an extensive comparison of the binding affinities of all PCNA partners does not exist, in collaboration with J. Delgado and L. Serrano at the CRG of Barcelona (Spain), we modelled the PIP box/PIP degrons of several PCNA partners into the hydrophobic pocket of PCNA, and calculated the corresponding interaction free energies (ΔGint) of each complex (Table 1). The obtained thermodynamic dissociation constants (KD) reveal roughly three hierarchical categories of clamp-binging peptides: I) PIP degrons, characterized by strong PCNA-interaction, II) canonical PIP boxes that have moderate affinity for PCNA, and III) non-canonical PIP box motifs that have suboptimal PCNA-binding affinity. The differences in interaction-affinity can be explained in terms of structure.

The human DNA helicase FBH1 was also proposed to contain a PIP degron. However, unlike other CRL4Cdt2 substrates, FBH1 is degraded mainly in response to DNA damage though not very efficiently in S-phase (Bacquin et al., 2013). This might be due to the non-canonical sequence of its PIP degron that lacks the TD motif. In addition to the PIP degron, FBH1 also interacts with PCNA through an APIM peptide and can impair the recruitment of Pol η at sites of DNA synthesis in a manner dependent upon both interaction motifs (Bacquin et al., 2013). The APIM peptide probably compensates for the lack of a TD motif in the PIP degron of FBH1 by increasing its interaction-affinity for PCNA and allowing competition with Pol η. The function of FBH1 is still not completely understood, but is believed to regulate HR by resolving recombination intermediates (Chiolo et al., 2007; Fugger et al., 2009). Because Pol η also functions in HR by extending the invading strand in recombination intermediates in a reaction that also involves PCNA (Kawamoto et al., 2005; McIlwraith et al., 2005; Sebesta et al., 2013; Sneeden et al., 2013), it is not clear if competitive binding of FBH1 to PCNA interferes with TLS or HR. Besides DSBs repair, the HR pathway is also required for the recovery of collapsed forks (Carr and Lambert, 2013). It is then tempting to speculate that CRL4Cdt2-mediated removal of FBH1 might regulate both TLS and the repair of broken forks, by limiting the anti-recombinase activity of FBH1 and at the same time promoting the interaction of Pol η with PCNA.
The small p12 subunit of the human heterotetrameric Pol δ is also degraded in response to DNA damage in a PIP degron- and CRL4<sup>Cdt2</sup>-dependent manner in mammalian cells (Terai et al., 2013; Zhang et al., 2013). Interestingly, p12 was shown to regulate the fidelity of DNA synthesis by modulating the 3' → 5' proofreading exonuclease activity of Pol δ and its capacity to extend mismatched primers (Meng et al., 2010; Meng et al., 2009). In the absence of p12, the capacity of Pol δ to synthesize DNA across 8-oxoG and other non-blocking lesions is reduced and its proofreading activity is largely increased. This raises the interesting possibility that the destruction of p12 can reduce the TLS activity of Pol δ, preventing replication error when cells encounter DNA damage.

<table>
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</tr>
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Table 1. Hierarchical classification of PCNA-interacting motifs based upon the dissociation constants and free Gibbs PIP/PCNA binding energies (Tsanov et al., 2015).

PIP boxes of different PCNA substrates were locally aligned against the sequence of the wild-type peptide (p21 C-terminal region) bound to Human PCNA (pdbId: 1AXC, X-ray structure, 2.6Å resolution), then side chains of the substrates were modelled over the wild-type peptide (backbone and C-β) coordinates using Build Model FoldX command. For the resulting PCNA/PIP complexes, Gibbs interaction relative free energies were calculated by Analyse Complex FoldX command. Ranking of the interaction energies resulted in three major groups: I) Strong II) Moderate III) Low for non-canonical PIP boxes (as those found in Y-family DNA polymerases).
1.4. Consequences of disrupting the natural equilibrium of PCNA-partner interactions

The present work and a previous study on p21 (Soria et al., 2008), suggest that there is a pre-established competition between PIP box-containing proteins that governs the access of low-fidelity Y-family DNA polymerases to the sliding clamp PCNA based on relative affinities. At least two distinct mechanisms are involved in regulating the equilibrium between these interactions, including PCNA\textsuperscript{monoUb}, and CRL4\textsuperscript{Cdt2}-mediated degradation of PCNA partners. Disrupting PCNA-partners interactions might have an effect on the function of TLS polymerases. For instance, loss of p21 results in increased rates of error-prone TLS across benzo[a]pyrene and cisplatin adducts, indicating that a naturally occurring competition between p21 and translesion polymerases may limit error-prone TLS activity (Avkin et al., 2006). Interestingly, expression of a PIP box mutant p21 does not reduce error-prone TLS, confirming the PIP box-mediated competition. Furthermore, a mutant of PCNA that displays high binding-affinity for FEN1 (Rad27) was isolated in budding yeast and showed high rates of spontaneous mutation (Fridman et al., 2010). This phenotype was reversed by the deletion of the catalytic subunit of the error-prone translesion polymerase Pol ζ (REV3), suggesting that Polζ replaces Polη when FEN1 competes out its PCNA binding.

The effect of PCNA-partners interactions disruption can be addressed by measuring TLS efficiency and fidelity in cells expressing non degradable CDT1\textsuperscript{R+4A} mutant, using a TLS assay.

1.5. Perspectives and Future directions

Amongst CRL4\textsuperscript{Cdt2} substrates, the p21 PIP degron appears to have the strongest affinity for PCNA. We have shown that overexpression of the p21 PIP degron on its own strongly interferes with TLS Polη foci formation. Hence, in view of the wide implication of PCNA in DNA repair pathways, the generation of a stable cell line conditionally expressing the PIP degron of p21 (tetON-tetOFF, for instance) may constitute a valuable tool to study the dynamics and the molecular basis of TLS polymerases foci formation and other DNA repair mechanisms. CDT1 overexpression impairs the assembly of UV-induced GFP-Pol η foci, in a PIP box dependent manner. In addition to Pol η, Pol κ and Pol ι also rely on a PIP box for direct
interaction with PCNA. Our data show that cells overexpressing CDT1 display impaired GFP-Pol-κ foci formation. As CDT1 is an oncogene overexpressed in different cancers, the function of Pol-η, Pol-κ, and Pol-λ in TLS may be impaired in these cancers. In this situation, error-prone Pol-ζ in combination with REV1 will probably replace the impaired TLS polymerases. Pol-ζ was associated with the development of secondary malignancies and the resistance to cisplatin chemotherapy, in addition to increased cisplatin-induced mutagenesis (Doles et al., 2010). Thus, high levels of CDT1 could result into a negative outcome upon treatment with cisplatin or other platinum-based drugs. For all these reasons, assessing TLS efficiency and fidelity in cancer cell lines displaying high levels of CDT1, and the characterisation of its impact on tumour resistance is an important objective to pursue.

2. “RAD18 Is a Maternal Limiting Factor Silencing the UV-Dependent DNA Damage Checkpoint in Xenopus Embryos”

2.1. RAD18 silences the DNA damage checkpoint in early Xenopus embryos by reducing replication fork uncoupling

The results presented in this work indicate that in Xenopus extracts constitutive PCNA monoUb driven by high RAD6-RAD18 abundance is responsible for silencing the UV-dependent DNA damage checkpoint. In C. elegans, Pol-η was previously proposed as a repressor of the checkpoint based on genetic evidence (Holway et al., 2006; Ohkumo et al., 2006; Roerink et al., 2012a). Although we find that Pol-η is implicated, this polymerase is not limiting in Xenopus since it is not quantitatively depleted from the egg extracts at high N/C ratio. Active bypass of DNA lesions induced by constitutive Pol-η recruitment represses replication fork uncoupling and may avoid fork stalling in front of UV lesions, thus inhibiting the trigger of checkpoint response and consequent CHK1 phosphorylation. In line with this possibility, RAD51 foci formation is reduced in early C. elegans embryos (Holway et al., 2006). Moreover, recent data show that ssDNA formation at stalled forks is also repressed in human embryonic stem cells (Desmarais et al., 2012) consistent with an unexpected chromosomal instability of the early embryo genome (Vanneste et al., 2009), and suggesting a possible conservation of function. Constitutive TLS activity may facilitate bypass of DNA lesions during replication. Alternatively, this process may occur at lesions left behind forks uncoupled from DNA synthesis (Daigaku et al., 2010; Karras and Jentsch, 2011; Lopes et al., 2006). Our data suggest
that lesions are not actively bypassed by the recently discovered primase-polymerase Primpol (Garcia-Gomez et al., 2013; Im et al., 2013; Wan et al., 2013), as we did not see Primpol chromatin binding at low N/C ratio. This regulation may be important to tolerate not only external damage but also endogenous damage and replication stress generated, for instance by high concentration of nucleotides present in the early embryo. In yeast, there is evidence that TLS activity is required for replication resumption upon replication stress induced by rNTPs incorporation (Lazzaro et al., 2012a).

DNA damage checkpoint is also repressed in Xenopus embryos after γ-irradiation (Conn et al., 2004). Since RAD18 is also implicated in the repair of double strand breaks (Huang et al., 2009; Szuts et al., 2006; Watanabe et al., 2009), it would be interesting to determine its contribution to this checkpoint inactivation.

