THE ROLE OF CYCLIN O IN THE DNA DAMAGE RESPONSE

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A les dues dones de la meva vida

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ABSTRACT

Cyclin O, a novel identified CDK1 and CDK2-binding cyclin, has been demonstrated to be required for γ-radiation induced apoptosis in a lymphoid cell line. γ-radiation induces the formation of DSBs in the DNA what activate the DDR in order to mitigate the consequences of this insult and repair the DNA damage. The aim of this thesis has been to study the role of Cyclin O in activation of the DDR in response to γ-radiation and the consequences in the cell survival. Using Cyclin O deficient cells as a cellular model, we have found that the Cyclin O limits the DNA resection, a process that drives the cell to repair the DNA damage by HR. Moreover, we have seen that ATM activation and some of its downstream targets are not properly activated after DNA damage in Cyclin O deficient cells. We also have found that Cyclin O complexes are able to phosphorylate ATM *in vitro* opening the door to study a new mechanism of the DDR regulation by Cyclin O.

RESUM

La Ciclina O és una nova ciclina que interacciona amb CDK1 i CDK2, i que s'ha demonstrat ser necessària per l'apoptòsi induïda per radiació gamma en una linia cel·lular d'origen linfoide. La radiació gamma indueix la formació de talls de doble cadena (DSBs) al DNA activant la resposta per dany al DNA (DDR) per tal de reduïr-ne les conseqüències citotòxiques i reparar el dany al DNA. L'objectiu d'aquesta tesi ha esta el d'estudiar el paper de la Ciclina O en l'activació de la resposta per dany al DNA i les conseqüències sobre la supervivència cel·lular. Utilitzant cèl·lules deficients en Ciclina O com a model, hem trobat que la Cyclina O limita el processament dels talls de doble cadena necessaris per a la reparació del dany al DNA per recombinació homologa. També hem una deficient activació d'ATM i la fosforil·lació d'alguns substrats d'aquesta proteína en cèl·lules deficients per la Ciclina O. Finalment, hem vist que els complexes de Ciclina O fosforil·len ATM *in vitro*, un fet que obre una porta a l'estudi de nous mecanismes de regulació de la resposta per dany al DNA mitjançant la Ciclina O.

Index

ABBREVIATIONS

53BP1	p53 Binding Protein 1
9-1-1	Rad9-Hus1-Rad1
aa	amino-acid
ARCA	Apoptosis-Related CDK2 Activator
A-T	Ataxia Telangiectasia
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine triphosphate
ATR	Ataxia Telangiectasia and Rad3 related
ATRIP	ATR Interacting Protein
AU	Arbitrary Units
BER	Base-Excision Repair
BLM	Bloom syndrome, RecQ helicase-like
bp	base pair
BRCA1	Breast Cancer 1
BRCA2	Breast Cancer 2
BRCT	BRCA1 C Terminus
BrdU	Bromodesoxiuridina
BSA	Bovine Serum Albumin
CA1	Cornu Ammonis, subdivision 1
CA3	Cornu Ammonis, subdivision 3
CAD	Caspase-Activated Dnase
Cdc25	Cell Division Cycle 25
CDK	Cyclin Dependent Kinase
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CSR	Class Switch Recombination
CtBP	C-terminal-binding protein 1
C-terminal	Carboxy Terminal
CtIP	CtBP-interacting protein
DAPI	4 ',6-diamino-2-fenilindol
DDR	DNA Damage Response
DISC	Death-Inducing Signaling Complex

DNA	Deoxyribonucleic Acid
DNA-PK	DNA Dependent Protein Kinase
DNApol	DNA polymerase
DSB	Double Strand Break
dsDNA	double stranded Deoxyribonucleic Acid
DTT	Dithiothreitol
ES cell	Embryonic Stem cell
FACS	Fluorescence-Activated Cell Sorting
FANCM	Fanconi Anemia, Complementation group M
FCS	Fetal Calf Serum
ɣH2AX	Gamma (phosphorylated-S139) H2A histone family, member X
H&E	Hematoxilin and Eosin
HET	Heterozygous
HH3	Histone H3
HOM	Homozygous
HR	Homologous Recombination
HRP	Horseradish Peroxidase
IAP	Inhibitor of Apoptosis
ICAD	Inhibitor of CAD
IF	Immunofluorescence
IL2	Inteleukin-2
IR	Ionizing Radiation
IRIF	Ionizing Radiation-Induced Foci
KD	Kinase Dead
KDa	KiloDalton
MDC1	Mediator of DNA-damage checkpoint 1
MEF	Mouse Embryonic Fibroblast
MMEJ	Microhomolgy-Mediated End Joining
MOMP	Mitochondrial Outer Membrane Permeabilization
MRE11	Meiotic Recombination 11
MRI	Magnetic Ressonance Imaging
MRN/MRX	Mre11-Rad50-Nbs1/Mre11-Rad50-Xrs2
MS	Mass-Spectrometry
NBS1	Nibrin
NHEJ	Non-Homologous End Joining
NLS	Nuclear Localization Signal or Sequence
N-terminal	NH2-terminal domain
OB-fold	Oligonucleotide/Oligosaccharide-Binding fold

PARP	Poly-(ADP-Ribose) polimerase
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PEI	Polyethylenimine
PI	Propidium Iodide
Pi3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIKK	Phosphatidylinositol 3-kinase-related kinases
PIN1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
PTMs	Posttranslational Modifications
RDS	Radioresistant DNA Synthesis
RIF1	RAP1 (Ras-proximate-1 or Ras-related protein 1) interacting factor
RNA	Ribonucleix Acid
RNApol	RNA polumeras
RPA	Replication Protein A
SCE	Sister Chromatide Exchange
SCID	Severe Combined Immunodeficiency
SDS	Sodium Dodecyl Sulfate
SDSA	Synthesis-Dependent Strand Annealing
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SEM	Standard-Error of the Mean
SIRT6	Sirtuin 6
SMC	Structural Maintenance of Chromosomes
SSB	Single Strand Break
ssDNA	single-stranded DNA
Tag	SV40 T Antigen
TBS-T	Tris-Buffered Saline - Tween20
TdT	Terminal deoxynucleotidyl transferase
TopBP1	Topoisomerase (DNA) II binding protein 1
UV	Ultraviolet
WT	Wild Type
XLF	XRCC4-Like Factor
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4

INTRODUCTION

1. DNA DAMAGE AND REPAIR

1.1.Short introduction

Since the discovery of the structure of the DNA more than 50 years ago, the mechanisms that preserve the genetic information and guarantee its transmission across generations have been of special interest. To maintain the genomic integrity, the DNA must be protected from damage induced by environmental agents or generated spontaneously during DNA replication and as a consequence of the metabolism of the cell. In response to this damage, healthy cells activate different mechanisms to reduce the consequences of these insults. In this context, the cell cycle is stopped and the repair machinery is recruited to the damaged sites in order to repair the DNA. If the alterations can not be repaired, or if the damaging agent persist the cell activates pro-apoptotic mechanisms (Ciccia & Elledge, 2010). Maintaining the continuity and stability of each DNA molecule is fundamentally important in preventing chromosomal rearrangements that can lead to cancer through altered gene expression (Thompson, 2012).

1.2.DNA damage agents & types of DNA damage

DNA damage can be produced either spontaneously as a consequence of the endogenous cellular metabolism or induced by exogenous or environmental agents. Spontaneous DNA damage occurs continuously in actively dividing cells as a consequence of defects during DNA replication such as the misincorporation of dNTPs. Also chemical interconversion, loss or modification of the

nitrogenated bases can occur due to deamination, depurination or alkylation. Additionally, products derived from the normal cellular metabolism such as ROS can lead to the oxidation of the nitrogenated bases or generate DNA breaks.

Environmental or exogenous DNA damage can be produced by either physical or chemical agents. Among the physical agents that generate DNA damage we can find Ionizing (IR) and Ultraviolet radiation (UV). IR can generate SSBs or DSBs which are the most harmful types of DNA damage. The sources of IR can be from different origin, for example cosmic radiation, which is a type of γ-radiation from a natural origin. On the other side, the X-rays and radiotherapy are commonly used in medicine for diagnosis and treatment of diseases, respectively. Chemicals agents that change the native structure of the DNA are often used in chemotherapy and generate a broad spectrum of DNA lesions: alkylated DNA bases (i.e. MMS), covalently crosslinked bases from the same (intrastrand) or different (interstrand) DNA strands (i.e. MMC, cisplatin or nitrogen mustard). Special harmful types of chemical agents are the inhibitors of topoisomerases I or II (also called radiomimetic drugs) such as camptothecin (CPT) or etoposide, respectively. These types of chemical agents induce the formation of SSBs or DSBs by trapping topoisomerase-DNA covalent complexes (Ciccia & Elledge, 2010; Hoeijmakers, 2009).

Each type of DNA damage generated is preferentially repaired by a specific mechanism, depending on the cell cycle stage and other cellular circumstances (i.e. differentiation status, nutrient availability or persistence of the insult). The main correspondences between

the types of DNA damage and the repairing mechanisms used are summarized in Figure.I1.

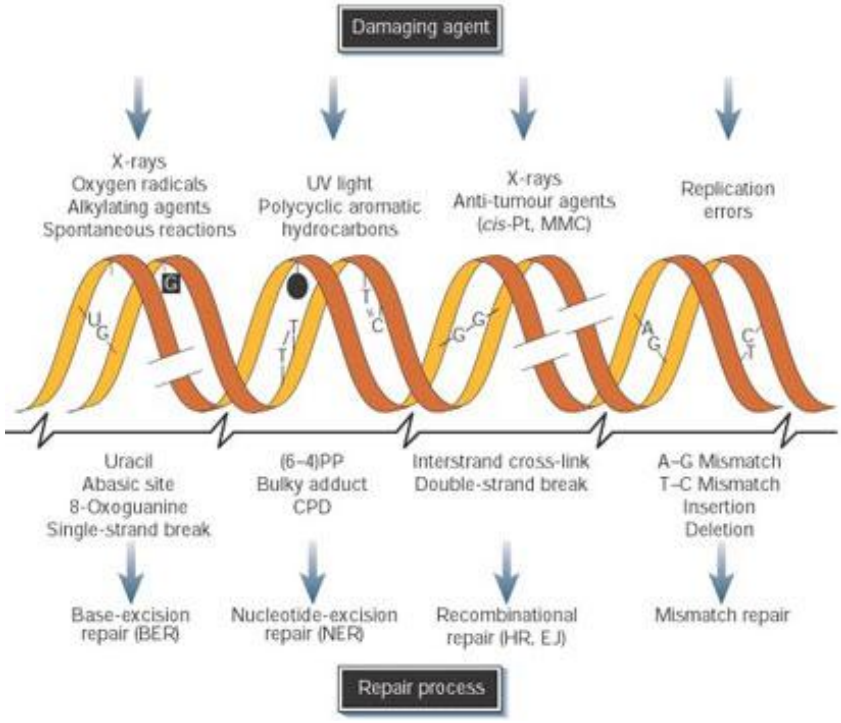


Figure.I1. – **Damaging agent, DNA damage type and repair mechanisms.** Adapted from (Hoeijmakers, 2001)

During the rest of the thesis I will focus on the DNA damage response (DDR) implicated in the repair of the DSBs.

1.3.The DNA damage response (DDR)

The DNA Damage Response is a group of molecular pathways implicated in DNA repair. It includes the biochemical events from the detection of the DNA lesion until the DNA is repaired and the normal cell cycle progression is resumed or the cell death

mechanisms are activated if the damage is severe or persistent. The proper activation of each DNA repair mechanism depends on the nature of the DNA lesion generated and requires a tight regulation of the molecular pathways that commit the cell to this particular mechanism. The molecular interconnections amongst all these pathways that inhibit or enhance each other are crucial to preserve the genomic integrity and prevent the cell to become transformed.

All these signaling pathways are mediated by proteins that can be classified in sensors (Ku, RPA, MRN complex), transducers (ATM, ATR and DNA-PK), mediators (53BP1, MDC1, BRCA1, TopBP1, etc.) signal amplification kinases (CHK1, CHK2) and effectors of the cellular responses (p53, CDC25, p21 etc.) (Figure.I2) (d'Adda di Fagagna, 2008; Zhou & Elledge, 2000).

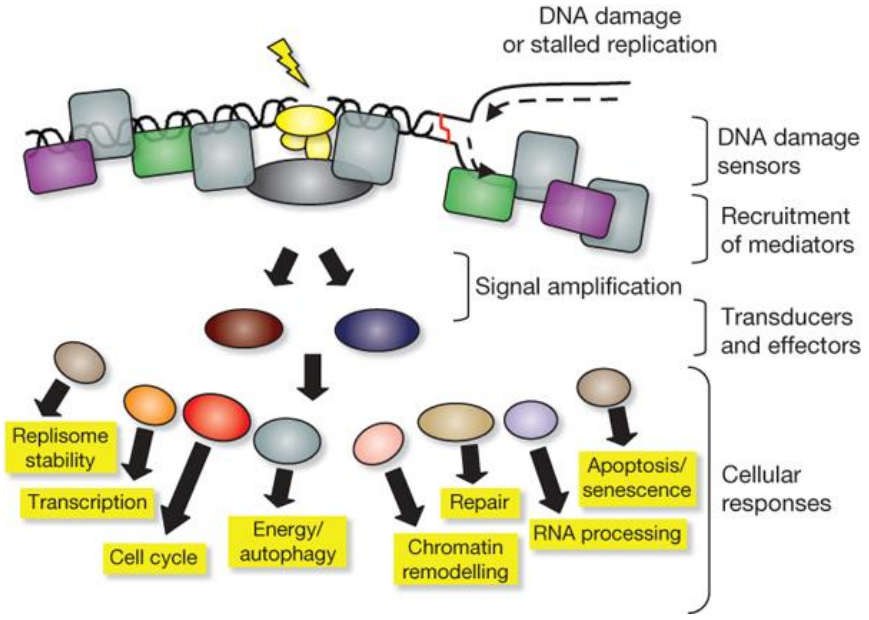


Figure.I2. – **Steps of the DDR and classification of the components** Adapted from (Jackson & Bartek, 2009)

There are two main mechanisms that are able to repair the DSBs: Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ). HR is based on the recruitment of members of the Rad and BRCA family of proteins and after detecting the DSBs there is a search for homologous regions in the genome to reconstitute the broken DNA. This mechanism prevents the errors generated by the DNA damage and the repair mechanism itself, but is less efficient than others and it is only possible during the S and G2/M phases of the cell cycle. On the other side, NHEJ is dependent on DNA-PKs and other detection proteins. During this mechanism the broken ends are processed for the ligation of independent Double Strand DNA ends. This mechanism is the most efficient way to repair DSBs but it is error prone and it can generate chromosomal aberrations.

1.3.1.Recognition of the DNA damage

Among the first proteins recruited to DNA breaks are those able to directly recognize DNA breaks. These proteins bind broken DNA in a sequence-independent manner and thus act as molecular sensors of DNA breaks. However, the activity of the DNA damage sensors is not only restricted to the recognition of the DNA lesions. They are also important for the stabilization of the DNA ends until they are repaired, in the scaffolding and recruitment of repairing proteins, the remodeling of the chromatin and they provide the

catalytic activities that prepare the DNA ends to be repaired (Figure.I3).

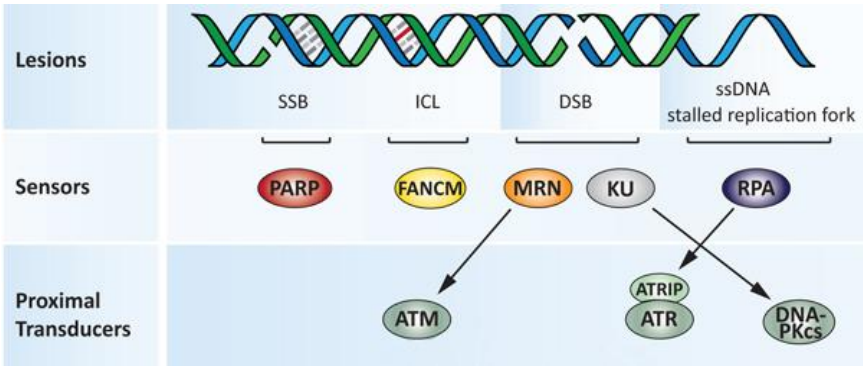


Figure.I3. – **Different types of DNA damage are recognized by different sensors and activate different DNA repair mechanisms.** Adapted from (Hühn, Bolck, & Sartori, 2013)

1.3.1.1.PARP

Poly (ADP-ribose) polymerase is a family of proteins which contain a catalytic domain that mediates the synthesis of PAR (poly (ADP-ribose)) chains. PAR is a PTM that mediates the recruitment of several proteins that execute the DNA repair. PARP family has 16 members but only PARP1, PARP2 and PARP3 have been related with DNA repair (Figure.I4a). PARP1 has been reported to bind several aberrant DNA structures and catalyze the addition of poly (ADP-ribose) chains on proteins to recruit DDR factors to chromatin breaks. Two zinc finger motifs (known as FI/Zn1 and FII/Zn2 motifs) of PARP1 recognize the DSBs and SSBs. On the other hand, the binding of PARP1 to the DNA relays on the CD domain, which is also a zinc finger motif (known as FIII/Zn3) (Figure.I4b/c). It is though that the tethering of PARP1 to the DNA lesions is enough to enhance PAR production, but the strength and duration

of the binding and activity correlate with the severity of the DNA damage (Ali et al., 2012; Sousa et al., 2012; Steffen, et al., 2013).

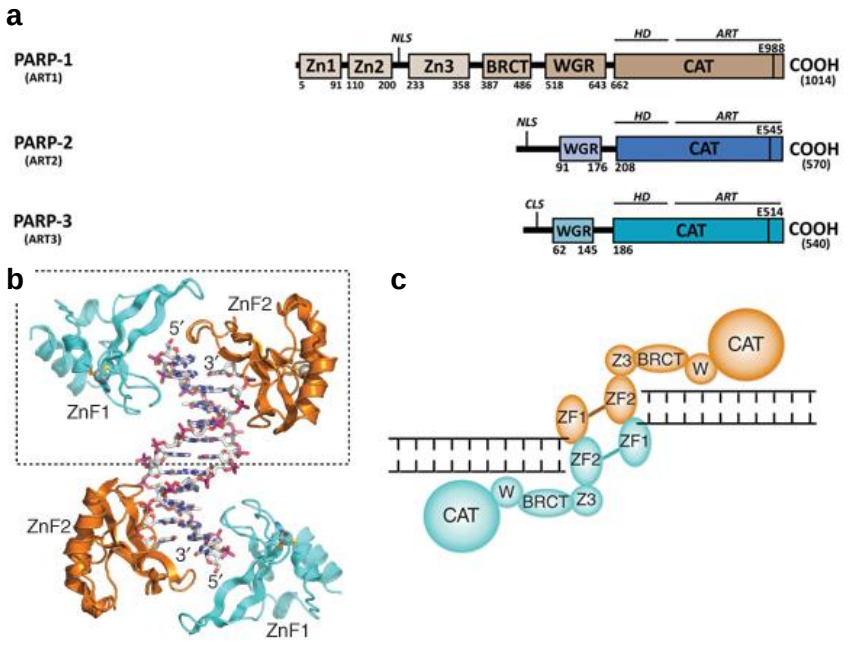


Figure.I4. – **PARP1/2/3 structure and catalytic domains (a)** Adapted from (Steffen et al., 2013)**. ZF1 (FI/Zn1) and ZF2 (FII/Zn2) motifs binding to DNA duplex (3D conformation) (b) and to DSBs (c)** Adapted from (Ali et al., 2012)**.**

The PARylation of target proteins such as Histones H1 and H2B contribute to chromatin reorganization and to the recruitment of important DNA repair and chromatin remodeling complexes at DNA damage sites such as ATM, ATR, DNA-PK, MRE11, NBS1, p53, Ku70/80 and DNA ligase IV. PARP1 promotes the recruitment of MRN complex and promotes HR. However, Ku (a sensor of DSBs that promotes NHEJ, as explained below) competes with PARP1 for DNA end binding. As PARP1, PARP2 also binds to SSBs but less efficiently through a basic DBD, and the activity of PARP2

accounts only for 10% of the total PAR production in response to DNA damage. Finally, PARP3 is activated *in vitro* by DSBs but its specific role has not been determined yet (Ciccia & Elledge, 2010; Mahaney, Meek, & Lees-Miller, 2009; Polo & Jackson, 2011).

1.3.1.2.Ku

Ku is necessary for the first step of the NHEJ, the detection of the DSBs, and is composed of Ku70 and Ku80 subunits, each of which contributes to a central DNA-binding core. Ku70-Ku80 forms a ring-shaped heterodimer which is among the earliest factors to directly bind DSBs. This property is provided by the structure of Ku70/80 center core, which adopts a pre-formed loop that encircles free dsDNA with high affinity and without sequence specificity as evidenced by its capacity to bind several DNA epitopes (Figure.I5) (Downs & Jackson, 2004; Mimori & Hardin, 1986). Ku70/80 is able to bind dsDNA fragments as short as 18bp and can also bind ssDNA but with a lower affinity. The binding of Ku70/80 to the DSB seems to assist in tethering broken ends together and protects them from DNA resection. Once bound to the DNA, Ku moves inwards the DNA fiber, in an ATP independent manner, allowing the binding of other proteins to the DNA termini, such as DNA-PK, XRCC4 and XLF. Ku also competes with PARP1 for DNA end binding in mammalian cells (Mahaney et al., 2009; Polo & Jackson, 2011).

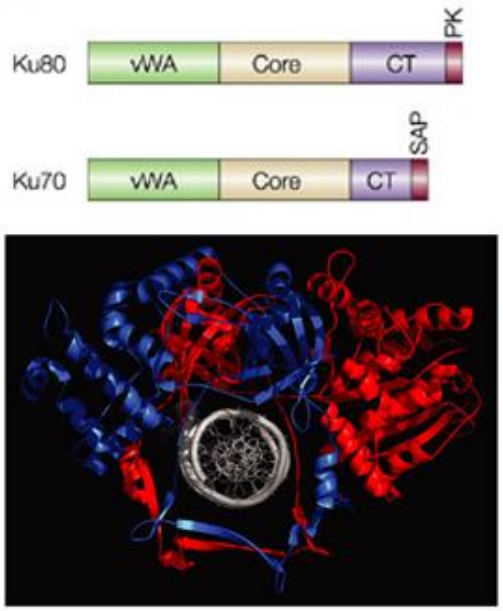


Figure.I5. – **Ku70 and Ku80 strtucture.** vWA:von Willbrand A domain; PK: DNA-PKcs binding domain. **(a). Crystal conformation of Ku bound to DNA.** Ku70 is indicated in blue, Ku80 in red and the DNA in grey **(b)**. Adapted from (Downs & Jackson, 2004)

1.3.1.3.MRN complex

DSBs can also be recognized by the MRN complex (Mre11-RAD50-NBS1) which promotes the activation of ATM. Mre11 associates with DNA using six DNA recognition loops at its N-terminus. In these conserved globular domains, 17 residues form sugar – phosphate interactions with the minor groove of the DNA (Stracker & Petrini, 2011). RAD50 is a member of the SMC family (Structural Maintenance of Chromosomes) and is a family of ATPases that participate in chromosome organization and dynamics (Nasmyth, 2011). Rad50 bears ATPase domains able to interact with MRE11 and to the DNA ends of the DSB. The dimerization of this protein is important for the tethering of DNA

ends together. Finally, NBS1 is a scaffolding protein that interacts with MRE11 and contains several protein-protein domains important for the DDR (Figure.I6). It is also important to note that Mre11 has endonuclease and exonuclease activities crucial for the initial steps of DNA resection and promoting HR. Of special interest is the association of NBS1 with ATM via the C-terminal region which promotes ATM recruitment to the DSBs. Importantly, the MRN complex compete for the DSBs with Ku70/80, which will be crucial to choose which DNA repair mechanism will repair the lesion (Ciccia & Elledge, 2010; Polo & Jackson, 2011; Stracker & Petrini, 2011).

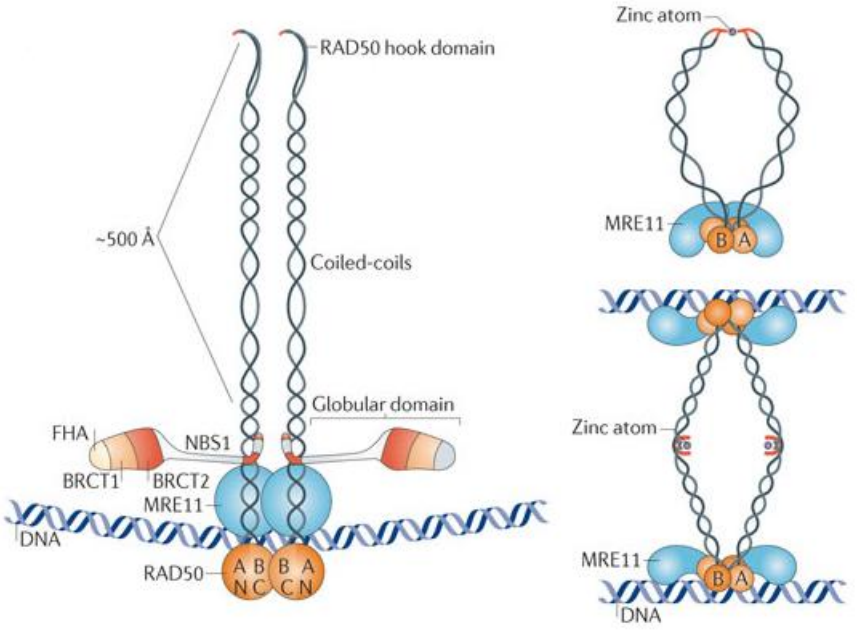


Figure.I6. – **MRN complex structure and association to the DNA.** Adapted from (Stracker & Petrini, 2011).

1.3.1.4.RPA

RPA is the most studied OB-fold containing protein. RPA accumulates at the resected DSBs (a process initiated by Mre11 and CtIP necessary for HR as it will be discussed later). RPA stabilizes ssDNA fibers and it is important for the recruitment of factors that promote HR (Y. U. E. Zou, Liu, Wu, & Shell, 2006). Proteins containing oligonucleotide/oligosaccharide-binding (OB)-fold motifs detect ssDNA fibers formed as a consequence of the processing of DSBs or SSBs after DNA resection and during gene transcription or replication. An OB-fold motif comprises β-strands arranged as cylindrical β-barrel, capped at one end by an α-helix (Figure.I7). This domain interacts with ssDNA through nucleotide base stacking with aromatic residues in the binding cleft and establishing electrostatic interactions with the phosphodiester backbone (Ashton, Bolderson, Cubeddu, O'Byrne, & Richard, 2013).

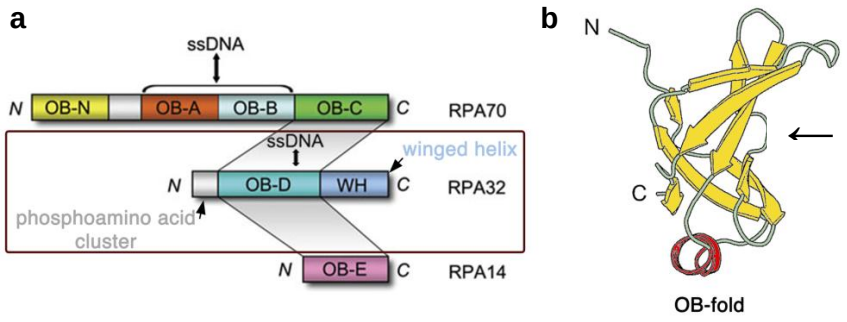


Figure.I7. – **RPA structure scheme (RPA70-RPA32-RPA14) (a)** Adapted from (J. Sun et al., 2011)**. 3D structure of the RPA OB-fold domain (b).** Adapted from AU2007268364.

The ssDNA fibers covered with RPA initiates the signaling pathway that will activate ATR and CHK1. RPA enhances the binding of Rad17 -RFC (Replication Factor C) and the recruitment of Rad9-Rad1-Hus1 complex (9-1-1 complex) (Bermudez et al., 2003; Doré, Kilkenny, Rzechorzek, & Pearl, 2009; Helt, Wang, Keng, & Bambara, 2005). 9-1-1 interacts with TopBP1, which binds the phosphorylated C-terminal tail of Rad9 and thereby activates the checkpoint kinase ATR (Figure.I8) (Delacroix, Wagner, Kobayashi, Yamamoto, & Karnitz, 2007; Joon Lee, Kumagai, & Dunphy, 2007).

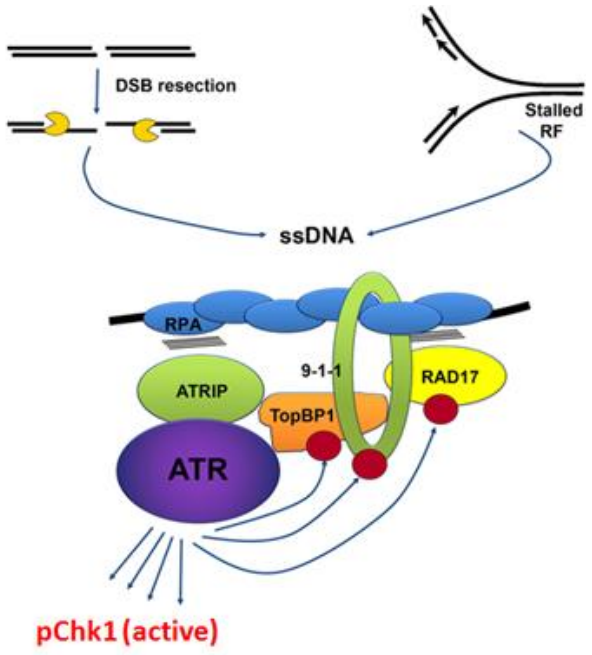


Figure.I8. – **Scheme of the RPA and 9-1-1 complex to the ssDNA and recruitment of ATR-ATRIP.** Adapted from (López-Contreras & Fernandez-Capetillo, 2010).

1.3.1.5.Other factors

Other OB-fold proteins are hSSB1 and hSSB2 and it has been suggested that they play a role in the response to stalled replication forks (Ashton et al., 2013). FANCM is an helicase/ATPase that also has an endonuclease activity that is able to recognize ICLs and stabilize stalled replication forks (Kee & D'Andrea, 2010). Other proteins such as helicases and nucleases involved in break repair are able to bind to the dsDNA-ssDNA junction (Polo & Jackson, 2011).

1.3.2.Transduction of DNA Damage Response

In response to DSBs, the main transducers activated are three members of the PIKKs (Phosphatidylinositol 3-kinase-related kinases) family: ATM, ATR and DNA-PK. PIKKs are a family of serine/threonine-protein kinases that contain a sequence similar to phosphatidylinositol 3-kinases (PI3Ks). Each PIKK is predominantly activated depending on the cell cycle stage and the nature of the DNA lesion, but all of them can be activated to some extent by the DNA damage. ATM and ATR are mainly related to HR during G2/M and S phases of the cell cycle, respectively. DNA-PK is related to NHEJ, but this mechanism can also be driven by ATM in the immune system (Ciccia & Elledge, 2010). The hierarchy of activation and the equilibrium amongst ATM, ATR and DNA-PK activities will determine the predominant signaling pathway and the mechanism of repair (Shiloh & Ziv, 2013). However, there is redundancy in their functions and the substrates activated. Although it has not been well clarified, the absence of one PIKK

could be replaced to some extend by another depending on the cell type and the cell cycle status, as seen in different reports (Callén et al., 2009; Serrano et al., 2013; Thomas Stiff et al., 2006; Tom Stiff et al., 2004).

1.3.2.1.ATM

ATM was identified as the gene mutated in ataxia-telangiectasia, a genetic disease characterized by severe neurodegeneration leading to ataxia, immunodeficiency, predisposition to lymphoma development and radiosensitivity (Savitsky et al., 1995). ATM is a 350KDa protein that contains a Pi3K signature at the C-terminal site, which is the most remarkable part of the protein. Separated by the kinase domain are located FAT and FATC domains which are likely to be involved in the regulation of the kinase activity (Bhatti et al., 2011). Moreover, rapidly after detecting DNA damage the FATC domain binds the HAT Tip60. The K3016 actylation of ATM activates the ATM kinase activaty (Y. Sun, Xu, Roy, & Price, 2007). Other domains are found in the rest of the ATM protein, among them, several repeats of the HEAT domain (common to Huntingtin, Elongation factor 3, protein phosphatase 2A (PP2A) and yeast Target of rapamycin 1 (TOR1), interaction sites with other proteins and a NLS. Other remarkable PTMs are represented in the Figure.I9 (Shiloh & Ziv, 2013). Of special interest are the autophosphorylation sites, specially the S1981 which is described later.

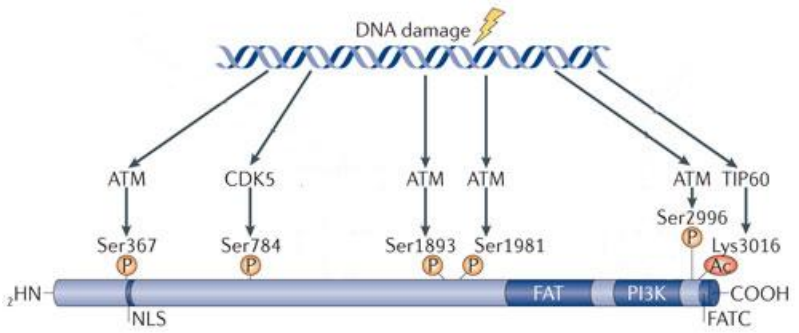


Figure.I9. – **Structure and PTMs of ATM after DNA damage.** Adapted from (Shiloh & Ziv, 2013)

ATM activation at the DBS depends on the MRN complex. In undamaged cells, ATM is a chromatin-associated protein that forms inactive dimers or multimers. Following DNA damage sensing by the MRN complex, ATM gets autophosphorylated on at least 4 residues (S367, S1893, S1981 and S2996) that promote its monomerization and activation of the kinase activity (Bakkenist & Kastan, 2003; Stracker, Roig, Knobel, & Marjanović, 2013). Phosphorylation of S1981 seems to be indispensable in human cells for ATM-dependent responses to DNA damage (Kozlov et al., 2011). However, it has also been demonstrated that the autophosphorylation of this residue (and also phosphorylaton of S367 and S1893) are dispensable to activate DDR in murine cells (Daniel et al., 2008; Pellegrini et al., 2006). Later on it was demonstrated that phosphorylation of the residue S1981 is necessary for the stabilization of ATM at the DNA damage sites (So et al.). Also, phosphorylation on ATM S794 by CDK5 in neurons has been shown to be a prerequisite for S1981 autophosphorylation and regulates neuronal cell death (Tian, Yang, & Mao, 2009).

ATM is recruited to the DNA damage sites through direct interaction with the C-terminal region of NBS1 which is, in turn, a phosphorylation target of ATM. Once activated, ATM phosphorylates several DNA associated proteins which are important mediators of the DDR. MDC1 interacts and is phosphorylated by ATM. MDC1 binds to the chromatin through the phosphorylated S139 of H2AX, which is also phosphorylated by ATM. This interaction promotes the recruitment of more active ATM at the DNA damage sites and the propagation of γH2AX phosphorylation. MDC1 anchored at γH2AX promotes the recruitment of RNF8 and RNF168 that ubiquitinate γH2AX promoting the recruitment of the mediator proteins 53BP1 and BRCA1. The recruitment of all this proteins and the concentration of PTMs surrounding the DSBs permit the detection of the sites of DNA damage/repair as discrete foci under microscope, the IRIF (Ionizing Radiation-Induced Foci) (Figure.I10) (Bekker-Jensen & Mailand, 2010; Belyaev, 2010). Other modifications at the level of the DNA include the relaxation of the chromatin mainly through the inactivation of KAP1 which is directly phosphorylated by ATM at S824 (Geuting, Reul, & Lo, 2013; J. Lukas, Lukas, & Bartek, 2011).

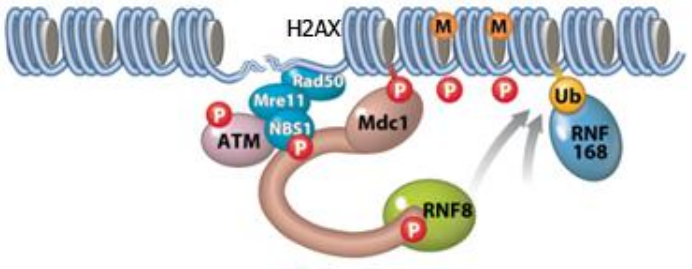


Figure.I10. – **Recruitment of proteins to IRIF in order to mediate DSB repair.** Adapted from (Weitzman, Lilley, & Chaurushiya, 2010).

To orchestrate the establishment of the scaffold to permit the recruitment of several DNA repair proteins is one of the functions of ATM. ATM is also important for the activation of the cellular checkpoints that will permit to stop the progression through to the next cell cycle phase until DNA damage is repaired. The pathways implicated to stop the cell cycle progression will be discussed later on, but it is important to note at this point that ATM phosphorylates effector proteins that either amplify the signal, such as CHK2, or change its transcriptional activity, such as p53. The phosphorylation of CHK2 at T68 by ATM is a prerequisite for its subsequent activation step, which is attributable to autophosphorylation at residues T383 and T387 in the activation loop of the kinase domain (Stracker and Petrini). Phosphorylation of p53 at S15 by ATM blocks the interaction of p53 with MDM2 and thus stabilizes the protein. p53 is a transcription factor that changes the transcriptional program after DNA damage activating the expression of genes implicated in the cell cycle stop, the DNA repair and apoptosis if the DNA damage can not be repaired. By repressing the expression of genes implicated in cell cycle progression, p53 safeguards the integrity of the genome by preventing the cell to transmit damaged DNA and mutations to daughter cells, and its activation is commonly used a readout of a proper ATM activation (Donehower, 2009; Goldstein et al., 2011).