2.2. The role of RAD6 in this regulation

Our observations show that RAD18 overexpression is sufficient to induce constitutive PCNA_{monoUb} independently of RAD6, in line with a recent report (Zeman et al., 2014), although we could not formally prove it in Xenopus, since we failed to express active, recombinant RAD18 without RAD6. When overexpressed RAD18 can either bypass the requirement of RAD6, or RAD18 could use another abundant E2 to catalyse PCNA_{monoUb}. This may explain why in our experiments overexpressing RAD18 on its own is sufficient to induce PCNA_{monoUb}, whereas RAD6 overexpression does not give the same phenotype. Consistent with this, mutations in RAD6 in C. elegans did not delay mitotic entry in early embryos (Holway et al., 2006). Surprisingly, when RAD6 and RAD18 were co-expressed in HEK293T cells, we observed neither GFP-Pol η foci formation nor constitutive PCNA_{monoUb}. A possible explanation is that, when overexpressed, RAD6 promotes the dimerization of RAD18, inducing its auto-ubiquitination and inactivation as the ubiquitinated form of RAD18 is mainly cytoplasmic (Zeman et al., 2014). In this work, the authors have observed that RAD6 overexpression reduces GFP-RAD18 foci formation and strongly abolish PCNA_{monoUb} upon UV or MMS treatment (Zeman et al., 2014). It would be interesting to repeat this experiment using a RAD18 mutant that cannot be auto-ubiquitinated (C207F) co-expressed with RAD6. Also, one can speculate that the low level of RAD6 that we have observed in glioma sample in
comparison with progenitors or Hela cells (Figure 4E in the article), might favours RAD18 activity in these cells. This possibility has to be experimentally investigated by knocking-down RAD6 in cells overexpressing RAD18.

2.3. RAD18 interacts with Dfr1 only in early embryos

Recent data propose that the DNA damage checkpoint affects TLS, through regulation of a RAD18-CDC7-DBF4 complex (Yamada et al., 2013a). Our observations show that deregulation of TLS affects the function of the DNA damage checkpoint, suggesting a cross-talk between these two pathways. We have shown that high RAD18 abundance at low N/C ratio in *Xenopus* eggs promotes complex formation with the DBF4-related protein DRF1 in early *Xenopus* embryos, to constitutively target RAD18 to replication forks, while in somatic cells the abundance of this complex increases only after DNA damage (Yamada et al., 2013a). Moreover, we have also observed that ectopic expression of a RAD18 mutant that cannot be phosphorylated by CDC7 (RAD18Δ401-445) does not suppress CHK1 phosphorylation upon UV damage, and does not induce spontaneous GFP-Pol η nuclear foci in mammalian cells (Figures S3C, S3D in the article). Although the abundance of DRF1 has been reported to be developmentally-regulated (Collart et al., 2013; Takahashi and Walter, 2005b), titration of DRF1 and three others replication factors (Cut5, RecQ4 and TRESLIN) in egg extracts occurs at N/C ratio higher than that observed for onset of the DNA damage checkpoint ((Chang et al., 2006; Collart et al., 2013; Dasso and Newport, 1990; Kappas et al., 2000) and this work). Of note, we did not observe destabilization of DRF1 in *Xenopus* embryos close to MBT, in contrast to what previously reported (Collart et al., 2013). This suggests that titration of RAD18 and not DRF1 may be responsible for checkpoint activation, and in this respect, RAD18 may be more limiting than DRF1. Consistent with this possibility, while DRF1 titration coincides with spontaneous developmental activation of CHK1 (Collart et al., 2013), we observed DNA damage-dependent checkpoint activation at an N/C ratio that results in titration of RAD18. Importantly, upon expression of wild-type RAD18 or a catalytic inactive mutant (RAD18C28F) in *Xenopus* embryos, we did not observe CHK1 phosphorylation in the absence of external damage (Figure S2K in the article). This result clearly suggests that RAD18 is not involved in the spontaneous developmental activation of the checkpoint.
2.4. RAD18 might be involved in the resistance to therapy of cancers

Translesion synthesis has been found upregulated in a number of cancer or cancer-associated diseases (Albertella et al., 2005). This has been explained as enhanced repair capacity and increased mutation rate intrinsic to the error-prone nature of TLS. Our results provide evidence for how unscheduled onset of TLS driven by deregulated RAD18 expression may also affect the function of the DNA damage checkpoint and constitute a mechanism by which damaged cells can override cell cycle delay and/or apoptosis and be positively selected for anarchic proliferation. These observations suggest that deregulated RAD18 expression might have a previously unrecognized oncogenic potential relevant to the observed resistance to the therapy of subtypes of cancers, such as those of embryonic origin or those generated by dedifferentiation of somatic cells.

RAD18 is involved in the resistance to therapy of different cancers. In addition to glioblastoma (Xie et al., 2014a; and the present work), elevated RAD18 expression has been found in primary and metastatic melanomas and correlated with bad prognosis (Wong et al., 2012). In this cancer, RAD18 knock-down reduces the expression of the proliferative proteins pAkt and Cyclin D1 (Wong et al., 2012), suggesting that the rapid cell proliferation in melanoma is sustained by high RAD18 levels. Moreover, significantly high mRNA expression of RAD18, Pol ξ, Pol ι and Pol κ was found in oesophageal carcinomas in comparison with normal oesophageal tissue (Zhou et al., 2012). Recent work has shown that RAD18 is highly expressed in human 5-fluorouracil (5-FU)-resistant colorectal cancer cells after 5-FU treatment (Liu et al., 2015), and that the tumour suppressor miR-145 directly targets RAD18 and suppresses the drug resistance of colorectal cancer cells (Liu et al., 2015). In addition, a Single Nucleotide Polymorphism (SNP) at Arg302Gln in the RAD18 gene is associated with high risk of colorectal cancer (Kanzaki et al., 2007). Another study reported a correlation between the same RAD18 SNP and non-small-cell lung cancer (Kanzaki et al., 2008), and siRNA-mediated RAD18 silencing potentiate the effect of cisplatin on these cells (Chen et al., 2016). Strikingly, a recent work suggested a tumour-suppressive role of RAD18 in the case of B-cells malignancies (Yang et al., 2016). Upon treatment with the myelosuppressive carcinogen DMBA (7,12-Dimethylbenz[a]anthracene), Rad18−/− mice showed higher propensity to develop this malignancies than wild type mice (Yang et al., 2016). This is probably due to the
fact that bone marrow multipotent progenitors are more sensitive to DMBA-induced genome instability in $Rad18^{-/-}$ mice, conferring “malignant potential” to subsequent lineages. In light of these observations, RAD18 appears to be as a double edged sword; on one hand when it is expressed at normal levels, it contributes to genomic stability upon DNA damaging conditions and has a tumour-suppressor role. On the other hand, when it is overexpressed, RAD18 promotes tumour progression and resistance to therapy by enhancing tolerance and repair capacities of the cells, and silencing the DNA damage checkpoint.

2.5. Perspectives and Future direction

This work has provided new insights onto how the DNA damage checkpoint is silenced in early embryos. This regulation might be conserved in other organisms, such as in $Drosophila melanogaster$, as demonstrated by the high level of PCNA$_{\text{monoUb}}$ observed during early embryonic stages (Lo Furno, Busseau & Maiorano, unpublished data). Also, in $C. elegans$ as previously suggested (Holway et al., 2006) and in mammalian cells (Desmarais et al., 2012). Intriguingly, a RAD18 orthologue cannot be identified in $Drosophila$ based on sequence homology. It would be interesting to identify the E3 ubiquitin ligase(s) responsible for PCNA$_{\text{monoUb}}$ in $Drosophila melanogaster$. Recently, a RING domain-containing protein called NOPO has been shown to promote Pol$\zeta$ ubiquitination and its localization to nuclear foci (Wallace et al., 2014). NOPO is the Drosophila homolog of human TNF Receptor associated factor (TRAF)-Interacting Protein (TRIP). Interestingly, whereas orthologues of the TLS polymerases Pol$\eta$, Pol$\iota$ and REV1 have been identified in $Drosophila$, Pol$\kappa$ orthologue is not yet known (Ishikawa et al., 2001).

In our model (see graphical abstract in the article), TLS polymerases, including Pol$\eta$, are constitutively recruited to replication forks allowing an active bypass of the lesions without stalling the replication machinery. This might constitute an adaptation mechanism in order to allow rapid proliferation and hatching of vulnerable embryos in a hostile environment. However, TLS polymerases are not accurate and are therefore expected to introduce mutations, implying that early embryos may accumulate mutations at a higher rate than somatic cells where TLS is regulated. Since the processivity of TLS polymerases is low, and because DNA replication rates are not slower in early embryos compared to somatic cells, it is likely that TLS
polymerases are not involved in bulk DNA synthesis but are only employed to bypass lesions or difficult to replicate DNA sequences that interfere with the progression of replicative polymerases, thus minimizing the mutation load. Moreover, in vitro data suggest that TLS polymerases are accurate when replicating certain kinds of lesions. For instance, Polη is capable of error-free bypass of the major UV-photoproduct CPD in vitro (Johnson et al., 2001). Notwithstanding, whether these features are also observed in vivo is not known. Hence, it would be important to assess the contribution of TLS polymerases to mutagenesis in early embryos. Preliminary data suggest that the mutation load is lower in flies homozygous mutants for the Polη gene in comparison to heterozygous flies, in addition to a reduced hatching rate (Lo Furno, Busseau, Zuchner & Maiorano, unpublished data). Moreover, mutations can be detected in a reporter gene injected into one blastomer of Xenopus embryos (Lo Furno & Maiorano, unpublished data), suggesting that indeed TLS are also mutagenic in vivo in the early embryo. It is then tempting to speculate that constitutive TLS activity in early embryos might constitute a novel mechanism generating genetic variability. Another question is why embryos do not use rather the error-free pathways of DNA damage tolerance, such as recombination-mediated template switch? One possibility is that template switch is a complex process that involves many proteins and thus causes fork pausing for a long period which is not compatible with high replication rates observed in early embryos. Indeed, in our experimental set-up, we could not detect the presence of PCNApolyUb which is the signal that recruits template switch proteins. Interestingly, a recent report has shown that mouse embryonic stem cells use fork reversal to cope with replication stress, and accumulate DNA damage (Ahuja et al., 2016). It would be then interesting to determine the frequency of fork reversal in Xenopus embryos prior and post MBT using electron microscopy-based technics.