ATM also regulates the last steps of the DNA repair and phosphorylates direct players in the process such as the nuclease Artemis, CtIP, DNA-PKcs or RAD9 (Shiloh & Ziv, 2013). Although the DNA repair mechanisms activated by ATM depend on the cellular context, it is commonly related with the activation of HR.

A crucial event in HR is the initiation of DNA end resection by CtIP. CtIP/SAE2 (Sae2 in yeast) was originally identified as being required to complete meiotic recombination in *S. cerevisiae*. Subsequent genetic and biochemical studies in yeast and mammalian cells have shown that Sae2 and its human counterpart CtIP cooperate with the nuclease activity of the MRX/MRN complex to initiate resection of DSBs (Ferretti, Lafranchi, & Sartori, 2013). DNA resection, mediated by CtIP and Mre11, definitively commits the cell to use HR instead of NHEJ. CtIP interacts with BRCA1 and the MRN complex to initiate DNA resection in S or G2/M cell cycle stages. CtIP has also been shown to have transcriptional regulation properties and the capacity to regulate the DNA damage checkpoints. At the MRN complex binding site of CtIP there are two residues putative phosphorylation targets of ATM (S664 and S745). However, it is not clear the importance of these residues in the regulation of CtIP (You & Bailis, 2010).

We can conclude that ATM is a central protein in the DDR at several steps ranging from the sensing to the final steps of the DNA repair as summarized in the Figure.I11. However, mouse models completely lacking ATM show a mild phenotype when compared to A-T human patients. Two different groups have created mouse models where ATM is present in an inactive form or express a kinase dead protein (Yamamoto et al.; Daniel, Pellegrini, B.-S. Lee et al.). These animal models are embryonic lethal with inherent genomic instability, at variance with what is observed in $ATM^{-/-}$ mice. It is possible, therefore, that the presence of an inactive form of ATM at the DDR hubs severely hampers the ability of the cell to respond to the DNA damage probably by blocking side pathways and redundancies among PIKKs or by interfering with the

spatiotemporal organization of the IRIF and the response (J. Lukas et al., 2011). However, as explained before, abolishing the phosphorylation sites in mouse Atm, whose equivalents in human ATM are phosphorylated during its activation, did not result in any discernible phenotype (Daniel et al., 2008; Pellegrini et al., 2006).

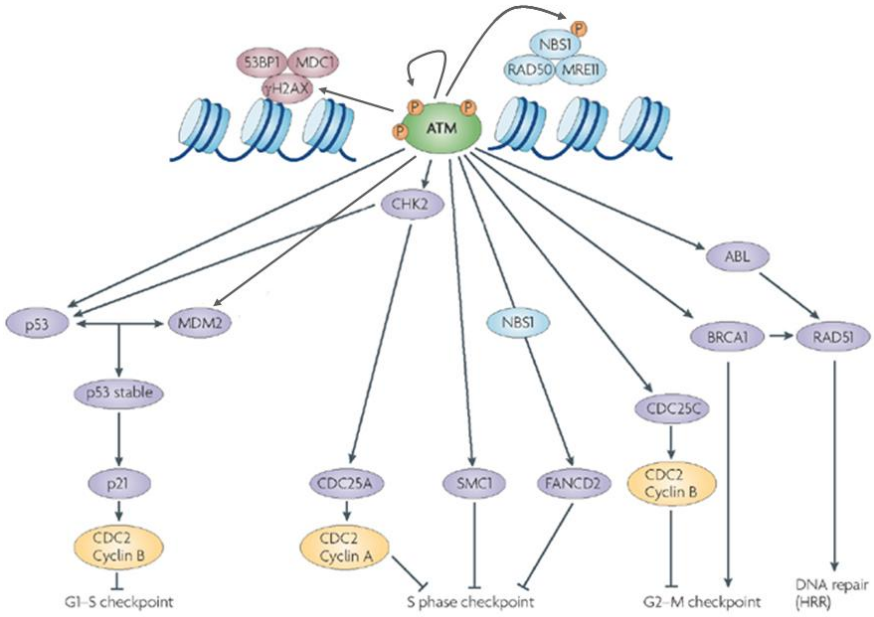


Figure.I11. – **ATM substrates and cellular response to ATM signaling.** Adapted from (Lavin, 2008)

1.3.2.2.DNA-PK

DNA-dependent Protein Kinase is a protein of more than 4000aa and 460kDa of molecular weight (Figure.I12). DNA-PK works as the central protein repairing DSBs by NHEJ especially during G1. DNA-PK stabilizes DSB ends preventing end resection after the Ku70/80 complex has detected the damage. Ku80 interacts with DNA-PK through the conserved C-terminal domains of both

proteins. DNA-PK contains a protein kinase catalytic domain which is activated after the binding to Ku80 and autophosphorylates T2609 and at least 5 other residues in the ABCDE domain (a protein region that contains the residues S2612, S2624, T2609, T2620, T2638 and T2657) in the center of the protein sequence. These residues from the ABCDE domain have been found to be also phosphorylation targets of ATM and ATR in response to IR and UV, respectively, depending on the cell type, cell cycle stage and extent of DNA damage (B. P. C. Chen et al., 2007; Yajima, Lee, & Chen, 2006). After phosphorylation, DNA-PK gets released from the DNA providing access to DNA processing enzymes, such as ARTEMIS. Excessive end processing is prevented by DNA-PK autophosphorylation at the PQR cluster (Mahaney et al., 2009; Meek K, Dang V, 2008). Importantly, phosphorylation of the ABCDE domain has been shown to permit the access of DNA ends to DSBs resecting enzymes in order to promote HR when NHEJ fails (Shrivastav, De Haro, & Nickoloff, 2008) while phosphorylation of PQR has an inhibitory effect on HR by preventing end resection (Meek K, Dang V, 2008) .

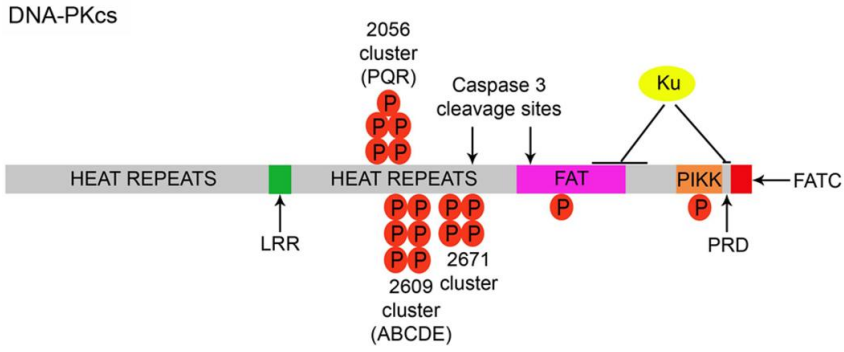


Figure.I12. – **Structure and PTMs of DNA-PKcs after DNA damage.** Adapted from (Mahaney et al., 2009).

These observations evidence how important is the fine tuning regulation through the redundancy and reciprocal inhibition of the DDR pathways to promote the proper mechanism of repair in a given cell type and context. *In vitro*, DNA-PK phosphorylates several substrates implicated in the NHEJ such as Ku70, Ku80, XRCC4, XLF, ARTEMIS and DNA ligase IV. However, there is little evidence that any of these events is required for NHEJ *in vivo* (Mahaney et al., 2009). ARTEMIS for example, has been identified as a substrate of ATM implicated in the repair of some complex DNA lesions *in vivo* (Jeggo & Löbrich, 2005). On the other side, DNA-PKcs phosphorylates γH2AX in response to DNA damage at S139 participating together with ATM in the formation of IRIF and recruitment of mediator proteins (Tom Stiff et al., 2004).

Mice lacking DNA-PKcs are highly radiosensitive and present defects in the development of the immune system due to a defect in the V(D)J recombination, as it is the case of the SCID mice (Meek K, Dang V, 2008). Moreover, in line with the case of ATM, mice expressing a mutant version of DNA-PKcs lacking 3 phosphorylation sites associated with its activation, die shortly after birth due to bone marrow failure, evidencing the importance of having an active form of DNA-PK (S. Zhang et al., 2011). This data from genetically modified mice somehow confirm the redundancy among the PIKKs in the response to DNA lesions.

1.3.2.3.ATR

The third member of the PIKKs implicated in the DDR is the protein ATM and Rad3-related (ATR). ATR is a 300KDa protein which is activated by several types of DNA damage such as DSBs, base adducts, crosslinks and replication stress (stalled replication forks)

(Nam & Cortez, 2011). However, it is believed that the common initiator is ssDNA coated by RPA, including the ssDNA formed during DNA repair, as a consequence of resection of the DSBs (Huertas, 2010). ATR recognition of ssDNA-RPA is dependent on ATRIP that should be considered a subunit of ATR (Figure.I13). The interaction take place through the acidic α-helix of ATRIP with the N-terminal of the OD-fold domain in the large RPA subunit (Ball, Myers, & Cortez, 2005; L. Zou & Elledge, 2003). However, this interaction is not sufficient for the activation of ATR and requires the colocalization with the 9-1-1 complex (Rad9-Rad1-Hus1). This is a ring-shaped molecule that recognizes the DNA end adjacent to a stretch of ssDNA-RPA in an ATP-dependent manner (Delacroix et al., 2007; Joon Lee et al., 2007). 9-1-1 complex binds and recruits TOPBP1 at the DNA damage sites, a protein necessary for ATR activation. TOPBP1 BRCT domains I and II interact with S387 of Rad9. TOPBP1 contains an activation domain that binds and activates ATR-ATRIP. Overexpression of this activation domain is sufficient to activate the phosphorylation of some ATR substrates and induce senescence bypassing the requirement for DNA damage. In turn, TOPBP1 is phosphorylated by ATM at S1131 and this phosphorylation is important for its interaction with ATR, thus allowing its activation at the DNA damage sites and permitting amplification of the signal (Kumagai, Lee, Yoo, & Dunphy, 2006; Mordes, Glick, Zhao, & Cortez, 2008).

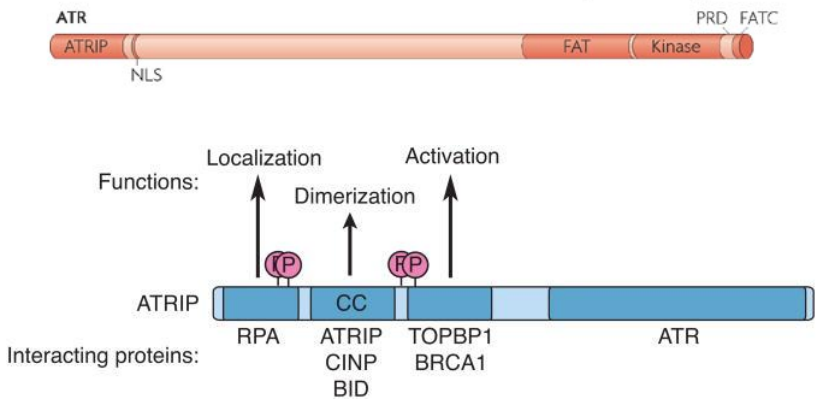


Figure.I13. – **Structure, PTMs and interactions of ATR (up)** (K. a Cimprich & Cortez, 2008) **and ATRIP (down)** (Nam & Cortez, 2011) **after DNA damage.**.

Some phosphorylations have been identified on ATR-ATRIP. However, none of them has been demonstrated to be a reliable marker of ATR activation. Thus, the way to detect ATR activation is to analyze the phosphorylation of its downstream targets. The most widely used substrate is CHK1 which is phosphorylated at S317 and S345. CHK1 is activated at the DNA damage sites and then it is released and amplifies the signal all throughout the nucleus (Q. Liu et al., 2000; H. U. I. Zhao & Piwnica-worms, 2001). Claspin is a mediator that interacts with CHK1 via phosphorylated S864 and S895 of Claspin and holds CHK1 and ATR together. Activated CHK1 phosphorylates and inhibits the CDC25 phosphatase, preventing the removal of inhibitory phosphorylations from the CDKs. This prevents the entry into mitosis and the regulation of replication origin firing (K. a Cimprich & Cortez, 2008; Travis H. Stracker, Takehiko Usui, 2010). (Figure.I14).

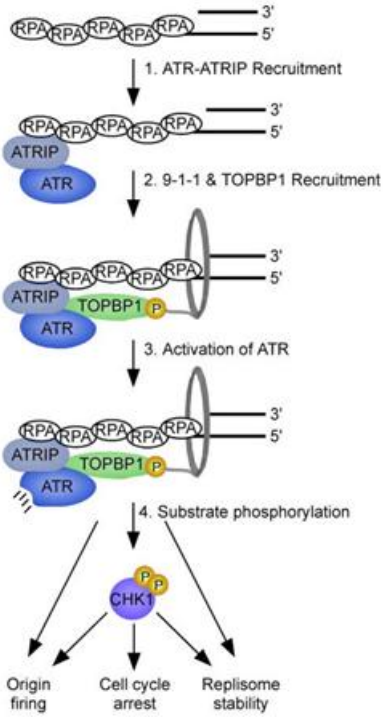


Figure.I14. – **Scheme of the ATR activity in the DDR.** Adapted from (Nam & Cortez, 2011).

ATR activity at the sites of DNA damage promotes the stabilization of the replication forks already initiated and the recovery of the stalled replicated forks to ensure completion of DNA replication. The ATR substrates present at the fork are the replication factor C complex, RPA1 and 2, MCM (microchromosome maintenance) 2 to 7 (helicases that unwind DNA duplex) and DNA polymerases. Of special interest is the phosphorylation of MCM2 on S108 by ATR. This phosphorylation permits the recruitment of Plk1 to the DNA damage sites and promotes the creation of new replication origins locally close to the DNA damage sites. Thus, Plk1 inhibits CHK1 locally at the DNA damage sites allowing to the completion the replication in these problematic areas (Ge & Blow, 2010).

In contrast to what is seen in the case of the loss of ATM, the complete loss of ATR in the mouse is embryonic lethal. However, a mouse model bearing a hypomorphic mutation in the ATR locus that reduces the abundance of the protein to undetectable levels permits the viability of the mice. These mice show high levels of replicative stress during embryogenesis and accelerated aging in adults. This phenotype is reminiscent of the human Seckel's Syndrome characterized by a sever deficiency in ATR (Murga et al., 2009).

A schematic summary of the responses initiated by the PIKKs in response to the DNA damage can be seen in the Figure.I15.

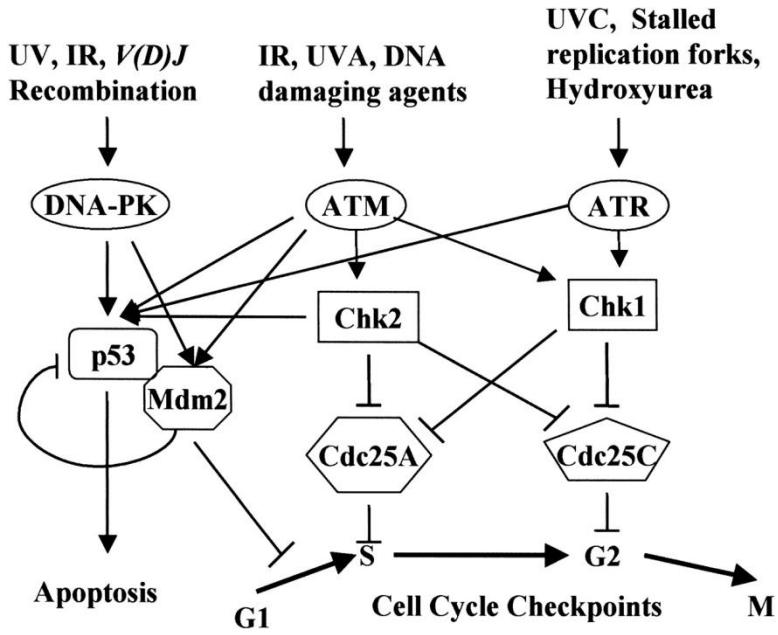


Figure.I15. – **PIKKs signaling in response to different types of DNA damage.** Adapted from (J. Yang, Yu, Hamrick, & Duerksen-Hughes, 2003)

1.4.Cell cycle checkpoints and the DDR.

Cell cycle progression is strictly regulated by different CDKs and cyclins. Cyclin expression is tightly regulated through the cell cycle what ensures a proper CDK activation depending on the cell phase. However, cytotoxic insults, such as radiation or drugs, can stop the cell cycle progression leading to an active and regulated status that permits to repair the damage before the cell cycle is resumed. These mechanisms are the cell cycle checkpoints (Figure.I16). Three of these checkpoints (G1-S, intra-S and G2/M checkpoints) are activated by DNA damage, but there is another checkpoint during M phase which is activated by condensed chromosomes with kinetochores unattached to the mitotic spindle or anti microtubule drugs such as nocodazole (Gabrielli, Brooks, & Pavey, 2012; Rieder & Maiato, 2004).

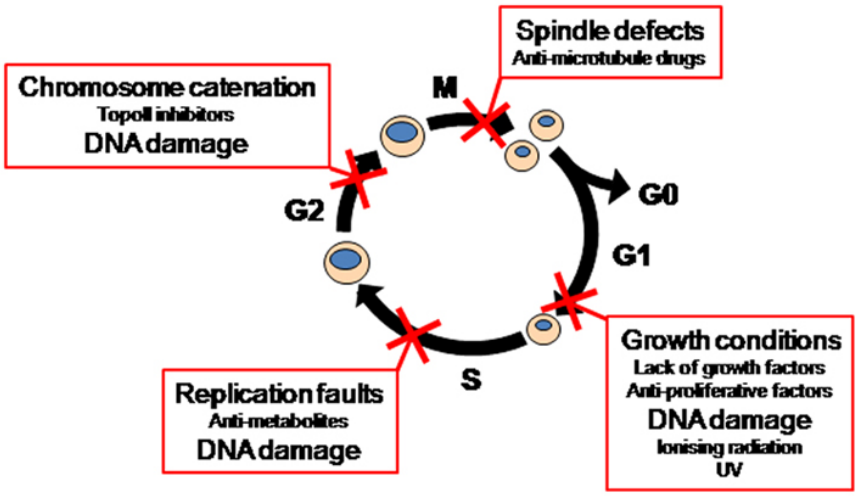


Figure.I16. – **Cell cycle checkpoints are triggered by different agents.** Adapted from (Gabrielli et al., 2012)

The signals that link DNA damage detection to the checkpoint activation depend on the ATR-CHK1 and ATM-CHK2 pathways. These two kinases have overlapping activities depending on the cell cycle stage. CHK2 is activated by ATM regardless of the cell cycle stage but CHK1 is preferentially activated by ATR during S and in G2. In G2, the activation of ATR is due to the formation of ssDNA fibers as a consequence of DNA resection (Bartek & Lukas, 2007). In general, cell cycle stop triggered by checkpoint activation is mainly regulated by two different mechanisms (Figure.I17). The first involves the Cdc25 family of phosphatases which are essential for the removal of the inhibitory phosphorylations from the CDKs (Yata & Esashi, 2009). On the other hand, the p53 tumour suppressor that integrates several stress signals, including DNA damage, regulates the expression of the CDK inhibitor $p21^{CIP1/WAF1}$ (CDKi) (Sperka, Wang, & Rudolph, 2012).

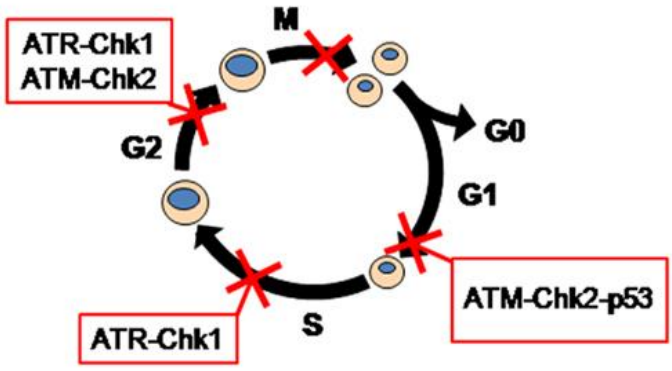


Figure.I17. – **Cell cycle checkpoints regulators.** Adapted from (Gabrielli et al., 2012)

1.4.1.The G1/S checkpoint induced by IR

G1 checkpoint is driven by ATM phosphorylation of CHK2 and p53 leading to the activation of two independent pathways (Figure.I18). In the p53-independent signalling arm, CHK2 activation in late G1 phosphorylates the Cdc25A phosphatase. This leads to Cdc25A ubiquitylation and proteasome-mediated degradation. Cdc25A is a member of the Cdc25 familiy of dual phosphatases able to dephosphorylate Ser, Thr and Tyr aminoacids. The degradation of Cdc25A results in persisting inactivating phosphorylations of CDK2 at T14/Y15 by Myt1 and Wee1 (J. Lukas, Lukas, & Bartek, 2004; Mailand, 2000).

In the p53-dependent arm of the G1 checkpoint, DNA damage results in ATM- and CHK1/2- mediated phosphorylation of p53 at S15/S20 and T18/S20 respectively leading to the stabilization of p53. These phosphorylations disrupt the interaction of Mdm2 with p53 resulting in the accumulation of p53 in the nucleus and in the change of the transcriptional program. Mdm2 is the main responsible of the control of the p53 levels via ubiquitylation, sending it to proteasomal degradation. Moreover, ATM phosphorylates Mdm2 permitting its ubiquitylation and degradation (Sherr & Roberts, 1999). The expression of $p21^{CIP1/WAF1}$ (CDKN1A/p21) in response to the ATM/Chk/p53 pathway promotes G1 arrest by inhibiting mainly CDK2. Other proteins necessaries for a proper G1 to S checkpoint activation, probably enhancing ATM activity, are the mediators TOPBP1 and 53BP1 (Cescutti, Negrini, Kohzaki, & Halazonetis, 2010). Thus, damaged cells that fail to arrest at G1 enter S-phase with unrepaired DSBs that give rise to chromosomal breaks in the G2 phase.

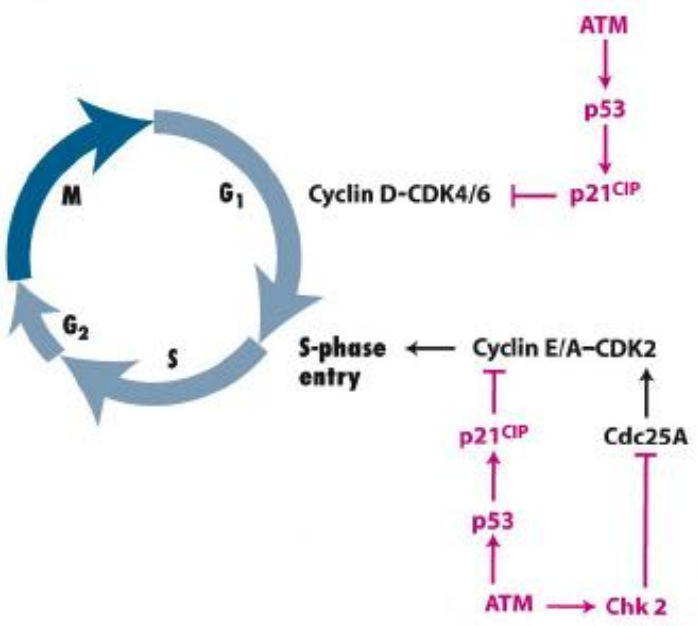


Figure.I18. – **Regulation of the G1-S checkpoint.**

1.4.2.The intra-S checkpoint

Cells in S-phase when detect DNA damage activate the intra-S phase checkpoint, leading to a transient reduction in DNA synthesis and to the suppression of replication origin firing. Defects in this checkpoint are characterized by radioresistant DNA synthesis (RDS) (Bartek, Lukas, & Lukas, 2004). The intra-S phase checkpoint is controlled by ATM and ATR which activate two parallel pathways (Figure.I19). When DNA damage is sensed, ATM and ATR activate both CHK2 and CHK1 kinases that phosphorylate the Cdc25A phosphatase leading to its degradation by ubiquitylation and proteasome-mediated degradation and resulting in the inhibition of CDK2 activity and origin firing (Falck, Petrini, Williams, Lukas, & Bartek, 2002). Moreover, SMC1 is also

implicated in this checkpoint. SMC1 is a member of the SMC family necessary for chromatid cohesion after DNA replication during S-phase (Nasmyth, 2011). Phosphorylation of SMC1 at S957 and S966 by ATM is important to prevent RDS although the specific mechanism is not yet known (Kitagawa, Bakkenist, McKinnon, & Kastan, 2004; Luo, Deng, Cheng, Li, & Qiu, 2013).

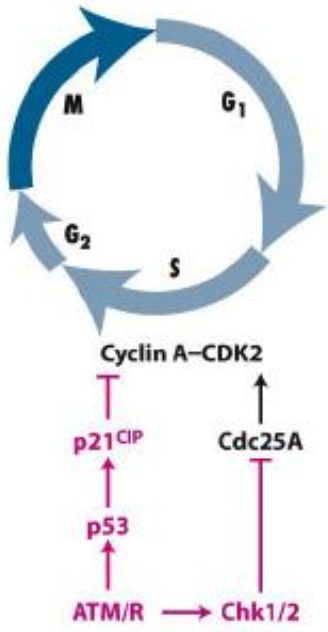


Figure.I19. – **Regulation of the intra S checkpoint.**

1.4.3.The G2/M DNA damage checkpoint

DSBs generated during the G2 phase of the cell cycle initially activate ATM which is sufficient to initiate checkpoint activation (Stracker et al., 2013). However, as a consequence of the generation of ssDNA due to DSB resection, ATR is rapidly activated rapidly after DNA damage (Figure.I20) (MacDougall, Byun, Van, Yee, & Cimprich, 2007). After that, CHK2 and CHK1

are activated and phosphorylate the Cdc25C phosphatase generating a binding site for 14-3-3, a regulatory protein that has affinity to bind and regulate the activity of several signalling proteins. The binding to 14-3-3 leads to the exit of Cdc25C from the nucleus, keeping the phosphatase away from its substrates, notably CDK1 (Matsuoka, 1998; C. Peng, 1997; Sanchez, 1997). Moreover, ATR-CHK1 has been show to be the main regulator of CDK1-cyclin B activity, via control of the Cdc25A protein levels (Sørensen et al., 2003; H. Zhao, Watkins, & Piwnica-Worms, 2002). Also, CHK1 phosphorylation of Cdc25A leads to its ubiquitylation and proteasomal degradation after DNA damage but, in the case of CHK1 it does so only during the S and G2 phases of the cell cycle (Busino et al., 2003; J. Jin et al., 2003). In conclusion, both CHK1 and CHK2 regulate Cdc25A/C and the G2/M checkpoint. However, the importance of CHK2 in the G2-M checkpoint is under debate since CHK2 deficient cells activate properly the G2-M checkpoint, probably due to the redundant activity with CHK1 in phosphorylating Cdc25A during this phase of the cell cycle (Lossaint, Besnard, Fisher, Piette, & Dulić, 2011; Takai et al., 2002).

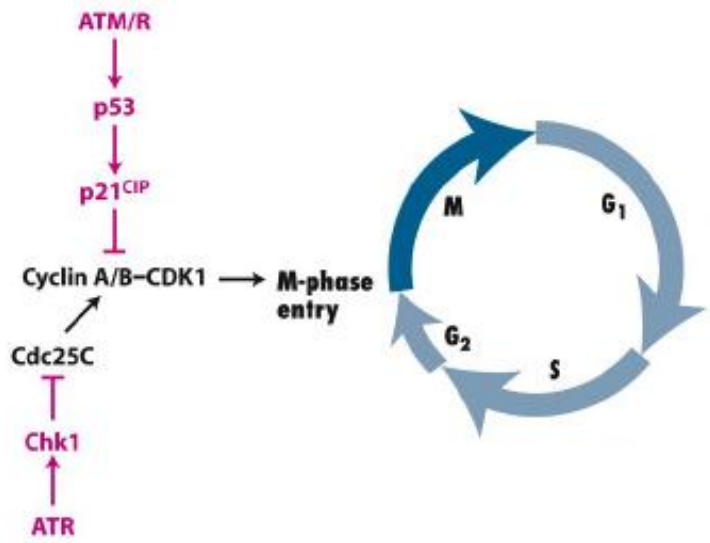


Figure.I20. – **Regulation of the G2-M checkpoint.**

For the checkpoint maintenance, it is necessary CtIP-mediated DNA resection (Figure.I21). This generates ssDNA leading to a switch from ATM to ATR dependency in the checkpoint control. CtIP deficiency or inactive mutant variants of the protein leads to the premature entry into mitosis with the DNA repaired by NHEJ instead of HR (A. N. Kousholt et al., 2012; Shibata et al., 2011).

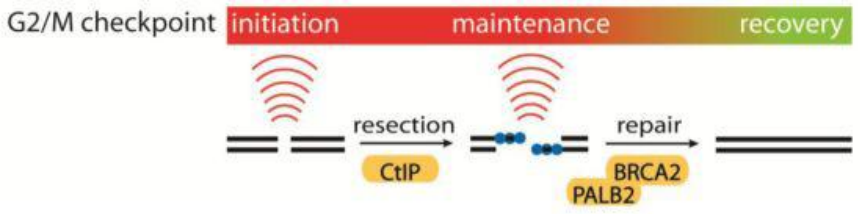


Figure.I21. – **Regulation of the G2-M checkpoint maintenance by CtIP.** Adapted from (A. Kousholt, Menzel, & Sørensen, 2012).

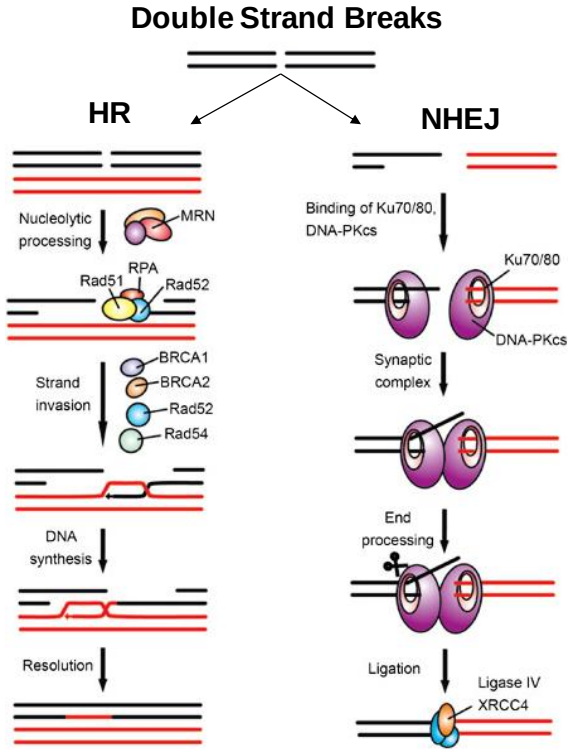
Another layer of control of the maintenance of the G2/M checkpoint originates from the p53 pathway. Through transcriptional induction of $p21^{CIP1/WAF1}$, p53 suppresses CDK activity, which allows to the activation of the pRb tumour-suppressor (Bunz, 1998). Active pRb reduces E2F activity and as a consequence the levels of its transcriptional targets such as the APC inhibitor EMI1 drop. This promotes premature activation of APC in G2 resulting in the degradation of key mitotic proteins such as Cyclin A and Cyclin B (Jinho Lee, Kim, Barbier, Fotedar, & Fotedar, 2009).

Checkpoint recovery is an active process necessary to resume the cell cycle after DNA repair. For this process it is required the phosphorylation of Plk1 by AURORA A/BORA complex (Fang, 2008; Macůrek et al., 2008). Plk1 modulates regulators of CDK1 to promote mitotic entry. First, it phosphorylates the CDK inhibitory kinases Wee1 and Myt1 promoting their degradation and inhibition, respectively. Finally, it phosphorylates and promotes the accumulation in the nucleus of the CDK1 activator Cdc25C (A. Peng, 2013; Yaffe, 2010).

1.5.DNA repair mechanisms

DNA repair is carried out by a plethora of enzymatic activities that chemically modify DNA to repair the DNA damage, including nucleases, helicases, polymerases, topoisomerases, recombinases, ligases, glycosilases, demethylases, kinases and phosphatases. Previously I have described the molecular mechanisms that sense the DNA damage and prepare the cell for DNA repair (cell cycle checkpoint activation and recruitment of mediators).

There are two main mechanisms in the cell to repair double-strand breaks: Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ) (Figure.I22). HR requires an homologous template strand of DNA to mend a lesion whereas NHEJ repairs a double-strand gap in DNA without a homologous template (Ciccia & Elledge, 2010). In addition to NHEJ and HR, recent studies demonstrate the operation of a third pathway of DSB processing called Microhomology-Mediated End Joining (MMEJ), functioning on simple end-joining principles, but repairing DSBs slower (Dueva & Iliakis, 2013).



Homologous **R**ecombination **N**on-**H**omologous **E**nd **J**oining

Figure.I22. – **Mechanisms and proteins involved in the repair of different types of DSBs.** Adapted from (Weterings & Chen, 2008).

1.5.1.Homologous recombination (HR)

Homologous recombination provides an important mechanism to repair both accidental and programmed DSBs during mitosis and meiosis. HR is active in the S and G2 phases of the cell cycle where it promotes repair of a broken chromatid from an intact sister chromatid, ensuring error-free repair (Moynahan & Jasin, 2010). This specific activity of HR during S and G2 is provided by the specific activation of enzymes involved in DNA resection (Rupnik, Lowndes, & Grenon, 2010; Shibata et al., 2011).

As explain before, the MRN complex senses DSBs, which promotes the activation of ATM and the generation of ssDNA, phenomenon called DNA resection, initially mediated by CtIP and Mre11 (L. Chen, Nievera, Lee, & Wu, 2008). The mediator protein BRCA1 associates directly with the MRN complex in an ATM- and CHK2- dependent manner that is enhanced after γ-radiation (Greenberg et al., 2006).

CtIP is a protein initially identified as a regulator of the transcription as a cofactor of the transcriptional repressor CtBP and also by binding to pRB (You & Bailis, 2010). CtIP interacts directly with both BRCA1 and the MRN complex to promote end resection and checkpoint activation (Huen, Sy, & Chen, 2010). CtIP is phosphorylated at S327 by CDK2 and this is a requisite for its interaction with BRCA1 (Huertas, Cortés-Ledesma, Sartori, Aguilera, & Jackson, 2008). This interaction enhances the ubiquitination of CtIP by the N-terminal E3 ligase domain of BRCA1, necessary for the recruitment of CtIP to the DSBs (Yu, Fu, Lai, Baer, & Chen, 2006) Localization of CtIP to damage sites is

mediated by a damage recruitment motif (aa 509-557) that can bind DNA (You et al., 2009). Recent observations indicate that the phosphorylation of the S327 residue is not essential for CtIP mediated resection (Reczek, Szabolcs, Stark, Ludwig, & Baer, 2013). However, this phosphorylation and the interaction with BRCA1 impair the recruitment of 53BP1 to the DSBs and promote HR. 53BP1 has been proposed to negatively regulate HR and promote NHEJ in the G1 phase through direct interaction with RIF1 (RAP1 interacting factor homolog, originally identified as a regulator of telomere length in *S. cerevisae* and recently it has been shown to be important in the regulation of NHEJ) in an ATM dependent manner (Chapman et al., 2013; Escribano-Díaz et al., 2013). Moreover, phosphorylation of CtIP S327 and DNA resection is facilitated by Mre11, which directly interacts with CDK2 and CtIP, thereby bringing CDK2 in proximity with its substrate (Buis, Stoneham, Spehalski, & Ferguson, 2012). Another residue of CtIP phosphorylated by CDK2 is T847 which has been described to be necessary for the initiation of DNA resection in eukaryotic cells. Thus, CDK2 controls CtIP-mediated resection activity at least through these two phosphorylations (Huertas et al., 2008; Huertas & Jackson, 2009; Huertas, 2010). However, it was recently reported that the phosphorylation of a cluster of five additional S/T-P motifs located in the central region of CtIP is important for DNA-end resection (H. Wang et al., 2013). In addition to this, CtIP has two phosphorylation sites targets of ATM, but their physiological relevance is not known (You & Bailis, 2010). Schematic details of the CtIP domains and PTMs can be seen in the Figure.I23.

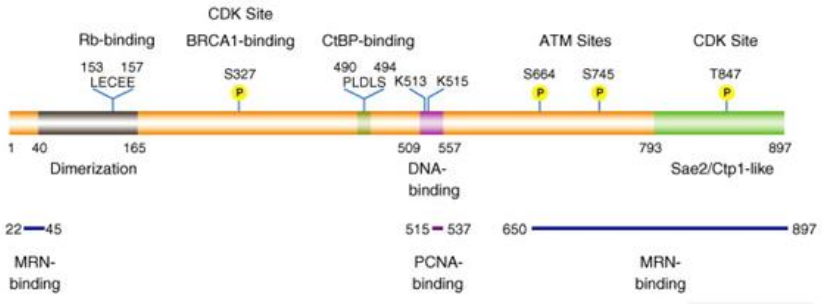


Figure.I23. – **CtIP structure and PTMs.** Adapted from (You & Bailis, 2010).

Another level of regulation of CtIP is the deacetylation by SIRT6. CtIP is constituvely acetylated and the deacetylation by SIRT6 promotes DNA resection (Kaidi, Weinert, Choudhary, & Jackson, 2010). Recently, it has been identified a mechanism by which CtIP turnover is regulated by the peptidyl-prolyl *cis-trans* isomerase PIN1. This mechanism consists in the targeting of CtIP to proteosomal degradation by PIN1-mediated isomerization of CtIP that requires CtIP phosphorylation at two conserved S/T-P motifs (S276 and T315) and show that CDK2 activity is required for PIN1-CtIP interaction. This mechanism has been demonstrated to be important for the regulation of DNA resection after DNA damage (Steger et al., 2013).