RAD18 is overexpressed in few cancers and cancer cell-lines. We have shown that expressing ectopic RAD18 in NIH3T3 cells makes them more resistant to the DNA damaging agents UV and cisplatin (Figures 4B and 4C in the article). Moreover, knocking-down RAD18 in U87 human glioblastoma cell-line is sufficient to sensitise them to cisplatin or to the alkylating agent MMS (this work and Kermi & Maiorano, unpublished data). Similarly, another group has shown that U87 and U251 human glioblastoma cell line are more sensitive to ionizing radiations upon RAD18 knock-down (Xie et al., 2014a). It would be interesting to determine if RAD18 mediates the resistance of therapy of other types of cancer. Recently, the deubiquitinating enzyme USP7 has been shown to be essential for maintaining RAD18 stability.
(Zlatanou et al., 2016). Using a previously described USP7 inhibitor P22077 (Altun et al., 2011), we were able to sensitize U87 cells to cisplatin (Kermi & Maiorano, unpublished data).

Glioblastoma is a cancer characterised by the cancer stem cell population responsible for the recurrence and the resistance to therapy of these tumours. Actually, the best model for studying glioblastoma are the gliospheres which are enriched in cancer stem cells. Unlike serum-cultured adherent cell lines, such as U87 cells, gliospheres share very similar global gene expression and phenotypes with human gliosblastosomas (Lee et al., 2006). Most importantly, when they are injected in the mouse brain they form an infiltrative and migratory tumour mirroring the in vivo features of glioblastoma (Lee et al., 2006). Preliminary data show that reducing RAD18 expression in these cells strongly affect their viability upon cisplatin treatment (Kermi & Maiorano, unpublished data). Moreover, knocking-down RAD18 on its own has already an effect on cell proliferation, suggesting a possible role of RAD18 in the self-renewal of these cells (Kermi & Maiorano, unpublished data). The molecular mechanisms behind RAD18-mediated resistance to therapy need to be clarified. This may involve the role of RAD18 in DNA damage tolerance and repair pathways (see paragraph 3.5.3), as well as in silencing the DNA damage checkpoint which is considered to serve as a barrier to malignant transformation (Halazonetis et al., 2008). For all these reason, I think that the next challenge is to resolve the crystal structure of full length RAD18, and set-up a chemical screen in order to identify specific inhibitors that might be relevant for cancer therapy.
Chapter IV
Additional Materials & Methods
IV. Additional Materials & Methods

1. Xenopus Egg Extract Preparation and Use

All the experiments with *Xenopus* were performed in accordance with current institutional and national regulations approved by the Minister of Research under supervision of the Departmental Direction of Population Protection (DDPP). Interphasic and cycling Xenopus egg extracts were prepared and used as described previously (Murray, 1991; Recolin et al., 2012), snap frozen in liquid nitrogen and stored at −80°C. Upon thawing, extracts were supplemented with cyclohexymide (250 µg/ml) and an energy regeneration system (1 mM ATP, 2 mM MgCl2, 10 mM creatine kinase, 10 mM creatine phosphate). When required egg extracts were supplemented with 100 µg/ml of aphidicolin (Sigma), and/or 5 mM caffeine (Sigma), and/or 600 µM of mitomycin C (Sigma). Sperm nuclei were irradiated at 300 J/m2 of UV-C using a Stratalinker (Stratagene). For experiments at low N/C ratio, sperm nuclei were diluted 10-fold in ice-cold XB buffer (100 mM KCl; 10 mM Hepes-KOH, pH 7.7; 2 mM MgCl2; 0.1 mM CaCl2; 50 mM sucrose, pH 7.7) supplemented with proteases inhibitors (leupeptin, aprotinin and pepstatin, 5 µg/ml each) and added to egg cytoplasm so to obtain a final concentration of 100 nuclei/µl.

1.1. Buffers

**HSB8x** pH=7.6

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total volume</strong></td>
<td>4 L</td>
</tr>
<tr>
<td>NaCl</td>
<td>205.72 g</td>
</tr>
<tr>
<td>KCl</td>
<td>4.76 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>7.9 g</td>
</tr>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td>5.73 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>5.37 g</td>
</tr>
<tr>
<td>Tris</td>
<td>58.14 g</td>
</tr>
<tr>
<td>CH₃COOH</td>
<td>25.2 mL</td>
</tr>
</tbody>
</table>
**Xb buffer** (make fresh) pH=7.7

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Volume for 100 mL Xb</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>3M</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.1M</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.5M</td>
</tr>
<tr>
<td>Hepes pH 7.7</td>
<td>1M</td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
</tr>
</tbody>
</table>

**2% cysteine hydrochloride** pH=7.9

2 g in 100 mL water. Dissolve the powder in 80 mL of water and add 1.4 mL of 7.5N NaOH. Solution should turn slightly red. Check pH with Ph paper and adjust to 7.9 only when the pH is around 7.00. Complete the volume to 100 mL with water. The solution is stable at room temperature for several hours.

**MMR 10x**

<table>
<thead>
<tr>
<th>MMR 10x 1L</th>
<th>Stock solutions</th>
<th>Volume for 1 L MMR 10x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepes pH7.8</td>
<td>50 mM</td>
<td>50 mL</td>
</tr>
<tr>
<td>NaCl</td>
<td>1 M</td>
<td>58.54 g</td>
</tr>
<tr>
<td>KCl</td>
<td>20 mM</td>
<td>6.7 mL</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>2 mL</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>10 mM</td>
<td>10 mL</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>20 mM</td>
<td>20 mL</td>
</tr>
</tbody>
</table>

1.2. Retrieving the eggs from *Xenopus* females and making the extracts

a. Inject 3 females with 200 u of gonadotrophin (Chorulon 1000 u/mL) and 600 u 6 hours later.

b. Frogs should be placed separately in tanks containing 4L of HSB 1x buffer each. The frogs are separated from the laid eggs by a plastic grid.
c. Leave the females in HSB 1x buffer overnight. Eggs are expected to be laid 14-16 hours after the second injection.

d. Collect the eggs in a Becher and rinse with HSB 1x.

e. Wait for the eggs to settle down, discard HSB 1x and then add 100 mL of cysteine solution and leave for 10 min at room temperature in order to dejell the eggs.

f. Swirl every 2 min. Eggs become packed with no space left between them.

g. Immediately rinse at least 5 times with HSB 1x, then twice with 0.2x MMR buffer to completely remove the cysteine.

h. At this step eggs are activated for replication kinetics experiments using 05 µg/mL of Calcium ionophore in 0.2x MMR for 5 min.

i. Rinse with room temperature Xb buffer.

j. Rinse with cold Xb and transfer to a petri dish.

k. Remove damaged of abnormal eggs using a Pasteur pipette.

l. Leave on ice for 10 min to cool down the eggs. Prepare 2 mL eppendorf tubes containing 1 mL of protease inhibitor (PI) and cytochalasin (actin inhibitor) diluted 1/1000 in Xb buffer.

m. Using a pipette aid and a reversely attached 10 mL pipette, collect the eggs and transfer them to eppendorf tubes. Pre-spin at 150g and remove most of the buffer.

n. Spin at 10 000 g for 10 min at 4°C in a pre-cooled centrifuge.

o. Three phases appear: an upper one with lipids, a grey interphase corresponding to the extract and a lower black phase of pigments and membranes.

p. Pass the pipette tip through the lipid layer and collect the extract using a P1000.

q. Transfer the extract to an ice cold 15 mL Falcon tube. Add Pi and cytochalasin (1/1000). Place in 2 mL tubes and spin again at 10 000 g for 10 min at 4°C.

r. Collect the extract using a pre-cooled syringe and G20 needle.

s. Measure the volume of the extract and add 33% of glycerol.

t. Snap-freeze in liquid nitrogen and make aliquots of 50 µL. Keep tubes at -80°C.
2. DNA replication assay

Egg extracts were supplemented with α-[32P]dATP (3000 Ci/mmol, Perkin Elmer). At the indicated time points samples were neutralized in 10 mM EDTA, 0.5% SDS, 200 µg/ml Proteinase K (Sigma) and incubated at 37°C over night. Incorporation of radioactive label was determined by TCA precipitation on GF/C glass fiber filters (Wathman) following by scintillation counting.

3. Xenopus Embryos and Microinjection Experiments

*Xenopus laevis* embryos were prepared by *in vitro* fertilization using standard procedures (Sive et al., 2000).

Embryos were UV-irradiated at 2-cell stage using a microprocessor-controlled crosslinker (BIO-LINK ®) at 300 J/m2 and microinjected using a Nanoject auto oocyte injector (Drummond Scientific Company). Total embryos protein extracts were obtained by collecting staged embryos, washing in XB buffer and centrifugation at 13000 rpm, for 10 min at 4 °C in a microfuge. The cytoplasmic interphase was recovered, denatured in *Laemmli* buffer and boiled.

3.1. Buffers

**MBS 10x** pH=7.8

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>880 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>10 mM</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>10 mM</td>
</tr>
<tr>
<td>HEPES</td>
<td>50 mM</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25 mM</td>
</tr>
</tbody>
</table>

Adjust final pH to 7.8 with NaOH, autoclave.

**MBS 1x:** Mix 100 mL of 10x solution with 7mL of 0.1 M CaCl₂, adjust the volume to 1 L with distilled water.
**3.2. Xenopus laevis embryos generation**

a. Collect fresh eggs laid within one hours in a Petri dish containing HS-MBS.

b. Remove most of the buffer (HS-MBS), while keeping the eggs floating.

c. Using a forceps, tease a piece of a freshly collected testis and rub the tissue over the eggs.

d. Leave the eggs for one minute without any manipulation.

e. Add 15 to 20 drops of tap water to activate the eggs with the Ca^{2+}.

f. Leave the eggs for one minute.

g. Add gently MBS 0.1x.

h. Leave the eggs for 35 min at room temperature, until the eggs rotate within the vitelline membrane so that the animal hemisphere faces upward.

i. Remove most of the MBS 0.1x, while keeping the eggs floating.

j. Pour some cysteine solution in the dish and swirl for 2-4 minutes (do not exceed 10 min) for dejellying the fertilized eggs.

h. Remove cysteine solution and wash the eggs extensively with MBS 1x.

i. Transfer to a new Petri dish containing fresh MBS 1x and leave them to divide at 23°C.
3.3. Production of mRNA for microinjection

XlRad18WT was cloned into the pCS2+ vector as BamHI-XhoI. pCS2+-Xl \textit{RAD18}^{C28F} mutant was generated by site directed mutagenesis. In vitro transcription of pCS2- Xl \textit{RAD18}^{WT} and pCS2+-Xl \textit{RAD18}^{C28F} was performed using mMESSAGE mMACHINE Kit sp6® (Ambion). mRNA was ethanol-precipitated and dissolved in water ready for microinjection.

3.4. Injection of the embryos

a. Transfer the embryos to a small Petri dish containing a plastic grid in order to maintain the embryos immobilized.

b. Poor in the Petri dish a solution of Ficoll 400 (3% in HS-MBS), this polymer solution helps to maintain the structure of the embryos.

c. Gently introduce the needle into the animal pole of the embryo and inject the appropriate volume of mRNA at 20ng/µl (2 injections of 9 nl in each blastomer for our experiments).

d. After injection, gently withdraw the needle and move on to the next embryo.

e. Transfer the injected embryos to another Petri dish containing fresh MBS 1x.

7. Cell Culture

NIH3T3, HEK 293T, Platinum-E, HCT8, HCT116 and U87 cells were maintained in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics in a humidified atmosphere of 5% CO2 at 37 °C. For transient expression of RAD18 or empty vector (pcDNA3), HEK 293T cells were transfected using calcium phosphate. Twenty-four hours after transfection cells were mock- or UV irradiated using a microprocessor-controlled crosslinker (BIO-LINK ®). Cells were collected at indicated time points after treatment and rinsed once in PBS. Whole cell extracts were clarified by centrifugation at 12000 rcf for 10 min at 4°C. Protein concentration of the clarified lysates was
estimated using BCA method (Pierce). Equal amount of protein was used for western blot analysis.

8. Foci formation assay

Cells were co-transfected with eGFP-Pol η and RAD18 variants and incubated for 24 hours before UV-C irradiation. Four hours after irradiation, cells were fixed, washed three times with PBS, and mounted with Prolong Gold DAPI (Invitrogen). The percentage of eGFP-Polη-expressing cells displaying eGFP-Pol η foci was determined by scoring at least 200 nuclei for each condition. Nuclei containing under 30 foci were scored as negatives. Means and standard deviation (error bars) of three independent experiments are shown. For scoring RPA foci, cells were fixed with 4% paraformaldehyde and extracted with 0.5% Triton X-100 for 5 minutes at 4 °C, then blocked for 30 minutes at room temperature with PBS/3%BSA/15%FBS, followed by detection of RPA by indirect immunofluorescence with specific antibodies (ab2175).

9. siRNA

U87 cells were co-transfected either with siRNA Rad18 (GAG GAU UCU UCU AGC UGU A) or siRNA Luciferase (Luc) as a control and an empty vector (pcDNA3) or RAD18 expressing vector using JETPrime reagent (Polyplus). Twenty-four hours after transfection, cells were trypsinized and seeded in 12 wells plates at a density of 10^4 cells/well. Twenty-four hours later cells were treated with increasing concentration of cisplatin (Sigma).

10. RAD18 qPCR primers

Two pairs of primers were used for Hs RAD18:

a. Hs Rad18_L: 5’-CACGCGAAGAGAAGAAGGAA-3’
Hs Rad18_R: 5’-TTAAATCACGATCAGAGAGCAA-3’
Product length: 96 nt

b. Hs Rad18_L2: 5’-GCAATAGTTCCAGTCAGACATCA-3’

Hs Rad18_R2: 5’-TTTCTGTGTCTTGAAGATCGTTTT -3’

Product length: 93 nt
Chapter V
Résumé en Français
V. Résumé en Français

1. Résumé

Notre génome subit constamment les effets néfastes des agents endommageant de l'ADN d’origine exogène, notamment les rayons ultraviolets, mais également d’origine endogène générés par le métabolisme cellulaire. Afin de se protéger de ces effets délétères, les cellules disposent d’un système de détection des dommages à l’ADN (point de contrôle ou « checkpoint ») capable de signaler et faciliter l’élimination des lésions. Certaines lésions peuvent persister quand les cellules entrent en phase S et inhiber ainsi la synthèse de l’ADN en interférant avec les ADN polymérasases réplicatives. Ceci peut provoquer des arrêts prolongés des fourches de réplication ce qui fragilise l’ADN et met en péril la stabilité du génome. Pour préserver l’intégrité de l’information génétique, les cellules ont développé une voie de tolérance qui implique des ADN polymérasases spécialisées dans la réplication des lésions, appelées ADN Polymérasases translésionnelles (Pols TLS). Ces dernières ont la capacité unique de répliquer l’ADN endommagé grâce à leur site catalytique qui peut accommoder les bases endommagées.

Dans ce processus, la protéine PCNA joue le rôle de facteur d’échafaudage polyvalent pour de nombreuses autres protéines impliquées dans le métabolisme de l'ADN, notamment dans la réplication et la réparation. Les mécanismes de régulation des échanges entre les différents partenaires de PCNA ne sont pas très bien compris. Parmi les protéines qui interagissent avec PCNA, certaines comme CDT1, p21 ou encore PR-Set7/Set8 sont caractérisées par une forte affinité pour cette protéine. Ces dernières possèdent un motif d’interaction particulier avec PCNA, nommé « PIP degron », qui favorise leur protéolyse d'une manière dépendante de l’E3 ubiquitine ligase CRL4Cdt2. Après irradiation aux UV-C, le facteur d’initiation de la réplication CDT1 est rapidement détruit d’une manière dépendante de son PIP degron, Dans la première partie de mon travail, j’ai contribué à comprendre le rôle fonctionnel de cette dégradation. Les résultats obtenus ont fourni des évidences expérimentales qui montrent que l’inhibition de la dégradation de CDT1 par CRL4Cdt2 dans les cellules de mammifères compromet la relocalisation des TLS Pol η et Pol κ en foyers nucléaires induits par les irradiations UV-C. En élargissant cette étude à d'autres partenaires de PCNA, on a constaté que seules les protéines qui contiennent un PIP degron interfèrent avec la formation de foyers de Pol η. La mutagenèse du PIP degron de CDT1 a révélé qu’un résidu de thréonine conservé parmi les PIP degrons est...
essentiel pour l'inhibition de la formation des foyers d’au moins deux TLS Polymérasas. Les résultats obtenus suggèrent que l’élimination de protéines contenant des PIP degrons par la voie CRL4Cdt2 régule le recrutement de TLS Polymérasas au niveau des sites des dommages induits par les UV-C.