The abovementioned mechanisms provide limited DNA resection, but HR requires extensively resected DNA ends. This is done by additional nucleases such as Exo1 (exonuclease1) and the endonuclease DNA2 (DNA replication, ATP-dependent helicase-nuclease DNA2). Exo1 recruitment to the damage sites early after radiation depends on CtIP that modulates its activity. ATM phosphorylates Exo1 at S714 after recruitment to DSBs and

promotes the formation of RPA and Rad51 foci (Bolderson et al., 2010; Eid et al., 2010). BLM is a helicase that interacts and enhances the nucleolytic activity of Exo1 producing resection products ranging up to 2Kbp. RPA and the MRN complex also enhance the activity of the BLM-Exo1 complex (Nimonkar et al., 2011; Nimonkar, Ozsoy, Genschel, Modrich, & Kowalczykowski, 2008). BLM-Dna2 complex also regulates and enhances 5'→3' long range resection generating resected DNA regions up to several thousands of base pairs. The MRN complex and RPA are necessary for BLM-Dna2 mediated resection (Nimonkar et al., 2011). A schematic view of this process is shown in the Figure.I24.

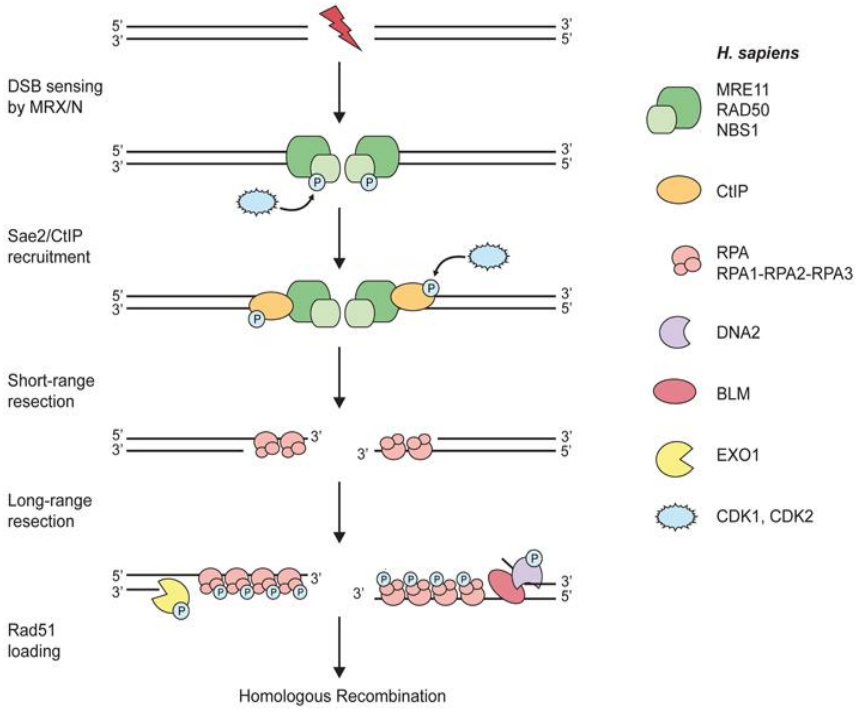


Figure.I24. – **Mechanisms and proteins involved in the short and long range resection during HR.** Adapted from (Ferretti et al., 2013).

After DNA resection, ssDDNA fibers have to be coated by Rad51 to proceed with HR (Figure.I25). Rad51 coating is necessary for the search of homologies and avoiding the formation of secondary structures in ssDNA. Rad51 filament formation is tightly regulated by BRCA2 (H. Yang et al., 2002) and dephosphorylation of RPA by PP4C and PP4R2 phosphatase subunits (D.-H. Lee et al., 2010). BRCA2 enhances Rad51 filament formation and exchange with RPA tails by promoting binding of Rad51 to ssDNA (Jensen, Carreira, & Kowalczykowski, 2010; J. Liu, Doty, Gibson, & Heyer, 2010; Thorslund et al., 2010). Rad51 recombinase recognizes homologous DNA sequences through a mechanism of "conformation proofreading", forming a D-loop structure with the help of the motor protein Rad54 (Savir & Tlusty, 2010).

In order to complete the repair, it is necessary to fill the gaps with the error-prone translesion DNA polymerases Polζ and REV1 which are able to extend 3' invading DNA containing base damage (Sharma et al., 2012). Finally Rad52 is the responsible to anneal the invading DNA with the receptor (Grimme et al., 2010; Stark, Pierce, Oh, Pastink, & Jasin, 2004).

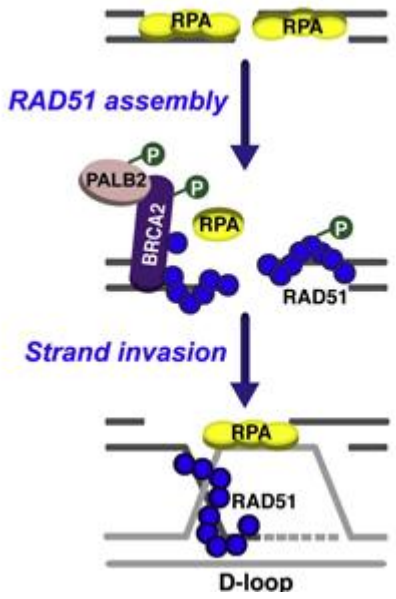


Figure.I25. – **Rad51 assembly and strand invasion during homologous recombination.** .Adapted from (Ciccia & Elledge, 2010).

The DNA intermediates generated as a consequence of the D-loop formation during the last steps of HR can be resolved by different ways as shown in the Figure.I26. The first is the Synthesis-Depending Strand Annealing (SDSA) and is regulated by the RTEL helicase where the 3' invading strand could be extended by polymerases and then reanneal to the processed second end of the break (Barber et al., 2008). Alternative to this pathway, double Holliday junctions could be formed and dissolved by the BLM/TOPOIII complex (generating noncrossover recombination products) or cleaved by the endonucleases GEN1, MUS8/EME1 or SLX1/SLX4 (generating either crossover (exchanges of chromosome arms between homologous chromosomes) or noncrossover products) (Andersen et al., 2009; Ciccia, McDonald,

& West, 2008; Fekairi et al., 2009; Ip et al., 2008; Muñoz et al., 2009; Svendsen et al., 2009).

Crossover events are highly regulated as they can lead to loss of heterozygosity and genomic rearrangements in mitotic cells and thus, defective crossover regulation is a major cause of genomic instability (Chu & Hickson, 2009).

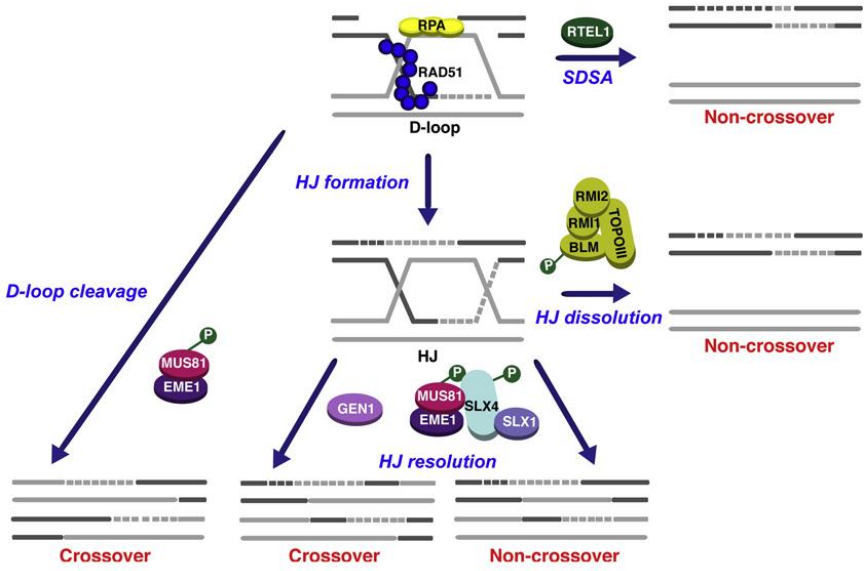


Figure.I26. – **Resolution of the DSB repair by homologous recombination.** HJ: Holliday Junction. Adapted from (Ciccia & Elledge, 2010).

1.5.2.Non-homologous end joining (NHEJ)

NHEJ is a fast and efficient mechanism to repair DSBs. However, when there is an extensive formation of DSBs in the genome, NHEJ generates chromosomal translocations. Since NHEJ occurs in the absence of a DNA template or extended regions of homology, processing of the DNA ends has the potential to result in

loss of nucleotides, making NHEJ an inherently error-prone process (Figure.I27) (Anthony J. Davis, 2013; Mahaney et al., 2009).

The first step in NHEJ is detection of the DSB by Ku proteins as explained before (page 10). Once bound, Ku translocates inwards from the DNA end making the extreme termini accessible to other proteins. The interaction of DNA-PKcs, XLF, DNA polymerase μ and DNA ligase IV with Ku is enhanced by the presence of DNA, indicating that the binding of Ku to DNA is a prerequisite for the interaction with other proteins necessary to proceed with NHEJ. DNA-PKcs interaction with Ku and the extreme termini of DNA allows to the formation o the “synaptic complex” that permits the binding of two DNA-PKcs molecules across the DSB (DeFazio, Stansel, Griffith, & Chu, 2002; S. Yoo & Dynan, 1999). This serves to tether the ends of the DSB together and is thought to protect the DNA ends form nuclease attack and, as a consequence, preventing HR.

However, NHEJ also requires processing of DNA ends to remove non-ligatable end groups and other lesions. Artemis is a 5’→3’ exonuclease and acquires endonuclease activity after the autophosphorylation of DNA-PKcs. This is necessary for the processing of DNA-containing dsDNA/ssDNA transitions, such as DNA hairpins. Artemis also interacts with DNA-PKcs providing a mechanism necessary for its recruitment to the DSBs (Goodarzi et al., 2006; Ma, Pannicke, Schwarz, & Lieber, 2002; Ma, Schwarz, & Lieber, 2005; Soubeyrand et al., 2006).

The processing of complex IR-induced DNA structures can lead to the creation of DNA gaps that require the action of DNA

polymerases for their repair. Two members of the X family of Polymerases, DNApol μ and λ, are widely expressed and have widespread roles in NHEJ. The X polymerases are involved in synthesis of short segments of DNA rather than in replication of whole chromosome DNA and play a role in DNA repair (Uchiyama, Takeuchi, Kodera, & Sakaguchi, 2009). These have highly conserved regions that include two helix-hairpin-helix motifs that are imperative in the DNA-polymerase interactions (Jennifer Yamtich, 2010). DNApol μ and λ are recruited to DSBs via interaction with Ku and X4-L4 complex (a complex that promotes the ligation of the DSBs as detailed below). Polymerase λ is largely template-dependent while polymerase μ is less dependent. A third member of this family, TdT (terminal deoxyribonucleotidyltransferase), is only expressed in lymphocytes and only plays a role in V(D)J recombination (Nick McElhinny & Ramsden, 2004).

The final step of the NHEJ is the ligation of the DNA ends. This is carried out by Ligase IV which acts in complex with XRCC4. Although no catalytic activity for XRCC4 has been described, it is necessary for both NHEJ and V(D)J recombination. It rather acts as a scaffolding protein and it only exists in homodimers that interact to form tetramers (Leber, 1998). It interacts with the C-terminal domains BRCT of DNA Ligase IV through its α-helical region forming a highly stable complex that stabilizes and stimulates its DNA ligase activity. The complex is also called X4-L4 and is recruited to DSBs in a Ku-dependent manner. XLF, a protein with similar structure to XRCC4 is also required for NHEJ. XLF has been shown to stimulate the ligase activity of DNA Ligase IV *in vitro*

suggesting that it regulates X4-L4 complex under some conditions (Grawunder et al., 1997; Gu et al., 2007).

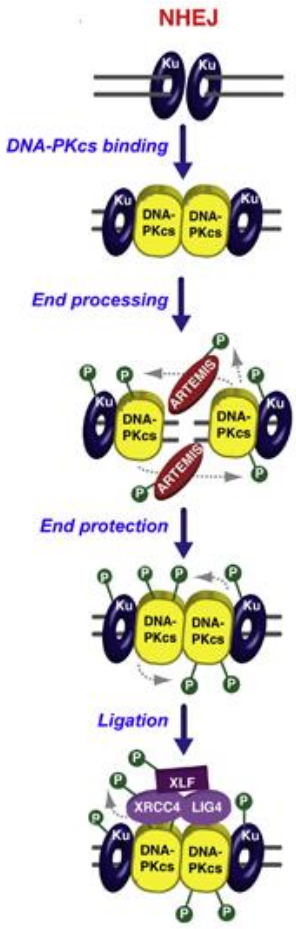


Figure.I27. – **Repair of DSBs by Non-homologous end joining.** Adapted from (Ciccia & Elledge, 2010).

Although the explained mechanism is used to rejoin DSBs, the high complexity of secondary DNA-structures formed after radiation can recruit other DNA repair mechanisms, such as BER and SSB

repair, which coordinate their action with NHEJ itself. Given that, it is likely that cross-talk among these pathways exists.

1.5.3.Alternative Non-homologous end joining (Alt-NHEJ)

In addition to NHEJ and HR, recent studies demonstrate the operation of a third pathway of DBS repair. This mechanism gains importance when HR or NHEJ processes fail to repair DSBs acting as a backup pathway (Figure.I28). It is facilitated by microhomologies present at the DNA ends and it is specially enhanced during S and G2 phases. When the engagement of alt-NHEJ follows a failure in NHEJ, the ligation under this conditions is performed by DNA ligase III (Paul et al., 2013). Alt-NHEJ can also backup HR in S and G2 phases of the cell cycle operating over resected ends mediated by the MRN complex, CtIP and BRCA1 (Lee-Theilen, Matthews, Kelly, Zheng, & Chaudhuri, 2011; Xie, Kwok, & Scully, 2009; Y. Zhang & Jasin, 2011; Zhong, Chen, Chen, & Lee, 2002).

This mechanism is highly error-prone on two counts: it can not restore DNA sequences in the vicinity of DSBs and it is able to catalyze the joining of unrelated DNA molecules generating loss of genomic information and translocations. Thus, alt-NHEJ is a major cause of genomic instability (Dueva & Iliakis, 2013; Mladenov & Iliakis, 2011).

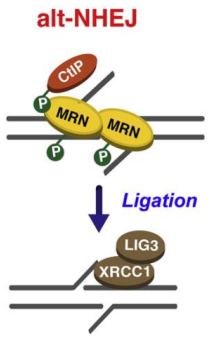


Figure.I28. – **Repair of DSBs by alt-NHEJ.** Adapted from (Ciccia & Elledge, 2010).

2.APOPTOSIS

2.1.Introduction to apoptosis

When in a given tissue the DNA repair mechanisms fail to restablish the integrity of the DNA, the cell has to be eliminated from the population. The current paradigm states that a cell suffering strong or persistent DNA damage overcoming its capacity to repair the lesions undergoes cell death by activating programmed cell death pathways (i.e. apoptosis) (Figure.I29). Defects in inducing apoptosis when DNA damage is not properly repaired are linked to increased incidence of cancer, probably due to the generation of genomic instability and the accumulation of chromosomal aberrations. Thus, apoptosis following DNA damage could be seen as a protective mechanism to prevent carcinogenesis (Hanahan & Weinberg, 2011; Roos & Kaina, 2013).

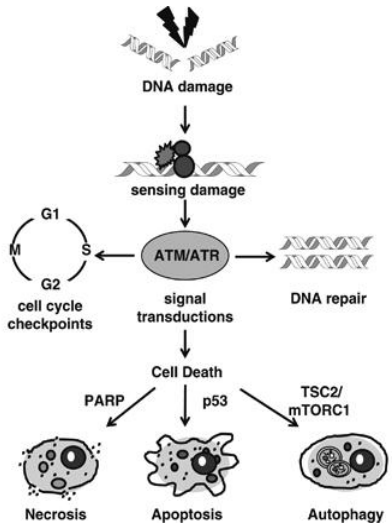


Figure.I29. – **Mechanisms of cell death in response to DNA damage.** Adapted from (Surova & Zhivotovsky, 2013).

2.2.Molecular regulation of apoptosis

2.2.1.Caspases

Caspases are cysteinyl aspartate proteinases belonging to the family of cysteine proteases which cleave their substrates after an aspartic acid (asp) residue and are known to participate essentially in the processes of programmed cell death and inflammation. Up to know, 14 different mammalian caspases have been identified. Caspases are synthesized as inactive proenzymes (zymogens) and only when they are cleaved at specific sites they become active enzymes during the induction of apoptosis. Structurally, caspases contain a prodomain followed by a large p20 and small p10 subunit. Caspases can be functionally classified into 3 groups: inflammatory caspases, apoptotic initiator caspases and apoptotic effector caspases (Figure.I30).

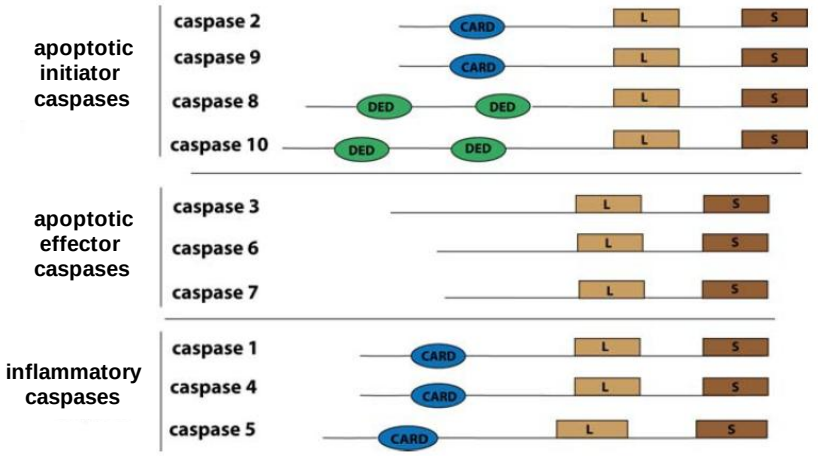


Figure.I30. – **Classification of mammalian caspases.** L corresponds to the p20 subunit and S corresponds to the p10 subunit. Adapted from (Z. Jin & El-deiry, 2005).

The inflammatory caspases include human caspase -1, -4, -5 and mouse caspase -1, -11 and -12 (Martinon, Boveresses, & Epalinges, 2004). The Caspases 2, 8, 9 and 10 account to the group of apoptotic initiator caspases. Apoptotic initiator caspases have long prodomains containing either a Death Effector Domain (DED) (Caspase -8 and -10) or a Caspase Activation and Recruitment Domain (CARD) (Caspase -2 and -9). Activation of initiator caspases requires oligomerisation of their monomeric inactive precursors. Formation of specific multimeric platforms such as the Death Inducing Signaling Complex (DISC) for caspase -8 and -10, the PIDDosome for caspase -2 and the apoptosome for caspase - 9, facilitates the oligomerisation of the initiator caspase leading to its activation by bringing multiple caspase molecules in close proximity forming a multiprotein complex called aggregosome. In this way, only when these platforms are present it is likely that activation of the caspase cascade is triggered (Park, 2012). Initiator caspases cleave and activate apoptotic effector caspases -3, -6 and -7, characterized by having very short prodomains. The effector caspases execute the downstream processes of apoptosis, such as promoting the degradation of chromosomal DNA and dismantling the physiology and architecture of the cell (Degterev, Boyce, & Yuan, 2003). The Caspase-Activated DNase (CAD) is responsible for this process. CAD builds cytosolic complexes with its inhibitor ICAD in order to keep it inactive. During apoptosis, active Caspase 3 cleaves ICAD at 2 positions and CAD is consequently released from the complex translocates to the nucleus and becomes able to cleave the chromosomal DNA linker in between the nucleosomes (Uegaki et al., 2000).

The inhibitors of apoptosis (IAPs) are proteins first identified in insect viruses where they prevent the infected cell from killing themselves by apoptosis. IAPs bind to activated caspases in order to inhibit their function. Some of them are able to mark the activated caspases for their destruction by the proteasome by inducing their polyubiquitination. IAPs include X-linked IAP (XIAP), cIAP-1, cIAP-2, Neuronal Apoptosis Inhibitory Protein (NAIP), Survivin and Livin carachterized by the presence of RING domains. IAPs are regulated by anti-IAP proteins which are released from the mitochondrial intermembrane space during the induction of the intrinsic apoptotic pathway. Anti-IAP proteins include Second Mitochondrial Activator of Caspases / Direct IAP Binding Protein with Low pI (Smac / DIABLO) and Omi / HtrA2 (High temperature requirement protein 2) (Richter & Duckett, 2000; Salvesen & Duckett, 2002; Vaux & Silke, 2003).

2.2.2.Bcl2 family of proteins

The proteins of the Bcl2 family have either pro- or anti-apoptotic functions that execute by regulating the Mitochondrial Outer Membrane Permeabilisation (MOMP). They regulate the release of mitochondrial proteins that are normally located at the intermembrane space of these organelles, such as Cytochrome c, endonuclease G, AIF, Smac/Diablo and HtrA2. Bcl2 family characteristically contain 1 up to 4 Bcl2 homology domains (BH) that are important for establishing homo- and heterodimeric interactions between the different family members (Figure.I31) (Youle & Strasser, 2008).

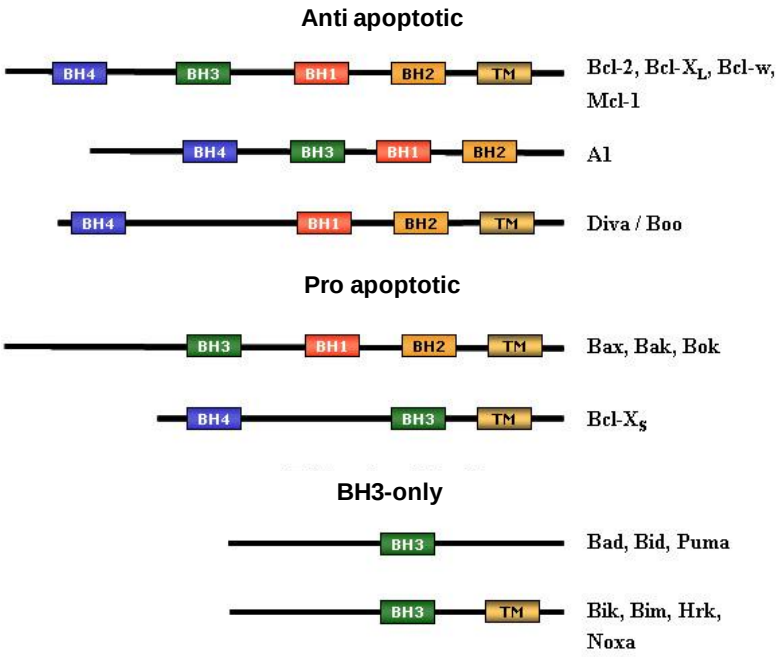


Figure.I31.- **The Bcl2 family of proteins.** (Ramon Roset, Laura Ortet, 2007).

There are 2 types of pro-apoptotic proteins that can be distinguished functionally. The pro-apoptotic effectors proteins Bax and Bak contain BH domains 1, 2 and 3 (Renault, Floros, & Chipuk, 2013). They undergo activation first by conformational change and then by mitochondrial translocation and by oligomerisation, getting integrated into the outer mitochondrial membrane leading to its permeabilisation. Other pro-apoptotic proteins such as Bid, Noxa or Bim contain only the BH3 domain and are thus called BH3- only proteins (Lomonosova & Chinnadurai, 2008). They seem to function upstream of Bax and Bak in order to regulate apoptosis. The anti-apoptotic Bcl2 proteins such as Bcl2 or Bcl-XL contain all 4 BH domains and are able to

inhibit apoptosis mainly by binding and inhibiting pro-apoptotic Bcl2 proteins (Chipuk & Green, 2008).

2.3.Apoptotic signalling pahtways

Caspase activation is a requirement to classify cell death as apoptosis. Activation of caspases can occur by two distinct pathways: an extrinsic, death receptor-mediated pathway and an intrinsic, mitochondrial-mediated pathway (Figure.I32). In either of these pathways, initiator caspases are activated by oligomerization following an apoptotic signal. Initiator caspases cleave and activate effector caspases which then cleave diverse cellular proteins resulting in apoptosis (Fulda & Debatin, 2006).

2.3.1.Intrinsic apoptotic pathway

The intrinsic apoptotic pathway signals apoptosis from inside the cell through the Mitochondrial Outer Membrane Permeabilisation (MOMP). This process is regulated by the Bcl2 family of proteins and induces the release of different mitochondrial intermembrane proteins such as Cytochrome c. Once it is released into the cytosol it participates in forming the apoptosome, a wheel-like heptamer of Apaf1 and Cytochrome c proteins. Once it is formed, the apoptosome recruits and activates initiator caspase 9 leading to the cleavage and activation of downstream executioner caspases, such as caspase - 3, -6 and -7 and consequently, to the induction of apoptosis (Renault et al., 2013; Reubold & Eschenburg, 2012).

2.3.2.Extrinsic apoptotic pathway

The extrinsic apoptotic pathway is triggered by extracellular proteins that bind to cell-surface death receptors, belonging to the superfamily of the Tumour Necrosis Factor (TNF) receptors (including Fas/CD95L/Apo-1,TNF-αR and DR4 and DR5). Ligands for the induction of this pathway include TNF-α, Fas ligand (CD95L) and TNF-Related Apoptosis Inducing Ligand (TRAIL). Binding of the ligand to the receptor triggers the recruitment of intracellular adaptor proteins to their cytosolic part via homotypic interaction of death domains (DD) which are found on the receptor as well as on the adaptor protein (Fulda & Debatin, 2006). The Fas Associated Death Domain protein (FADD) (E.-W. Lee, Seo, Jeong, Lee, & Song, 2012) and the TNF Receptor Associated Death Domain (TRADD) protein (Pobezinskaya & Liu, 2012) are the two main DD-containing adaptor proteins binding to the different death receptors, FADD mainly to Fas and TRAIL-R1 and -2, while TRADD preferentially binds to TNFR1, DR3 and DR6. Additionally, the adaptor proteins contain a Death Effector Domain (DED) necessary to recruit DED-containing procaspases -8, -10 and the caspase inhibitor c-FLIP to the aggregosome, in this case called Death-Inducing Signaling Complex (DISC). Binding FasL to the Fas receptor triggers DISC formation containing FADD and Procaspase 8. Oligomerisation and proteolytic cleavage of Procaspase 8 molecules due to their close proximity in the DISC leads to their activation. The activated initiator caspases signal by either direct cleavage of effector Procaspase 3 or indirectly by cleavage of the BH3-only protein Bid (depending on the cell type) generating truncated Bid (tBid). tBid is able to permeabilise the mitochondrial outer membrane leading to the formation of the apoptosome and

hence, activation of the effector caspases -3, -6 or -7 (Dickens, Boyd, et al., 2012; Li, Zhu, Xu, & Yuan, 1998).

c-FLIP is a catalytically inactive homolog of caspase 8 that binds DISC but inhibits the activation of Procaspase 8 and, consequently, inhibit death receptor-mediated apoptosis (Dickens, Powley, Hughes, & MacFarlane, 2012; Silke & Strasser, 2013).

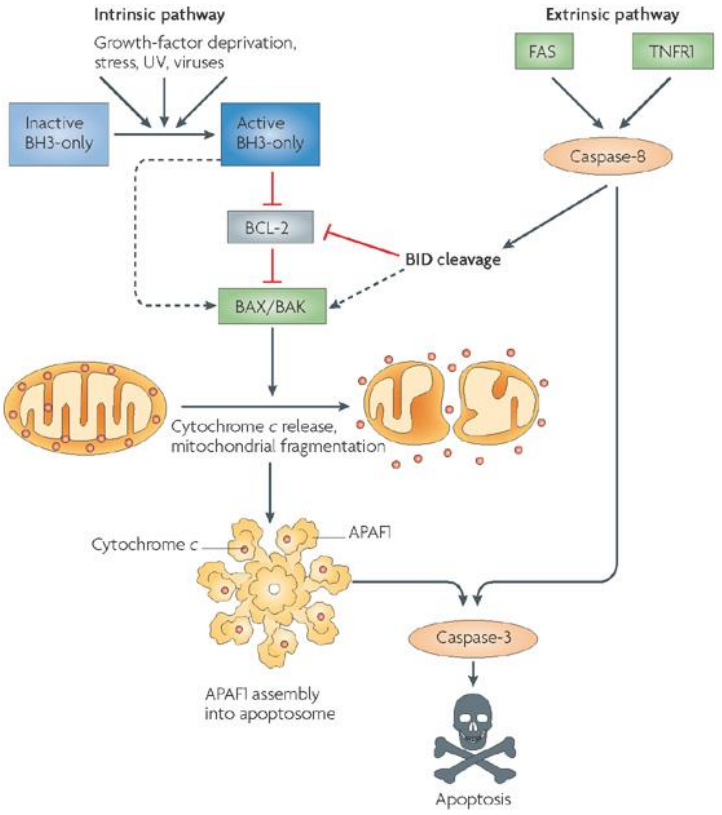


Figure.I32. – **Regulation of apoptotic intrinsic and extrinsic pathways regulation.** Adapted from (Youle & Strasser, 2008).

2.4.CDKs and apoptosis

Cell cycle progression is strictly regulated by different CDKs and cyclins. Cyclin expression is tightly regulated throughout the cell cycle what ensures a proper CDK activation depending on the cell cycle phase. However, a role for CDKs in apoptosis is not well defined due to the lack of clear genetic evidence (Golsteyn, 2005).

Thymocytes are a good model to study the role of CDKs in apoptosis since around 90% of the cells are quiescent. Previously in our lab it has been demonstrated that CDK2 in addition to its role regulating cell cycle progression it is also activated in quiescent thymocytes after treatment with external pro-apoptotic stimulus, such as gamma radiation and glucocorticoids, suggesting a possible role of CDK2 in apoptosis. Chemical inhibition of CDK2 has been to shown to block thymocyte apoptosis induced by intrinsic but not extrinsic apoptotic stimulus (Gil-Gómez, Berns, & Brady, 1998; Granes, F., Roig, M.B., Brady, H.J. & Gil-Gomez, 2004). Moreover, a correlation between $p27^{kip1}$ downregulation and CDK2 activation during apoptosis and the modulation of CDK2 activity by p53, Bax and Bcl2 has been demonstrated (Owen Williams, Gabriel Gil-Gomez, Trisha Norton & Brady, 2000).

2.5.Apoptosis in response to DNA damage

2.5.1.p53

In response to DNA damage, the transcription factor p53 plays a dual role: on the one hand it has a protective role increasing DNA repair but on the other hand sensitizes the cell by promoting

apoptosis execution. The balance between the two roles of p53 depends on which is the agent and the dose that generates the DNA damage. ATM, ATR and their targets CHK1 and CHK2 are able to phosphorylate p53. ATM and ATR directly phosphorylate p53 at S15 promoting its stabilization, tetramerization and the translocation to the nucleus where regulates gene transcription. CHK1 and CHK2 can phosphorylate p53 at S20 preventing its ubiquitylation by the ubiquitin E3 ligase Mdm2 and thus preventing its proteasome-mediated degradation. Consequently, the nuclear localization of p53 and its binding activity increases and its target genes are induced. p53 regulates the expression of pro-apoptotic genes such as FasR, Bax, Puma, Noxa, Apaf-1 and PIDD (Canman, 1998; Donehower, 2009).

2.5.2.Transcriptional inhibition

Apoptosis may occur through this mechanism because vital proteins are no longer produced. Massive non repaired lesions have the ability to repress transcription and DNA replication and it is likely that apoptosis happens as a consequence of the accumulation of DNA breaks generated because of replication blockage. However, it is necessary more work to clarify this question (Ljungman & Lane, 2004).

2.5.3.JNK and p38 activation

DNA damaging agents activate p38 and SAPK/JNK (stress-activated protein kinase/c-Jun N-terminal kinase) kinases which result in an increase of active c-Jun levels leading to activation of the transcription factor AP-1. Sustained AP-1 activation increases

the levels of FasL expression. This, accompanied by a p53-dependent increase in the levels of Fas activates the extrinsic apoptotic pathway (Hamdi et al., 2005). Moreover, sustained activation of JNK also activates the mitochondrial apoptosis pathway via phosphorylation of Bax (Tournier, 2000).

2.5.4.Caspase-2 activation

This caspase is constituvely present in the nucleus and has been shown to play a role in IR-induced apoptosis. Caspase-2 leads to the activation of apoptosis through different ways: first, it is able to induce Bid cleavage, Bax translocation to mitochondria and Cytochrome c release (Lassus, Opitz-Araya, & Lazebnik, 2002). It can also directly activate the intrinsic pathway by disrupting the interaction of the Cytochrome c with cardiolipin enhancing its release from the mitochondria (Enoksson et al., 2004; Robertson et al., 2004). Finally, it is also able to activate apoptosis in response to IR independently of p53, Bcl-2 and caspase-3 in CHK1 defficient cells (Sidi et al., 2008).

2.5.5.NF-κB

NF-κB is a “rapid-acting” transcription factor that is found inactive in the cyctoplasm bound to the Inhibitor of κB (IkB). It becomes activated after DSB generation by NEMO (NF-κB Essential MOdulator). NEMO is localized in the nucleus in the presence of DSBs where is phosphorylated by ATM and shuttled to the cytosol where binds and activates IKK permitting the phosphorylation and degradation of IκBα. This permits the translocation of NF-κB to the nucleus where it performs its function as transcription factor and

promotes the expression of genes such as Bcl-xL and A1/Bfl-1, that inhibit the intrinsic apoptotic pathway, and c-IAP2, TRAF1 and TRAF2, that inhibit the extrinsic apoptosis pathway (C. Wang, 1998; Werner, de Vries, Tait, Bontjer, & Borst, 2002).

2.5.6.AKT

Activation of AKT by Pi3Ks causes suppression of apoptosis. AKT suppresses the apoptosis by 5 different mechanisms. The first is to phosphorylate BAD (a BCL-2 family member) impairing its binding to BCL-2 and BCL-xL. The second is to phosphorylate and inhibit Caspase-9. The third is the phosphorylation of ASK1, an upstream activator of p38 and JNK. The fourth is to promote the degradation of IκB, enhancing the NF-κB prosurvival activity. Finally, it prevents the nuclear localization of p53 via phosphorylating Mdm2 enhancing its ubiquitin ligase activity. The phosphatase PTEN counteracts the proapoptotic effect of AKT by preventing its phosphorylation, activation and reducing the threshold of DNA damage tolerance by the cell (Roos & Kaina, 2013; Yao & Cooper, 1995).

2.5.7.Survivin

Survivin is a member of the inhibitor of apoptosis (IAP) family. It directly inhibits the activity of caspases, notably caspase-3, and stimulates DSB repair by its interaction with DNA-PKcs (Altieri, 2010).

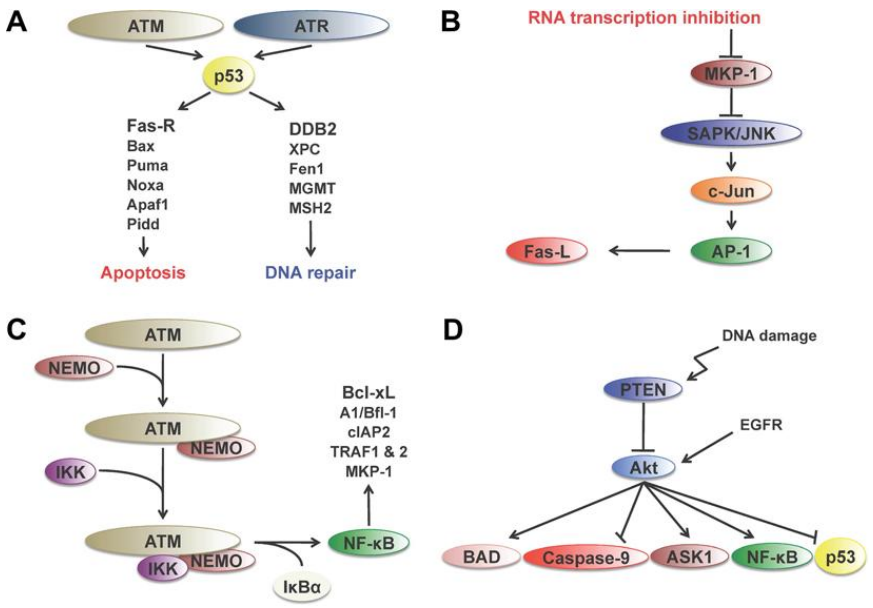


Figure.I33. – **Downstream signalling from the DNA damage response to the apoptosis machinery.** Adapted from (Roos & Kaina, 2013).

2.5.8.Concluding remarks for DNA damage induced apoptosis

From the previous discussion about the regulation of apoptosis we can conclude that in order to activate apoptosis after DNA damage, the checkpoints and the repair mechanisms have to be overwhelmed by the severity of the DNA damage. Some of the abovementioned factors such as p53 and NF-κB have a dual role enhancing DNA repair at low levels of DNA damage and promoting cell death at high levels of DNA damage.

Finally, it is important to note that the interplay between prosurvival and proapoptotic balance becomes more complex in cancer cells. The same mechanisms that promote the transformation of normal

cells in tumoral cell are those that protect cancer cells from apoptosis, such as AKT, loss of PTEN, loss of p53, NF-κB, etc.