Dans un second temps, on s’est intéressé à l’étude du point de contrôle « checkpoint » des dommages à l’ADN au cours de l’embryogénèse. En effet, dans les embryons précoces, le point de contrôle des dommages de l’ADN est silencieux jusqu’à la transition de mid-blastula (MBT), en raison de facteurs maternels limitants. Dans ce travail, nous avons montré, aussi bien in vitro qu’in vivo, que l’ubiquitine ligase de type E3 RAD18, un régulateur majeur de la transléson qui implique les TLS Pols de la famille Y, est un facteur limitant pour l’activation du checkpoint dans les embryons de xénope. Nous avons montré que l’inactivation de la fonction de l’ubiquitine ligase RAD18 conduit à l’activation des points de contrôle des dommages de l’ADN, entrainant la phosphorylation de CHK1 par un mécanisme qui implique l’inhibition de l’arrêt des fourches de réplication en face des lésions produites par les UV-C. De plus, nous avons montré que l'abondance de RAD18 et de PCNA monoubiquitiné (PCNA mUb) est régulée au cours du développement embryonnaire. À l’approche de la MBT, l’abondance accrue de l’ADN limite la disponibilité de RAD18, réduisant ainsi la quantité de PCNA mUb et induisant la dé-répression du « checkpoint ». En outre, nous avons montré que cette régulation embryonnaire peut être réactivée dans les cellules somatiques de mammifères simplement par l'expression ectopique de RAD18, conférant une résistance aux agents qui causent des dommages à l'ADN. Enfin, nous avons trouvé que l'expression de RAD18 est élevée dans les cellules souches cancéreuses de glioblastome hautement résistantes aux dommages de l'ADN. En somme, ces données proposent RAD18 comme un facteur embryonnaire critique qui inhibe le point de contrôle des dommages de l’ADN et suggèrent que le dérèglement de l’expression de RAD18 peut avoir un potentiel oncogénique inattendu.

2. Introduction

Les cellules sont constamment exposées à des agents qui induisent des endommagements de l’ADN et mettent ainsi en péril la stabilité du génome. Une forte instabilité génomique est caractéristique de la plupart des cancers. Pour pouvoir préserver
l’intégrité du génome, les cellules ont développé un système de surveillance connu sous le nom de point de contrôle, ou « checkpoint » (Hartwell and Weinert, 1989) qui constitue une barrière à la transformation maligne car les mutations dans les gènes du « checkpoint » sont caractéristiques en majorité, des carcinomes à un stade agressif (Bartkova et al., 2005; Gorgoulis et al., 2005). L’activation de ce système aboutit à l'inactivation des régulateurs majeurs de la phase S et de la mitose, les protéines phosphatases de la famille CDC25 et les CDKs. Deux voies de signalisation majeures ont été décrites chez les vertébrés et les mammifères ; les voies dépendantes des kinasés ATM et ATR. La kinase ATR est responsable de la signalisation du checkpoint en phase S.

Les endommagements de l’ADN peuvent provoquer l’arrêt de la réplication car les ADN polymérasas réplicatives ne peuvent pas synthétiser l’ADN à travers les lésions. L’arrêt prolongé des fourches de réplication (stress réplicatif) constitue la principale source d’instabilité génomique retrouvée dans la plupart des cancers sporadiques (Negrini et al., 2010), mais sa nature est très mal comprise. L’arrêt des fourches de réplication induit par les lésions de l’ADN provoque le découplage enzymatique des activités des ADN polymérasas et de l’hélicase. Par conséquent une quantité importante d’ADN simple brin est produite, ce qui déclenche le « checkpoint » (Byun et al., 2005; Zou and Elledge, 2003). L’activité kinase d’ATR se déclenche lors de la formation d’un complexe entre ATR avec son partenaire ATRIP, la protéine TOPBP1 et le complexe 9-1-1, constituant le complexe minimal pour l’activation du checkpoint (Cimprich and Cortez, 2008). Les lésions de l’ADN sont prises en charge au niveau des fourches de réplication bloquées par un système de tolérance des lésions qui implique des ADN polymérasas spécialisées, les ADN Polymérasas translésionnelles (Pols TLS) de la famille Y (Friedberg et al., 2005). L’ubiquitine ligase RAD18, associée à son partenaire RAD6, est requise pour le recrutement des Pols TLS au niveau des fourches de réplication bloquées. Cette activité produit la monoubiquitination (mUb) de la protéine de la fourche de réplication PCNA et constitue le signal qui permet le recrutement des Pols TLS (Kannouche and Lehmann, 2004). Ce mécanisme de tolérance des lésions aide la cellule à échapper à l’apoptose et il est donc très certainement impliqué dans la résistance thérapeutique de certains cancers. La dérégulation de l’expression des Pols TLS est associée au cancer (Albertella et al., 2005; Canitrot et al., 2000) mais le mécanisme moléculaire responsable n’a pas été clarifié.
De nombreuses protéines interagissent avec PCNA via une courte séquence peptidique conservée chez tous les métazoaires, appelée « PCNA-Interacting-Protein Box » ou « PIP Box » (Moldovan et al., 2007). Du fait de sa structure trimérique, PCNA peut en théorie interagir simultanément avec trois protéines au maximum, et de ce fait ses partenaires sont constamment en compétition pour la liaison à la poche hydrophobe. L’étude des mécanismes qui régulent les interactions entre PCNA et ses partenaires est importante pour comprendre comment les processus cellulaires essentiels pour le maintien de la stabilité du génome, notamment la réplication de l’ADN, la réparation, et la TLS, sont coordonnés. Par exemple, l’oncogène CDT1 et l’inhibiteur des cycline-dépendantes kinases (CDK) p21 interagissent avec PCNA via un PIP box. Après endommagement de l’ADN, CDT1 et p21 sont polyubiquitinés par la cullin 4-RING E3 ubiquitine ligase (CRL4), et sont dégradés par le protéasome (Abbas et al., 2008; Higa et al., 2003; Jin et al., 2006). Le rôle de cette dégradation est mal compris. Pour ce qui est CDT1, il a été précédemment proposé que sa dégradation après endommagement de l’ADN soit nécessaire pour inhiber la réplication de l’ADN (Higa et al., 2003).

Chez les embryons précoces, le point de contrôle de phase S retardant la division cellulaire suite à des dommages de l’ADN (Anderson et al., 1997; Hensey and Gautier, 1997) ou à la présence d’ADN non répliqué (Dasso and Newport, 1990; Kimelman et al., 1987) est inefficace. En effet, les divisions sont rapides, consistant en une alternance des phases S et M, avec des phases intermédiaires pratiquement absentes (Graham, 1966). Cela représente une sorte d’adaptation afin d’assurer la prolifération rapide des embryons dans un environnement extérieur hostile. Les mécanismes moléculaires responsables de l’inhibition du point de contrôle dans les embryons précoces sont mal compris. Des études antérieures chez le Xenopus laevis (Conn et al., 2004; Dasso and Newport, 1990; Kappas et al., 2000) ont montré que l’activation du point de contrôle dépend du rapport nucléo-cytoplasmique (N/C) en raison de l'absence de la croissance des cellules et ne dépend ni de la transcription ni de la traduction, ce qui suggère le titrage de facteurs maternels limitants dont l'identité est inconnue. Des données génétiques chez C. elegans (Holway et al., 2006; Ohkumo et al., 2006) ont mis en cause la TLS Pol η spécialisée dans la réplication de l'ADN endommagé (Sale et al., 2012). Pol η est recrutée aux sites de dommages à l'ADN par liaison à la protéine PCNA^mUb par le complexe ubiquitine ligase RAD6 (E2)-RAD18 (E3), alors que l'ubiquitine hydrolase USP1 catalyse la réaction inverse (Ulrich and Takahashi, 2013).
3. Hypothèse et objectifs

Notre hypothèse de travail est que la dérégulation du système de tolérance des lésions (translésion) contribue à l’instabilité génétique ainsi qu’à la transformation oncogénique par interaction avec le checkpoint des dommages à l’ADN.

Les objectifs de ma thèse étaient d'étudier les bases moléculaires et fonctionnelles de l’interaction entre la translésion et le checkpoint. L’enjeu est de déterminer comment la dérégulation fonctionnelle de ces interactions génère de l’instabilité génomique dans des systèmes modèles (xénope et cellules de mammifères).

Dans la première partie de ce travail, nous nous sommes intéressés au rôle de la dégradation de CDT1 après exposition aux UV-C. Nous avons fait l’hypothèse que la dégradation de CDT1 suite à l’endommagement de l’ADN pourrait réguler l’accessibilité des différentes protéines à PCNA et favoriser les interactions entre PCNA et des protéines essentielles au maintien de la stabilité du génome. Suite à un endommagement de l’ADN, l’accès de ces protéines (telle que la TLS Pol η) à la chromatine est régulé via l’interaction avec PCNA.

Le premier objectif de ce travail était de déterminer si CDT1 peut entrer en compétition avec TLS Pol η visualisée par la formation de foyers eGFP-Pol η, dans une manière dépendante de son PIP-box. Si tel est le cas, la dégradation CDT1 via l'E3 ubiquitine ligase CRL4Cdt2 serait nécessaire pour la localisation de Pol η dans des foyers subnucléaires. Une autre question était de savoir si la voie CRL4Cdt2 cible spécifiquement les protéines contenant un PIP-degron qui confère une forte affinité de liaison pour PCNA plutôt que tous les partenaires PCNA. Cette possibilité implique également que la voie de dégradation CRL4Cdt2 peut avoir une nouvelle fonction dans la régulation des interactions PCNA-Pol η. De plus, il serait intéressant de savoir si un court peptide contenant le PIP-degron est suffisant pour bloquer l'accès de Polη aux sites de réplication et d'identifier le motif minimal nécessaire à cette activité. Enfin, CDT1 peut également concurrencer d'autres polymérasases de la famille Y pour la formation des foyers nucléaires, pour cette raison, nous avons étendu notre étude à une autre polymérase TLS, Polk.