3. CYCLIN O

3.1.Introduction

CDK2 activation during apoptosis has been demonstrated that precedes apical caspase activation, mitochondrial disfunction and the loss of membrane asymmetry, indicating a role upstream of mitochondria (Granes, F., Roig, M.B., Brady, H.J. & Gil-Gomez, 2004). Importantly, it was demonstrated that this CDK2 activation in response to apoptotic stimuli was independent of Cyclin A and Cyclin E, its binding partners during cell cycle progression, suggesting that CDK2 is activated by an unknown Apoptosis Related CDK2 Activator (ARCA) (Figure.I34) (Gil-Gómez et al., 1998).

Using *in silico* analysis of the human genome, several non-characterised Cyclin-like proteins were identified, from which preliminary results encouraged us to further characterise one of them, located in human chromosome 5 in a region sintenic with mouse chromosome 13 that was annotated as Cyclin O. This Cyclin O was identified to be the activator of CDK2 in response to pro-apoptotic stimuli (Roig et al., 2009).

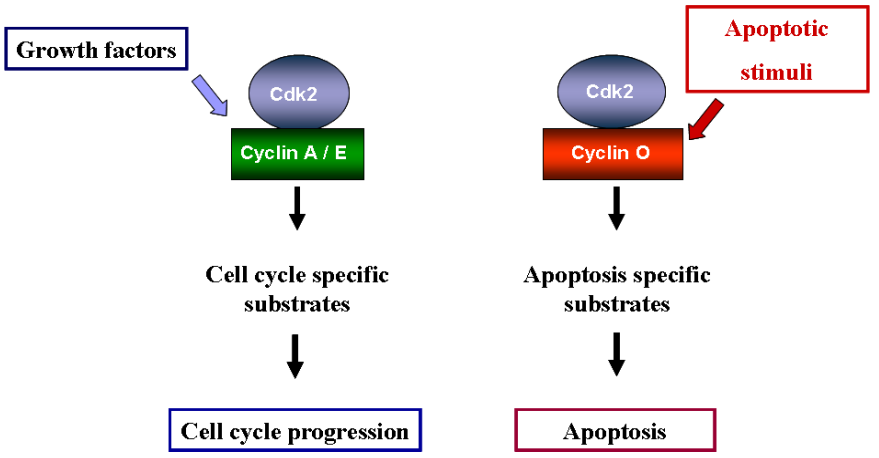


Figure.I34. – **Hypothesis of ARCA for the regulation of CDK2 in the intrinsic apoptotic pathway.** Adapted from (Roig et al., 2009).

3.2.Cyclin O locus and protein

Cyclin O was identified as a 40KDa and 350aa protein encoded by an mRNA constituvely expressed in the testis, ovaries, brain, lungs, liver and at lower levels in kidneys and heart. In other tissues, such as the thymus, it is expressed after treatment with proapoptotic stimulus, such as γ-radiation or glucocorticoids. Cyclin O has been shown to be required for the induction of the intrinsic apoptotic pathway (Roig et al., 2009) and has been suggested to have a putative functional role in the DNA damage response (DDR), in stress responses and colorectal cancer (unpublished results).

The Cyclin O gene is highly conserved and is present in all the vertebrate genomes sequenced until now. The generic Cyclin O locus encode for two splicing variants (Cyclin Oα and Cyclin Oβ) and two long non coding RNAs (Cyclin Oγ and Cyclin Oδ). About two-thirds of the Cyclin Oα (hereforth Cyclin O) sequence

encompasses a highly conserved cyclin box, sharing about 28% of identity with human Cyclins A2 and B1 (Roig et al., 2009). Details of the Cyclin O locus and protein can be seen in the Figure.I35.

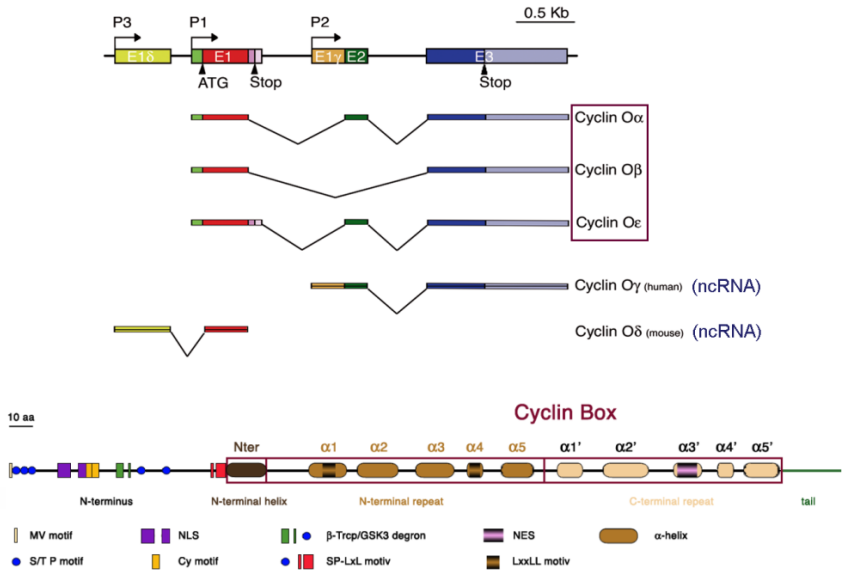


Figure.I35. – **Cyclin O locus and protein structure.** Adapted from (Roig et al., 2009).

3.3.Cyclin O in apoptosis.

Cyclin O is expressed in thymus after treatment with gamma radiation and its expression precedes the activation of Caspase-3. It was also demonstrated that the interaction of this novel cyclin with CDK2 and CDK1 leads to their activation unlike others CDKs. All these data suggested that Cyclin O was a proper candidate to regulate the intrinsic apoptosis pathway. It was demonstrated that the downregulation of Cyclin O in a cell line impairs DNA damage and glucocorticoid-induced apoptosis. Moreover, deficiency in Cyclin O leads to a block in the activation of the apical and

effectors caspases, and a deficient translocation of BAX (but not BAK) and subsequent Cytochrome c release from the mitochondria (Figure.I36) (Roig et al., 2009).

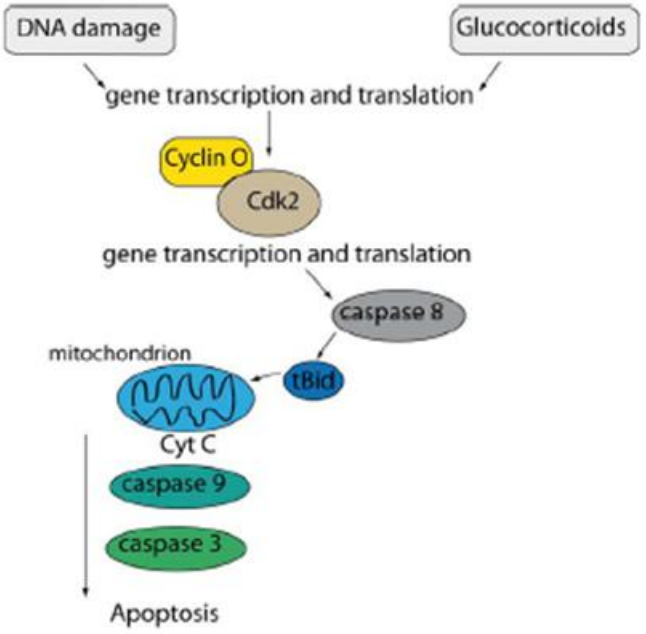


Figure.I36. – **Cyclin O in the intrinsic apoptotic pathway.**

OBJECTIVES

Objectives

Our laboratory research is focused on the study of the mechanisms that regulate the intrinsic apoptosis pathway and the implication of the CDK-cyclin complexes. Previously in the lab, it was identified a novel cyclin, the Cyclin O that binds and activates the CDK2 in response to γ-radiation, a mechanism necessary for the regulation of the intrinsic apoptotic pathway. Preliminary results in our lab suggest that Cyclin O affect the activation and the phosphorylation of some DDR factors. Given that, we have proposed the following objectives:

1. To study th role of the Cyclin O in the DNA Damage Response.
    a. Which DDR pathways are affected in Cyclin O deficient cells.
    b. Which are the molecular connections between the Cyclin O and the DDR.
2. To study the DNA damage-induced apoptosis in Cyclin O deficient cells.
3. To study the phenotype of the Cyclin O knock-out mice.

MATERIALS AND METHODS

1.CELL CULTURE AND TRANSFECTION

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with antibiotics (Penicillin and streptomycin) and 10% foetal calf serum (Biological Industries). Cells were maintained at 37 ºC with a humid atmosphere of 5% of CO2. Cells used were Human Embryonic Kidney cells (HEK-293T) obtained from the American Type Culture Collection (ATCC), primary Mouse Embryonic Fibroblasts (MEFs) and SV40 Tag immortalized fibroblasts obtained as explained below.

1.1.Culture of Primary MEFs

Heterozigous females were mated overnight with heterozygous males. Next morning plugs were checked and embryos from E.13.5 embryos isolated from pregnant females. Embryos were separated from its placenta and surrounding membranes. Each embryo was washed by transferring it to a petri dish containing clean PBS. The head was dissected and frozen for genotyping. Using a scalpel the embryos were cut in pieces as small as possible in a 0.5 mL of PBS. With the aid of a 1.0 mL syringe fitted with a 19G 1 ½" needle the embryo was aspirated and forced it in and out of the barrel several times. The suspension was transfered to a 15 mL sterile tube. 2mL of prewarmed trypsin was added to each tube and incubated for 15 minutes at 37ºC. Cell suspensions were centrifugated 5 minutes at 1000 rpm to pellet the cells. Trypsin was aspirated and the pellet resuspended in 5 mL of medium (DMEM, 1X GPS, and 10% FCS). The pellet was pippetted up and down gently until obtaining a cell suspension. DNA fibers were not discarded since they contain lots of trapped cells that also get

attached to the dishes eventually. The suspension of embryonic cells was plated out (passage 1). Primary MEFs cells were grown as described above using complete medium (DMEM, 1X GPS, and 10% FCS) plus 45 μM β-mercaptoethanol and used for experiments until passage 5 while still in the log phase of growth.

1.2.Immortalization of MEFs

Mouse Embryonic Fibroblasts were immortalized with the transfection of the SV40 large Tag. Primary MEFs were split at pass 2-4 into 6 well dishes. 1/4 and 1/6 dilutions from a confluent 10cm plate were made for each genotype. Cells were grown overnight in a 37C incubator. 25% confluent wells were transfected with 2μg SV40 Tag expression vector (pSG5-Tag) using Lipofectamine reagent (Invitrogen). When cells got confluent they were split into a 10cm dish. Additional 1/10 splitting was done when the plates were confluent at least 5 more times. The goal is to get a 1/100,000 fold splitting of the original cells.

1.3.Irradiation of primary and immortalized fibroblast

Primary MEFs and SV40 Tag immortalized fibroblasts were seeded into 10cm, 6-well or 24-well plates at a density of 17000 cell/cm^2 and cultured overnight. The cells were exposed to 2, 2,5 or 5Gy of γ-radiation depending on the experiment using a ^{137}Cs irradiator.

1.4.Transfection of HEK-293T cells with PEI

$2x10^6$ 293T cells were seeded in 10cm plates and 24 hours later transfection was performed using the PEI method. A mixture was done with 11,25μg of DNA, 58,5μl of PEI (1mg/mL) and the volume

completed up to 1170µl of 150mM NaCl. The mixture was incubated for 15 minutes at RT and added drop by drop to the target cells. 48 hours later, cells were analysed as required.

2.PROTEIN EXTRACTION AND WESTERN BLOTTING

2.1.Whole cell lysates

Cells to be used for immunoblotting were washed with cold PBS, scrapped and dry pellets frozen at -80 ºC until used. Cell pellets where extracted with lysis buffer (10 mM Tris-HCl pH 7.4, 1% NP-40, 1% sodium deoxycolate and 0.15 M NaCl) to which protease and phosphatase inhibitors were added (2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml antipain, 20 µg/ml soybean trypsin inhibitor, 1 mM DTT, 1 M NaF, 0.5 M β-glycerol phosphate, 0.1 M sodium pyrophosphate, 1 mM Pefablock and 20 mM sodium ortovanadate). Cell pellets were incubated for 30 minutes on ice and centrifuged at 10.000rpm for 15 minutes at 4ºC. Supernatants were collected and placed in new eppendorf tubes. Remaining pellets were used for acid histone extraction as explained below. Protein sample concentration was quantified using the Bradford Protein Assay (BioRad).

2.2.Acid histone extraction

Remaining detergent-insoluble pellets from whole cell lysates were resuspended in 90 µl of 0.1M HCl, incubated on ice for 30 minutes and centrifuged at 10.000rpm for 15 minuts at 4ºC. Supernatants

(containing histones) were transfered to a new 1.5 ml tube and 10µl of 1M Tris pH9.0 were added. Protein sample concentration was quantified using the Bradford Protein Assay (BioRad).

2.3.Subfractionation of soluble and chromatin bound proteins

Cells were washed with PBS, scraped and pelleted, Cell pellets were resuspended in solution A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 1 mM Na3VO4, protease inhibitors), and Triton X-100 was subsequently added to a final concentration of 0.1%. After a 5 min incubation on ice, the samples were spun at low speed (1300g for 4 min) to separate soluble proteins and permeablized nuclei. The resulting nuclei were lysed with solution B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT), and a chromatin enriched fraction was isolated by centrifugation (1700g for 4 min). These pellets were subsequently extracted with solution C (50 mM Tris [pH 8.0], 600 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF, protease inhibitors) to release the chromatin-bound proteins (Shiotani & Zou, 2009). Protein sample concentration was quantified using the Bio-Rad DC Protein Assay / Lowry reagent (BioRad).

2.4.Western blotting

Equal amounts of protein were loaded on each lane (20 to 70 µg / lane), resolved by SDS-Polyacrylamide Gel Electrophoresis (SDS PAGE) and transferred into a nitrocellulose membrane (Protran). Membranes were blocked with 5 % non-fat milk prepared in TBS-T

(20 mM Tris HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20) either for 1 hour at RT or overnight at 4 ºC under constant agitation. Primary antibodies were diluted in TBS-T containing 2% BSA and incubated for 1h at RT or overnight at 4 ºC. After extensive washing with TBS-T, membranes were incubated for 1 hour with the corresponding secondary HRP (Horseradish Peroxidase)- conjugated antibody. Membranes where then incubated with either ECL chemiluminiscent substrate (Pierce) or Immobilon Western Chemiluminescent HRP Substrate (Millipore) for low abundace proteins. For stripping off the antibody the membranes were incubated for 30 minutes at 50 ºC with 62.5 mM Tris-HCl pH 6.8, 2% SDS, 100 mM β-mercaptoethanol.

3.IMMUNOFLUORESCENCE

Cells grown on coverslips in 24-well plates were washed with cold PBS and fixed with cold Metanol for 10 minutes at -20ºC. Permeabilization of the cells was performed with 0.1 % Triton X-100 dissolved in PBS for 10 minutes at RT. Blocking was carried out for 30 minutes with 5% horse serum dissolved in PBS-1% BSA. Then, the coverslips were incubated with the primary antibody diluted in TBS-1% BSA overnight at 4 ºC in a humidified chamber. After washing six times with PBS, coverslips were incubated for 40 minutes at RT with the secondary antibody diluted in PBS-BSA 1%. Then, cells were washed four times with PBS and afterwards with miliQ water and mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories). The cells were examined by confocal microscopy (Leica TCS SPE).

For ssDNA detection the cells were cultured for 24h before the treatment with 10µM of BrdU to allow homogenous incorporation of the thymidine analogue to the DNA of replicating cells. Immunofluorescence was performed as explained above in non-denaturing conditions (Beck et al., 2010).

4.IMMUNOPRECIPITATION AND KINASE ASSAY

HEK-293T cells were transfected with empty vector or expression vectors for myc-tagged Cyclin O and HA-ATM-KD (Canman, 1998) using the PEI method. 48 hours after transfection cells were collected by scrapping, washed with PBS and lysed with Lysis Buffer (50 mM Tris HCl pH 7.4, 0.5% NP-40, 20 mM EDTA, 0.15 M NaCl) to which protease and phosphatase inhibitors were added (2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml antipain, 20 µg/ml soybean trypsin inhibitor, 1 mM DTT, 1 mM NaF, 1 mM β-glycerol phosphate, 1 mM sodium orthophosphate, 1 mM Pefablock and 200 mM sodium ortovanadate). For immunoprecipitation, first 50 µl of the supernatant of the 9E10 anti-myc hybridoma or 100ul of the 12CA5 anti-HA hybridoma were bound to 10 µl of washed Protein-G Sepharose beads (GE Healthcare). As a negative control, 50 µl of an irrelevant hybridoma of the same immunoglobulin isotype were used. 750 µg of cell extract was then mixed with the antibody beads and incubated for 3 h at 4 ºC with constant agitation. Beads were then washed extensively with Kinase Buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT). Beads with the myc-Cyclin O or the negative controls were finally resuspended in 30 µl of Hot Mix (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 20 µM ATP, 10 µCi [$\gamma-^{32}P$]

ATP, 1 mM DTT) and added to the immunoprecipitated HA-ATM-KD substrate beads. When Histone H1 was used as a substrate (positive controls), we added 2µg of calf thymus Histone H1 (Roche) instead of the HA-ATM-KD beads. Kinase reactions were incubated 30 minutes at 30ºC and then stopped by adding 30 µl of Laemmli Buffer 2X. 20 µl of the reaction were loaded on a SDS-PAGE, Coomassie Blue stained, drained and the signal detected by autoradiography (Roset R; Gil-Gómez G., 2009).

5.CHECKPOINT ANALYSIS

5.1.G1/S checkpoint

One million of primary MEFs were seeded in 10cm plates and cultured overnight. The next day the cells were treated with 5Gy of γ-radiation. One hour prior to harvesting, the primary MEFs were pulsed for one hour with 10 µM BrdU. Cells were harvested at the required times and centrifuged 5 minutes at 300g at RT. Supernatant was aspirated and the pellet loosened by tapping the tube. While vortexing, ice cold 70% ethanol was added dropwise to the cells to a final concentration of 1 x 10^6 cells/100 µl and incubated 20 minutes at RT. Cells were then washed with 1 ml of wash buffer (PBS containing 0.5% BSA). Pellets were resuspended in denaturing solution (2M HCl) and incubated 20 minutes at RT. After that, cells were washed, resuspended and incubated 2 minutes at RT in 0.5 ml of 0.1 M sodium borate ($Na_2B_4O_7$), pH 8.5, to neutralize any residual acid. Cells were washed again and incubated 20 minutes at RT in 50µl of anti-BrdU monoclonal

antibody (Cat. No. 555627) diluted 1/100 in PBS containing 0.5% Tween-20, 0.5% BSA. Cells were washed again one time and pellets resupended in FITC-conjugated goat anti-mouse Ig (Cat. No. 554001) in dilution buffer and incubated 20 minuts at RT. Cells were washed again and resuspended in 0,5mL of 10µg/ml propidium iodide in PBS and incubated 30 mintues at RT protected from light. Finally, cells were analyzed by flow cytometry (FACScan – Becton Dickinson). The percentage of BrdU positive cells in the entire alive cell population were measured using the FlowJo software.