Dans la deuxième partie de ma thèse, le principal objectif était de comprendre la base moléculaire de l’inhibition du point de contrôle dans les embryons précoces. Pour réaliser cela, nous avons utilisé comme système des extraits dérivés d'œufs de xénope activés. Ce système in
vitro reproduit fidèlement la régulation développementale de l'activation du point de contrôle des dommages de l'ADN observée in vivo (Anderson et al., 1997; Conn et al., 2004; Kappas et al., 2000; Newport and Dasso, 1989). Les premiers résultats ont montré que la répression du point de contrôle dans des œufs de xénope est une conséquence d'une forte inhibition du découplage de la fourche de réplication médiée par la monoubiquitination constitutive de PCNA, et le recrutement de la TLS Pol η sur la chromatine, indépendamment des dommages à l'ADN. Puisque RAD6-RAD18 est l'ubiquitine ligase responsable de la PCNA<sub>monoUb</sub>, nous avons vérifié si c’était un facteur maternel limitant, responsable de l'inactivation du point de contrôle dans les embryons précoces. Un autre point important était de vérifier si cette régulation est réversible. De plus, il était également intéressant de savoir comment RAD18 et PCNA<sub>monoUb</sub> sont régulés au cours du développement dans les embryons de xénope. L'étape suivante était de déterminer si cette inhibition embryonnaire du point de contrôle pourrait être réactivée dans les cellules somatiques de mammifères en surexprimant RAD18. A partir des données indiquées ci-dessus, il est prévisible que les cellules surexprimant RAD18 peuvent présenter une résistance à certains agents endommageant l'ADN, tels que les rayons UV ou le cisplatine. L'un de nos objectifs était de mesurer la sensibilité des cellules exprimant des niveaux élevés de RAD18 sauvage ou de mutants spécifiques de RAD18<sup>(C28F et C207F)</sup>, exposées à des agents endommageant l'ADN en effectuant des essais de survie.

Comme la surexpression de RAD18 dans les cellules pourrait être responsable de l'inactivation du « checkpoint » qui est considéré comme une barrière à la transformation maligne (Halazonetis et al., 2008), nous avons exploré la possibilité que RAD18 peut être surexprimé dans les cancers. En d'autres termes, il est possible que l'expression forcée de RAD18 peut reproduire les phénomètes des mutations dans les gènes du point de contrôle et favoriser la prolifération et la résistance des cellules aux agents endommageant de l'ADN ou des cellules qui subissent un stress réplicatif généré par l'activation d'un oncogène.
4. Résultats et Discussion

4.1. Caractérisation d’un nouveau mécanisme de recrutement des TLS Pol η et κ

Nous avons mis en évidence un nouveau mécanisme moléculaire de recrutement des TLS pols de la famille Y, dépendant de la dégradation par la voie de l’ubiquitine ligase CRL4Cd2 de protéines possédant un PIP-degron (tel que CDT1) après irradiation aux rayons UV, et ceci indépendamment de PCNAmUb.

Pour savoir si CDT1 peut interférer avec le rôle de la Pol η dans la voie de la TLS, on a étudié le recrutement de la TLS Pol η en foyers nucléaires dans des cellules exprimant eGFP-Pol η et qui surexpriment aussi CDT1. Après irradiation de ces cellules aux UV-C, on a observé une réduction importante de la formation des foyers d’eGFP-Pol η dans les cellules surexprimant CDT1. Des résultats similaires ont été obtenus dans les cellules humaines de la lignée U2OS issue d’ostéosarcome, montrant que cet effet de CDT1 sur la localisation de la Pol η n’est pas spécifique du type cellulaire. Les cellules NIH3T3 qui surexpriment CDT1 n’ont pas montré un défaut du cycle cellulaire, ni un défaut de l’activation du checkpoint de l’endommagement de l’ADN. Plus important encore, dans ces cellules le checkpoint ne s’active pas en absence d’irradiation aux UV. Ces observations suggèrent que le défaut de la localisation de la Pol η n’est pas dû à un stress réplicatif provoqué par la surexpression de CDT1, mais à un effet direct de CDT1 sur la Pol η.

Nos observations montrent également que les cellules surexprimant CDT1 ne présentent pas de défaut de PCNAmUb. Par contre, la surexpression d’un mutant de CDT1 dont le PIP box est supprimée n’avait aucun effet sur la formation de foyers de Pol η. Ces résultats suggèrent que CDT1 agit sur Pol η par un effet direct de compétition au niveau de l’interaction avec PCNA et indiquent que la PIP box de CDT1 joue un rôle essentiel dans ce mécanisme. Ceci est confirmé par le fait que l’expression d’un mutant de CDT1 qui se lie à PCNA mais qui ne peut être dégradé par la voie de l’ubiquitine ligase CRL4Cd2 après irradiation aux UV-C (CDT1R4A), inhibe fortement la formation des foyers GFP-Pol η. Nous avons aussi montré que l’expression du PIP box de CDT1 est suffisante pour inhiber la localisation de Pol η en foyers. En élargissant cette étude à d’autres protéines possédant une PIP-box, nous avons montré que l’inhibition de
la formation des foyers Pol η est une caractéristique spécifique des protéines possédant une PIP-box spéciale appelée PIP-degron (PIP-box qui permet de cibler les protéines liées à PCNA pour une dégradation par le protéasome). Ces protéines sont caractérisées par une forte affinité de liaison avec PCNA. En collaboration avec J. Delgado et L. Serrano au CRG de Barcelone (Espagne), nous avons modélisé les PIP box et PIP degrons de plusieurs partenaires PCNA dans la poche hydrophobe de PCNA. Les constantes de dissociation thermodynamiques obtenus (KD) révèlent l’existence de trois catégories hiérarchiques de protéines en fonction de leur affinité de liaison: I) Les PIP degrons : caractérisés par une forte interaction avec PCNA, II) Les PIP box canoniques qui ont une affinité modérée pour PCNA et III) PIP box non-canonique qui ont une affinité sub-optimale pour la liaison avec PCNA.

4.2. L’ubiquitine ligase RAD18 supprime le checkpoint des dommages à l’ADN dans les embryons de xénope

Dans les embryons précoce de xénope, le checkpoint des dommages à l’ADN est inactif jusqu’avant le stade de midblastula suite à la titration d’un facteur maternel dont l’identité reste inconnue. Pour comprendre ce phénomène, j’ai utilisé comme modèle l’extrait d’œufs de xénope qui reproduit fidèlement la régulation embryonnaire du checkpoint observée in vivo (Anderson et al., 1997; Conn et al., 2004; Kappas et al., 2000; Newport and Dasso, 1989).

On a pu montrer que la protéine Rad18 est titrée sur la chromatine au fur et à mesure que le rapport N/C augmente, en raison de l’absence de croissance de l’œuf lors des divisions embryonnaires. On a aussi observé qu’à faible rapport N/C, CHK1 n’est pas phosphorylé en présence d’endommagements à l’ADN, PCNA est constitutivement monoubiquitiné et Pol η est recrutée sur la chromatine. Dans ces conditions, très peu de RPA s’accumule après endommagement de l’ADN, ce qui suggère l’inhibition du découplage des fourches de réplication. Nous avons également montré in vitro que RAD18 est responsable de ce phénotype et par conséquent, de l’inactivation du checkpoint des dommages à l’ADN.

Par des expériences d’immunoprécipitation à partir des extraits de xénope, j’ai réussi à démontrer que RAD18 interagit avec DRF1, une sous-unité activatrice de la protéine kinase CDC7, qui a été démontrée être nécessaire à la phosphorylation de RAD18 et le recrutement de Polη sur PCNA (Yamada et al., 2013b). Cette interaction n’est observée qu’à faible rapport
N/C et suggère que l’abondance de RAD18 dans cette condition fait qu’il forme un complexe avec DRF1 pour le lier à la chromatine même en l’absence d’endommagements.

J’ai aussi analysé les niveaux de RAD18 et DRF1 dans les embryons de xénope, et montré que la quantité de RAD18 diminue progressivement à partir du stade 4 jusqu’à devenir presque nulle aux alentours du stade 6,5 (avant le stade de midblastula) alors que DRF1 diminue plus tardivement vers le stade 7 ce qui est en accord avec les données de la littérature (Collart et al., 2013; Takahashi and Walter, 2005a).

Pour démontrer que RAD18 inactive aussi le checkpoint in vivo, j’ai surexprimé RAD18 sauvage ou le mutant RAD18C28F (mutant au niveau du site catalytique) par microinjection des ARNm correspondants dans des embryons de xénope au stade de deux blastomères. Les embryons injectés avec RAD18 sauvage se développent normalement et atteignent le stade 6,5 après irradiation aux UV-C, alors que très peu de ceux injectés avec RAD18C28F atteignent ce stade. De plus et en accord avec les résultats in vitro, le checkpoint des dommages à l’ADN est inactif dans les embryons injectés avec RAD18 sauvage alors qu’il est fonctionnel dans ceux injectés avec le RAD18C28F. L’ensemble de ces résultats démontrent pour la première fois que RAD18 est un facteur responsable de l’inactivation du checkpoint des dommages à l’ADN au cours de l’embryogenèse précoce du xénope.

4.3. Réactivation d’un état embryonnaire du checkpoint par surexpression de RAD18 dans les cellules de mammifères

En ligne avec des observations antérieures (Bi et al., 2006; Daigaku et al., 2010), j’ai aussi démontré que la surexpression de RAD18 dans les cellules de mammifères est suffisante pour activer constitutivement la TLS en induisant une monoubiquitination de PCNA, la formation de foyers Polη en l’absence de dommages externes, ainsi que l’inactivation du checkpoint après endommagement aux rayons UV.

J’ai aussi montré que l’expression de RAD1818A 401-445, un mutant au niveau du site de sa phosphorylation par CDC7 (Day et al., 2010a) et d’interaction avec Pol η (Watanabe et al., 2004) n’est pas capable d’induire la formation de foyers Polη aussi bien en absence qu’en présence d’endommagements à l’ADN. Ce mutant ne supprime pas la phosphorylation de CHK1 suggérant que cet effet dépend de la phosphorylation de RAD18 par la kinase CDC7.
Enfin, en cohérence avec les résultats obtenus chez le xénope, j’ai pu montrer que la surexpression de RAD18 sauvage et pas celle de RAD18C28F dans les cellules HEK293T inhibe la formation des foyers de RPA après endommagement de l’ADN, ce qui suggère l’inhibition du découplage des fourches de réplication.

4.4. RAD18 induit une résistance cellulaire aux dommages à l’ADN

Puisque les cellules qui surexpriment RAD18 semblent être en mesure de passer outre l’activation du checkpoint, et que le recrutement constitutif des TLS pols a pour effet d’introduire plus de mutations, j’ai exploré la possibilité que RAD18 peut avoir un potentiel oncogénique auparavant inconnu. En d’autres termes, il est possible que l’expression élevée de RAD18 puisse donner le même phénomène que les mutations dans les gènes du point de contrôle et favoriser ainsi la prolifération des cellules exposées à des lésions de l’ADN ou des cellules en situation de stress réplicatif généré par l’activation d’un oncogène. Pour cela, j’ai généré des cellules (NIH 3T3) qui expriment stablement RAD18 sauvage, RAD18C28F ou RAD18C207F (ce dernier étant un mutant qui ne peut pas être ubiquitiné et qui se localise principalement dans le noyau de la cellule) et j’ai procédé à des tests de viabilité cellulaire en exposant les cellules à différents agents endommageant de l’ADN (UV-C, MMC, Bléomycine, Camptothecine, Cisplatine). Les résultats que j’ai obtenus montrent que la surexpression de RAD18 sauvage ou de RAD18C207F confère une résistance aux agents endommageant de l’ADN contrairement à RAD18C28F.

4.5. RAD18 est surexprimé dans le glioblastome et induit une résistance à la thérapie

J’ai ensuite exploré une relation possible entre l’expression élevée de RAD18 et la résistance aux agents endommageant de l’ADN des cellules cancéreuses. Pour cela, j’ai analysé l’expression de RAD18 dans différentes lignées cellulaires de cancer et observé des niveaux très faibles de RAD18 dans la lignée HCT8 qui montre une sensibilité à l’oxaliplatine (un dérivé du cisplatine couramment utilisé en chimiothérapie) par rapport à la lignée HCT116 qui est résistante (Balin-Gauthier et al., 2008).

De façon intéressante, on a observé une surexpression de RAD18 dans des biopsies de glioblastome (au niveau de l’ARNm et de la protéine) en comparaison avec les progéniteurs
correspondants ou les cellules Hela. Cette tumeur cérébrale montre une résistance extraordinaire aux traitements thérapeutiques (radiations ionisantes et agents alkylants) dont le mécanisme moléculaire demeure inconnu. Récemment, il a été suggéré que RAD18 est impliquée dans la résistance des glioblastomes aux radiations ionisantes (Xie et al., 2014). En accord avec ces résultats, j’ai pu montrer que la lignée de glioblastome U87 devient sensible au cisplatine après inhibition de l’expression de RAD18. De plus, la reconstitution des cellules U87 traitée avec un petit ARN interférant (siRNA) dirigé contre RAD18 avec RAD18 exogène résistant au siRNA induit une très forte résistance au cisplatine. Ces résultats suggèrent une forte implication de RAD18 dans la survie de cette tumeur ainsi que dans sa résistance aux agents endommageants de l’ADN.

5. Perspectives

5.1. Conséquence de la surexpression de CDT1 dans les cancers

La surexpression de CDT1 affecte la formation de foyers nucléaires de Polη induits par l’irradiation aux UV-C, d’une manière dépendante de sa PIP-box. En plus de Polη, Polκ et Polλ utilisent également leurs PIP-box pour une interaction directe avec PCNA, et nos données montrent que les cellules qui surexpriment CDT1 présentent aussi une réduction de la formation de foyers de GFP-Polκ. Comme CDT1 est un oncogène surexprimé dans différents cancers, la fonction de Polη, Polκ et Polλ pourrait être altérée dans ces cancers. Dans cette situation, une TLS pol plus mutagène telle que la Polζ en combinaison avec REV1 pourrait probablement remplacer les autres TLS pols. Polζ a été associée au développement de tumeurs malignes secondaires et la résistance à la chimiothérapie par cisplatine, en plus de l'augmentation de la mutagenèse induite par le cisplatine (Doles et al., 2010). Ainsi, des niveaux élevés de CDT1 pourraient d’une part entraîner une prolifération plus accrue vue sa fonction oncogénique comme régulateur positif de la réplication de l’ADN, et aussi des mauvais résultats lors d'un traitement thérapeutique. Pour toutes ces raisons, l'évaluation de l'efficacité et de la fidélité des polymérasas TLS dans des lignées cellulaires de cancer présentant des niveaux élevés de CDT1, et la caractérisation de son impact sur la résistance de la tumeur est un objectif important à poursuivre.
5.2. Etude de l’oncogénicité de Rad18

Ce travail a fourni de nouvelles données sur la façon dont le point de contrôle des dommages de l'ADN est régulé dans les embryons précoces. Cette régulation pourrait être conservée dans d'autres organismes. Chez la mouche *Drosophila melanogaster*, nos données non publiées suggèrent que le même mécanisme est présent dans les embryons précoces, comme le démontre le niveau élevé de PCNA mUb au cours des stades embryonnaires précoces (Lo Furno, Busseau and Maiorano, données non publiées). Cependant, l’orthologue de RAD18 n'a pas encore été identifié chez la drosophile. Il serait intéressant d'identifier la ou les E3 ubiquitine ligase(s) responsable(nt) de la monoubiquitination de PCNA chez la drosophile. Récemment, une protéine contenant un domaine RING, appelée NOPO a été montré comme induisant l'ubiquitination de Polη et sa localisation en foyers nucléaires (Wallace et al., 2014). De plus, alors que des orthologues des TLS Polη, Polε et REV1 ont été identifiés chez la drosophile, l’orthologue de Polκ n’est pas encore connu (Ishikawa et al., 2001).

Dans notre modèle (voir le résumé graphique), les polymérasases TLS, dont Pol η, sont constitutivement recrutées à la fourche de réplication permettant un contournement actif des lésions sans bloquer la réplication. Cependant, les polymérasases TLS ne sont pas fidèles et peuvent introduire un grand nombre de mutations lors de la réplication de l'ADN non endommagé. Comme la processivité des TLS Pols est faible, et la vitesse de réplication de l'ADN n’est pas plus faible chez les embryons précoces, il est plus probable que la synthèse translésionnelle est utilisée uniquement pour contourner les lésions qui arrêtent la progression des polymérasases réplicatives même si les TLS polymérasases sont liés à la fourche de façon permanente. Si tel est le cas, les mutations devraient être moins fréquentes parce que les TLS Pols sont plus fidèles lors de la réplication de certains types de lésions. Par exemple Pol η est capable de répliquer sans erreur les dimères de thymine générés par l’irradiation UV *in vitro* (Johnson et al., 2001). Cependant, il serait important d'évaluer la contribution des TLS Pols à la mutagenèse dans les embryons précoces. Il est possible de spéculer que l'activité TLS constitutive dans les embryons précoces pourrait contribuer à la variabilité génétique des individus.

RAD18 est surexprimée dans différents cancers et lignées cellulaires cancéreuses. Nous avons montré que l'expression ectopique RAD18 dans des cellules NIH3T3 les rend plus résistants à aux agents endommageant de l’ADN dont les rayons UV et le cisplatine (figures 4B et 4C dans l'article). De plus, réduire l’expression de RAD18 dans la lignée cellulaire de
glioblastome humain U87 est suffisant pour les sensibiliser au cisplatine ou à l'agent alkylant MMS (Kermi & Maiorano, données non publiées). De même, un autre groupe a montré que les lignées cellulaires de glioblastome humain U87 et U251 sont plus sensibles aux rayonnements ionisants après réduction de l’expression de RAD18 (Xie et al., 2014b). Il serait intéressant de déterminer si RAD18 est responsable de la résistance au traitement dans d'autres types de cancer. Récemment, l'enzyme de désubiquitination USP7 a été montré être essentiel pour la stabilité des RAD18 (Zlatanou et al., 2016). En utilisant un inhibiteur d’USP7 décrit précédemment P22077 (Altun et al., 2011), nous avons pu sensibiliser les cellules U87 au cisplatine (Kermi & Maiorano, données non publiées).

Le glioblastome est un cancer caractérisé par la présence d’une population de cellules souches cancéreuses responsables de la récurrence et la résistance aux traitements thérapeutiques. Actuellement, le meilleur modèle pour l'étude du glioblastome est la culture des gliosphères qui sont enrichies en cellules souches cancéreuses. Contrairement à des lignées de cellules adhérentes cultivées dans du milieu contenant du sérum, telles que les cellules U87, les gliosphères présentent une expression génique globale et des phénotypes très similaire aux glioblastomes humains (Lee et al., 2006). Plus important encore, quand elles sont injectées dans le cerveau de la souris, elles forment une tumeur infiltrante et migratoire reflétant les caractéristiques in vivo du glioblastome (Lee et al., 2006). Des expériences récentes que nous avons effectuées sur les gliosphères montrent que la réduction de l'expression de RAD18 dans ces cellules affecte fortement leur viabilité suite à un traitement au cisplatine (Kermi & Maiorano, données non publiées). De plus, il semble que réduire l'expression de RAD18 en-soi a déjà eu un effet sur la prolifération, ce qui suggère un rôle potentiel de RAD18 dans l'autorenouvellement de ces cellules (Kermi & Maiorano, données non publiées).

Les mécanismes moléculaires responsables de la résistance à la thérapie médiee par RAD18 doivent être clariés. RAD18 est impliqué dans de nombreuses voies de tolérance et de réparation des dommages à l'ADN, ainsi que dans l’inhibition du checkpoint des dommages de l’ADN qui est considéré comme une barrière à la transformation maligne (Halazonetis et al., 2008).

Pour toutes ces raisons, je pense que notre prochain défi est de résoudre la structure cristalline de RAD18, et d’essayer de mettre en place un criblage chimique afin d'identifier des inhibiteurs spécifiques qui pourraient être pertinents pour la thérapie anti-cancer.
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Abstract

Our genome is continuously exposed to DNA damaging agents. In order to preserve the integrity of their genome, cells have evolved a DNA damage signalling pathway known as checkpoint. DNA lesions that persist when cells enter the S-phase halt the progression of replicative DNA polymerases. This can cause prolonged replication forks stalling which threaten the stability of the genome. To preserve the integrity of replication forks, cells have developed a tolerance pathway which involves specialized DNA polymerases, called translesion DNA polymerases (TLS Pols) that have the unique ability to accommodate the damaged bases. In this process, the replication factor PCNA acts as a scaffold for many proteins involved in DNA metabolism. The mechanisms governing the exchanges between different PCNA partners are not well understood. Among the proteins that interact with PCNA, CDT1, p21 and PR-Set7/set8 are characterized by a high binding affinity. These proteins have a particular interaction domain with PCNA, called "PIP degron", which promotes their proteasomal degradation via the E3 ubiquitin ligase CRL4Cdt2. After UV-C irradiation, the replication initiation factor CDT1 is rapidly degraded in a PIP degron-dependent manner. During the first part of my work, we wanted to understand the functional role of this degradation. Our results have shown that inhibition of CDT1 degradation by CRL4Cdt2 in mammalian cells, compromises the relocation of TLS Pol η and Pol κ to nuclear foci after UV-C irradiation. We also found that only the proteins which contain a PIP degron interfere with the formation of Pol η foci. Mutagenesis experiments on CDT1 PIP degron revealed that a threonine residue conserved among PIP degrons is essential for inhibiting foci formation of at least two TLS polymerases. This results suggest that CRL4Cdt2-dependent degradation of proteins containing PIP degrons regulates the recruitment of TLS polymerases at sites of UV-induced DNA damage.

During the second part of my thesis, we studied DNA damage checkpoint regulation during embryogenesis. Indeed, in early embryos, the DNA damage checkpoint is silent until the mid-blastula transition (MBT) due to maternal inhibiting factors. In this work, we have shown, both in vitro and in vivo, that the E3 ubiquitin ligase RAD18, a major regulator of translesion DNA synthesis, is a limiting factor for the checkpoint activation in Xenopus embryos. We have also shown that RAD18 depletion leads to the activation of DNA damage checkpoints by inducing replication fork uncoupling in front of the lesions. Furthermore, we showed that the abundance of RAD18 and PCNA monoubiquitination (PCNAmonoUb) is regulated during embryonic development. Near the MBT, the increased abundance of DNA limits the availability of RAD18, thereby reducing the amount of PCNAmonoUb and inducing the de-repression of the checkpoint. Moreover, we have shown that this embryonic-like regulation can be reactivated in somatic mammalian cells by ectopic expression of RAD18, conferring resistance to DNA damaging. Finally, we found high RAD18 levels in glioblastoma cancer stem cells highly resistant to DNA damage. All together, these data propose RAD18 as a critical factor that inhibits DNA damage checkpoint in early embryos and suggests that dysregulation of RAD18 expression may have an unexpected oncogenic potential.
**Résumé**

Notre génome subit constamment les effets néfastes des agents endommageant l'ADN. Afin de se protéger de ces effets délétères, les cellules disposent d'un système de détection des dommages à l'ADN (point de contrôle ou «checkpoint»). Certaines lésions peuvent persister quand les cellules entrent en phase S et inhiber ainsi la synthèse de l'ADN en interférant avec les ADN polymérases réplicatives. Ceci peut provoquer des arrêts prolongés des fourches de réplication ce qui fragilise l'ADN. Pour préserver l'intégrité de l'information génétique, les cellules ont développé une voie de tolérance qui implique des ADN polymérases spécialisées dans la réplication des lésions, appelées ADN Polymérases translésionnelles (Pols TLS). Dans ce processus, le facteur de réplication PCNA joue le rôle de facteur d'échafaudage pour de nombreuses protéines impliquées dans le métabolisme de l'ADN. Les mécanismes de régulation des échanges entre les différents partenaires de PCNA ne sont pas très bien compris. Parmi les protéines qui interagissent avec PCNA, CDT1, p21 ou encore PR-Set7/Set8 sont caractérisées par une forte affinité pour cette protéine. Ces dernières possèdent un motif d’interaction particulier avec PCNA, nommé « PIP degron », qui favorise leur protéolyse d'une manière dépendante de l’E3 ubiquitine ligase CRL4\textsubscript{Cdt2}. Après irradiation aux UV-C, le facteur d’initiation de la réplication CDT1 est rapidement détruit d’une manière dépendante de son PIP degron. Dans la première partie de mon travail, j’ai contribué à comprendre le rôle fonctionnel de cette dégradation. Les résultats obtenus ont fourni des évidences expérimentales qui montrent que l’inhibition de la dégradation de CDT1 par CRL4\textsubscript{Cdt2} dans les cellules de mammifères compromet la relocalisation des TLS Pol \(\eta\) et Pol \(\kappa\) en foyers nucléaires induits par les irradiations UV-C. On a constaté que seules les protéines qui contiennent un PIP degron interfèrent avec la formation de foyers de Pol \(\eta\). La mutagenèse du PIP degron de CDT1 a révélé qu'un résidu de thréonine conservé parmi les PIP degrons est essentiel pour l’inhibition de la formation des foyers des TLS Polymérases. Les résultats obtenus suggèrent que l’élimination de protéines contenant des PIP degrons par la voie CRL4\textsubscript{Cdt2} régule le recrutement de TLS Polymérases au niveau des sites des dommages induits par les UV-C.

Dans un second temps, on s’est intéressé à l’étude du checkpoint des dommages à l’ADN au cours de l’embryogénèse. En effet, dans les embryons précoces, le checkpoint est silencieux jusqu’à la transition de mid-blastula (MBT), en raison de facteurs maternels limitants. Dans ce travail, nous avons montré, aussi bien in vitro qu’in vivo, que l’ubiquitine ligase de type E3 RAD18, un régulateur majeur de la translésion, est un facteur limitant pour l’activation du checkpoint dans les embryons de Xénope. Nous avons montré que l’inactivation de la fonction de l’ubiquitine ligase RAD18 conduit à l’activation du checkpoint par un mécanisme qui implique l’arrêt des fourches de réplication en face des lésions produites par les UV-C. De plus, nous avons montré que l’abondance de RAD18 et de PCNA monoubiquitiné (PCNA\textsubscript{monoUb}) est régulée au cours de l’embryogénèse. À l’approche de la MBT, l’abondance de l’ADN limite la disponibilité de RAD18, réduisant ainsi la quantité de PCNA\textsubscript{monoUb} et induisant la dé-répression du checkpoint. En outre, nous avons montré que cette régulation embryonnaire peut être réactivée dans les cellules somatiques de mammifères par l’expression ectopique de RAD18, conférant une résistance aux agents qui causent des dommages à l’ADN. Enfin, nous avons trouvé que l’expression de RAD18 est élevée dans les cellules souches cancéruseuses de glioblastome hautement résistantes aux dommages de l’ADN. En somme, ces données proposent RAD18 comme un facteur embryonnaire critique qui inhibe le point de contrôle des dommages de l’ADN et suggèrent que le dérèglement de l’expression de RAD18 peut avoir un potentiel oncogénique inattendu.