5.2.G2/M checkpoint

250.000 SV40 Tag transformed MEFs were seeded on a 6 cm plate and cultured overnight. The cells were harvested by trypsinization at different time points after treatment with γ-radiation, washed with PBS, and fixed in suspension by the addition of 2 ml of 70% ethanol followed by incubation at −20°C for 24 h. After fixation, the cells were washed twice with 1mL of PBS containing 0.25% Triton X-100. After centrifugation, the cell pellet was suspended in 100 µl of PBS containing 1% BSA and 10ug/ml anti phosphor-S10 Histone H3 (1:200 of commercial Millipore Ab – ref: 06-570) and incubated for 1h at room temperature. The cells were then rinsed with PBS containing 1% BSA and incubated with FITC-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories) diluted at a ratio of 1:200 in PBS containing 1% BSA. After 1 hour of incubation at room temperature in the dark, the cells were washed again, resuspended in PBS containing 25 µg/mL of propidium iodide (PI) and 0.2 mg/mL of RNase A (Sigma), and incubated at room temperature for 30 min

before the fluorescence was measured (Xu, Kim, Kastan, & Xu, 2001). Finally, cells were analyzed by flow cytometry (FACScan – Becton Dickinson). The percentage of phospho-S10 Histone H3 positive cells in the entire alive cell population were measured using FlowJo software.

6.APOPTOSIS ASSAYS

6.1.Apoptosis assay in thymocytes

Thymuses were obtained from 9 weeks old mice. The organs were washed with PBS, disaggregated using a 5mL syringe barrel and the thymocytes separated filtering through a 70µm nylon filter into 15mL conical tubes. Cells were pelleted and resuspended in 5mL of Blood Cell Lysis Buffer (Sigma) for 5 minutes in order to eliminate the erythrocytes. Cells were pelleted and resuspended in RPMI-1640 supplmented with 10% FBS, antibiotics (Penicillin and Streptomycin) and 45 µM β-mercaptoethanol. Cells were then cultured at a density of $1x10^6$ cells/mL and harvested at different time points after treatment with γ-radiation. For the staining, cells were washed twice with PBS and resuspended with Nicoletti solution (0.1% tri-sodium citrate, 0.1% Triton X-100, 50 µg/ml propidium iodide) (Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, 1991). Samples were incubated for 30 minutes at RT and then analyzed by flow cytometry (FACScan – Becton Dickinson). The percentage of SubG1 cells in the entire cell population were measured using FlowJo software (Gil-Gómez et al., 1998).

6.2.Apoptosis assays in activated T-cell blasts

Spleens were obtained from 9 weeks old mice. The organs were washed with PBS, disaggregated using a 5mL syringe barrel and the spleenocytes isolated by filtering through a 70µm nylon filter into 15mL conical tubes. Cells were pelleted and resuspended in 5mL of Blood Cell Lysis Buffer (Sigma) for 5 minutes in order to eliminate the erythrocytes. Cells were pelleted, resuspended and cultured in RPMI-1640 supplemented with 10% FBS, X63-IL2 conditioned medium (1:50) as a source of IL2 (Brady, Gil-gomez, Kirberg, & Berns, 1996), 2µg/mL of Concanavalin A, antibiotics and 45 µM β-mercaptoethanol for 3 days. After this period, the cells debris was removed by centrifugation through a discontinous Percoll gradient. The living cells, corresponding to a T-cell enriched population, were cultured 2 more days in RPMI-1640 supplmented with 10% FBS, X63-IL2 conditioned medium (1:50) as a source of IL2 (Brady et al., 1996), antibiotics (Penicillin and Streptomycin) and 45 µM β-mercaptoethanol at a density of $1xE10^6$ cells/mL. The third day, the cells were treated with γ-radiation and harvested at different time points. For the staining, cells were washed twice with PBS and resuspended with Nicoletti solution (0.1% tris-sodium citrate, 0.1% Triton X-100, 50 µg/ml propidium iodide) (Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, 1991). Samples were incubated for 30 minutes at RT and then analyzed by flow cytometry (FACScan – Becton Dickinson). The percentage of SubG1 cells in the entire cell population were measured using FlowJo software.

7.COLONY ASSAY

The ability of immortalized MEFs to form colonies upon γ-radiation was tested by clonogenic colony forming assay. Non-treated or irradiated cells were plated on 10cm plate in triplicates at a density of 500cells/plate (in the case of 0, 2 and 4Gy) or 1000cells/plate (in the case of 6Gy). Cells were cultured for 13 days until cells in the control plates formed visible colonies. Colonies were stained with crystal violet and counted to calculate the survival ratio.

8.MASS SPECTROMETRY

Sample preparation

100µg of each sample was reduced with dithiothreitol (10 µM, 30min, 56°C), alkylated in the dark with iodoacetamide (5 µM mM, 30 min, 25 ºC) and digested with 2 µg of trypsin (Promega, cat # V5113) for eight hours at 37˚C following fasp procedure (Zougman, Nagaraj, & Mann, 2009). After digestion, peptide were eluted and cleaned up on a homemade Empore C18 column (3M, St. Paul, MN, USA) (Rappsilber J1, Mann M, 2007). 3µg of each sample was analyzed using a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled to a nano-HPLC (Agilent Technologies 1200 Series, CA, USA). Peptides were loaded onto a C18 Zorbax pre-column (Agilent Technologies, cat #5065-9913) and separated by reversed-phase chromatography using a 12-cm column with an inner diameter of 75 µm, packed with 5 µm C18 particles (Nikkyo Technos Co., Ltd. Japan). All data were acquired with Xcalibur software v2.2.

TiO2-Based Enrichment of Phosphopeptides

80 µg of the digested was enriched on TiO2-beads as previously described (Thingholm TE, 2009). Samples were analyzed using a LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled to an EasyLC (Thermo Fisher Scientific (Proxeon), Odense, Denmark). Peptides were loaded directly onto the analytical column at 1.5-2 µl / min using a wash-volume of 4 to 5 times injection volume and were separated by reversed-phase chromatography using a 12-cm column with an inner diameter of 75 µm, packed with 5 µm C18 particles (Nikkyo Technos Co., Ltd. Japan). All data were acquired with Xcalibur software v2.2.

Data Analysis

Proteome Discoverer software suite (v1.3.0.339, Thermo Fisher Scientific) and the Mascot search engine (v2.3, Matrix Science (Perkins DN, Pappin DJ, Creasy DM, 1999)) were used for peptide identification and quantification. The data were searched against a SwissProt database containing entries corresponding to Mouse (version of September 2012), a list of common contaminants, and all the corresponding decoy entries. Trypsin was chosen as enzyme and a maximum of three miscleavages were allowed. Carbamidomethylation (C) was set as a fixed modification, whereas oxidation (M), acetylation (N-terminal) and phosphorylation (STY) were used as variable modifications. Searches were performed using a peptide tolerance of 7 ppm, a product ion tolerance of 0.5 Da. Resulting data files were filtered for FDR < 5 % at the peptide level for not enriched proteome, and Mascot IonScore > 20 for the phosphorylated dataset.

The area under the curve for each peptide has been used for the quantitation analysis. The data have been normalized at the peptide level by median normalization. Normalized peptide areas were then used to calculate fold-change of the proteins or phospho peptides, proteome and phospho proteome respectively, with MSstat (v 1.0.0) (Clough, Thaminy, Ragg, Aebersold, & Vitek, 2012). Then, phosphorylated peptide abundance changes were corrected according to the observed protein changes.

9. ANTIBODIES USED

Antigen	Supplier	Reference	Clone	Techniques
BrdU	Beckton Dickinson	555627		FACS; IF
phospho-S10 H3	Millipore	06-570		FACS; IF; WB
Histone H3	Ab Cam	Ab 1791		WB
phospho-S1981 ATM	Millipore	05-740	10H11.E11	IF; WB
ATM	Santa Cruz	SC-7230	H-248	WB
phospho-T2609 DNA-PK	Ab Cam	Ab97611		WB
DNA-PK	Neo Markers	MS-423-P1	Cocktail	WB
ɣH2AX (phospho-S139 H2AX)	Millipore	05-636	JBW301	IF; WB
H2AX	Ab Cam	Ab11175		WB
phospho-S343 NBS_1	R&D Systems	AF-4944		WB
NBS_1 (Nibrin)	Santa Cruz	SC-8580	C-19	WB
Sin3A	Santa Cruz	SC-994		WB
phospho-CHK1	Cell Signaling	#2348	133D3	WB
CHK1	Santa Cruz	SC-7878	FL-476	WB
CHK2	Millipore	05-649	Clone-7	WB
phospho-S15 p53	Cell Signaling	#9286	16G8	WB
p53	Cell Signaling	#9282		WB
p21	Santa Cruz	SC-397	C-19	WB
RNA-Pol II	Santa Cruz	SC-899	N-20	WB
Actin	Sigma	A5441	AC.15	WB
53BP1	Novus B	NB100-904		IF

Antigen	Supplier	Reference	Clone	Techniques
phospho-S4/S8 RPA	Bethyl Laboratories	A300-245A		IF
myc antigen	Hibridoma		9E10	IP
HA antigen	Hibridoma		12CA5	IP
Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L)	Invitrogen		A11029	IF
Alexa Fluor® 488 Donkey Anti-Rabbit IgG (H+L)	Invitrogen		A21206	IF
Alexa Fluor® 555 Goat Anti-Mouse IgG (H+L)	Invitrogen		A21424	IF
Rabbit IgG, HRP-linked whole Ab (from donkey)	GE Healthcare Life Sciences		N934V	WB
Mouse IgG, HRP-linked F(ab)2 fragment (from sheep)	GE Healthcare Life Sciences		N931V	WB

10. GENERATION OF A CYCLIN O DEFICIENT MOUSE

Design and construction of a Cyclin O-targeting vector, ES cell transfection and identification of correctly targeted cell clones were done by KOMP (Knock-Out Mouse Project). In Figure.M1 is shown the strategy used to target the Cyclin O gene. The Cyclin O ORF was replaced by homologous recombination by the LacZ coding sequence preserving the Cyclin O regulatory elements of the promoter. The inserted sequence also includes a floxed neomycin resistance cassette that could be removed later on by intercrossing with transgenic mice expressing the Cre recombinase.

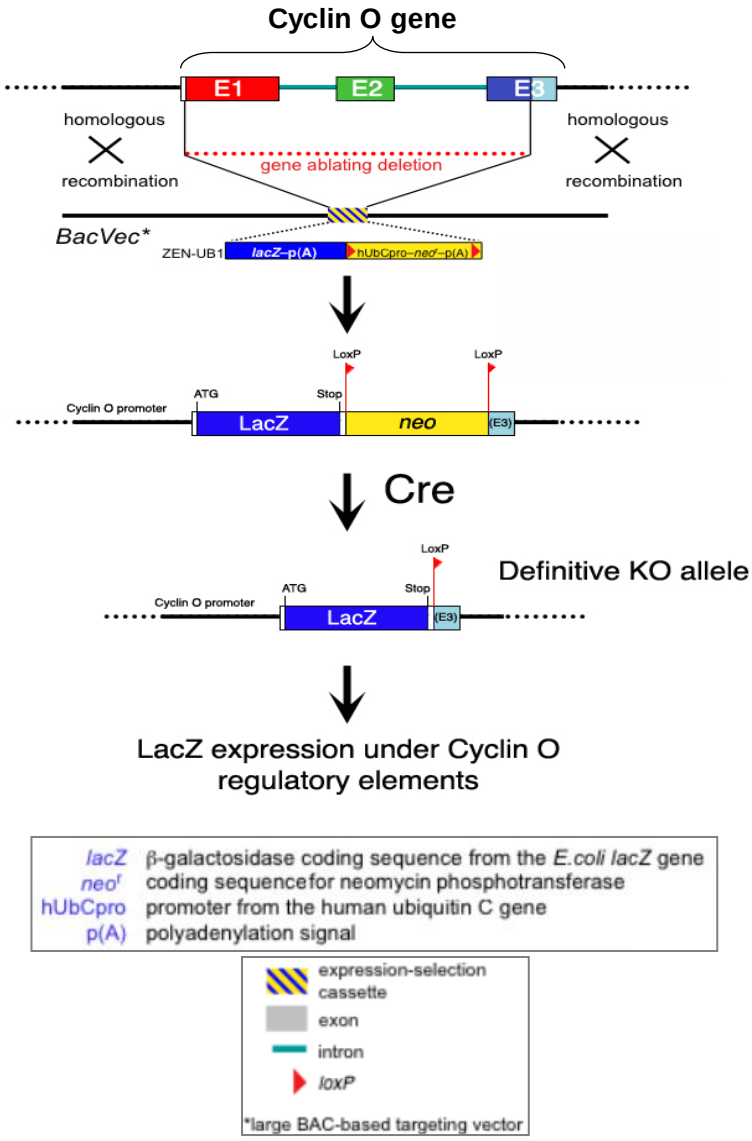


Figure.M1. – **Targeting strategy of the Cyclin O gene in ES cells.**

Three ES targeted clones were purchased and injected into into C57Bl/6N blastocysts and these implanted into foster mothers. Six alive male chimaeras were obtained from two of the clones

(Table.M1). The ES microinjection into pre-implantation stage mouse embryos and the generation of chimeras were done at the Transgenesis Unit of CNIO (Madrid).

Clone	Euploidy	DOB	Chimeras	% coat color
Ccno-AC4	81-90%	18/01/2011	4	100/100/70/50
Ccno-AD7	41-50%	02/02/2011	0	Dead pups had black eyes
Ccno-AD9	71-80%	09/02/2011	2	80/70

Table.M1. – **KOMP ES cell clones and chimaeras obtained.**

All six male chimaeras were bred to C57Bl/6N females and the offspring screened for germ line transmission by PCR. All the chimaeras transmitted the knockout allele and then we established two lines: AC4/ALO and AD9/ALN that showed identical phenotype. Animals were kept under pathogen-free conditions and all procedures were approved by the Animal Care Committee.

To obtain the definitive KO line, the heterozygous mice were crossed to Cre-deleter mice (Schwenk F, Baron U, 1995) to remove the floxed neo cassette (Figure.M1). After getting Cre-mediated excision of the neo marker, the transgene was segregated from the targeted allele by crossings with wild type C57Bl/6N mice.

Since all this process takes a long time, we intercrossed the Cyclin O null, neo-containing alleles to obtain the cellular models used in this work.

10.1.Genotyping strategy

For the purpose of genotyping we have designed primers for PCR that allowed us to distinguish WT and KO alleles based on unique

amplification products (Table.M2). Primer sequences are shown in the Table.M3 and PCR conditions in Table.M4. Genomic DNA used for genotyping was isolated from tail biopsies and extracted by proteinase K digestion over night at 55ºC in tail buffer (10mM Tris ph:8, 100mM NaCl, 10mM EDTA pH:8, 0,5% SDS). The next day, the DNA was isolated by isopropanol precipitation and rehydrated with TE solution (10mM Tris·Cl pH8 and 1mM EDTA).

Alleles	Fragment Size
KO allele (NeoF - SD)	471 bp (KO)
WT allele (WTE3F1 - SD)	652 bp (WT)

Table.M2. – **Fragments size obtained with the primers designed for genotyping.**

Primer Name	Sequence
WTE3F1	GCTGAGCCTAACGGATTACG
SD	CAGTCAGGAGGCTGAGTTCC
NeoF	TCATTCTCAGTATTGTTTTGCC

Table.M3. – **Primer sequence used to genotype Cyclin O KO mice.** WTE3F1 is only present in the WT allele and the NeoF only in the KO.

	PCR conditions	
	Initial Denaturation	2' 93ºC
Cycles x40	Denaturation	30" 93ºC
	Annealing	30" 55ºC
	Extension	50" 72ºC
	Final Extension	10' 72ºC

Table.M4. – **PCR conditions for genotyping.**

10.2.Histochemistry

Immunohistochemical analysis was performed using 4 μm sections of paraformaldehyde-fixed, paraffin-embedded mouse tissue blocks. The tissue sections were hydrated and stained with haematoxylin, dehydrated and mounted. Samples were analyzed under the microscope (Olympus BX61).

10.3.MRI analysis of adult mice brains

We performed T2-weighted MRI scans while each mouse was under isoflurane-induced anesthesia using a BioSpec 70/30 USR MRI system at the IDIBAPS (Barcelona). There was an in-plane resolution of 0.13 × 0.25 mm^2 and an approximate slice thickness of 0.6 mm acquired in the axial, sagital and coronal planes.

11.STATISTICAL ANALYSIS

Data were expressed as the mean ± S.E.M. (Standard Error of the Mean). Data were evaluated statistically by Student's t-test.

Survival data were analyzed according to the method of Kaplan-Meier and tested for significance between the groups with the log rank test

Significance levels: P=0.05, *: 0.05 >p>0.01, **: 0.01>p>0.005.

RESULTS

1.CYCLIN O AND THE CELL CYCLE CHECKPOINTS

The first part of the results deals with the analysis of the participation of Cyclin O in the regulation of the cell cycle checkpoints. Determining if any checkpoint is affected by the Cyclin O deficiency in response to DNA damage will permit us to identify biochemical pathways regulated by Cyclin O.

The cellular model used to evaluate the cellular checkpoints was the MEFs obtained from Cyclin O WT, HET and KO E13.5 embryos as explained in Material and Methods. We used primary MEFs to analyze the G1/S checkpoint, since it is dependent on p53 function, and SV40 Tag immortalized MEFs to evaluate the G2/M checkpoint. We observed comparable growth rates of primary and immortalized MEFs regardless of the genotype indicating that they do not present gross growth or checkpoint defects during normal cell cycle progression (data not shown).

1.1. Activation of the G1/S cell cycle checkpoint after γ-radiation in Cyclin O deficient cells

In order to ensure a correct cell division and to avoid the accumulation of mutations in the daughter cells, the cell cycle has different regulatory mechanisms that make it to stop upon sensing DNA damage altogether known as cell cycle checkpoints. These checkpoints prevent the execution of the molecular events that determine the irreversible progression from one cell cycle stage to the following. Three of these checkpoints (G1/S, intra S and G2/M) are activated by DNA damage.

The first checkpoint analyzed regulates the G1 to S transition, which is activated after DNA damage and it is dependent on ATM activity. The activation of this checkpoint prevents the entry into the cell cycle phase were DNA is going to be duplicated. Thus, we can analyze the entry into S-phase by measuring the incorporation of the thymidine analogue BrdU.

We treated primary MEFs with 5Gy of γ-radiation and added BrdU to the medium 1h prior to collecting the samples in order to allow to the incorporation of this thymidine analogue into the cells that are actively replicating their DNA. We collected the cells at different time points and analyzed the BrdU incorporation by FACS. We observed a time-dependent reduction in the percentage of cells positive for BrdU, indicating the activation of the G1/S phase checkpoint. As it is shown in the Figure.R1, we observed up to 4 fold reduction of the BrdU incorporation rate 24h after irradiation, but we did not detect any significant difference between WT and KO cells at any time point.

This result indicates that the activation of the G1/S checkpoint and the transition G1 to S-phase is not affected by the loss of Cyclin O.

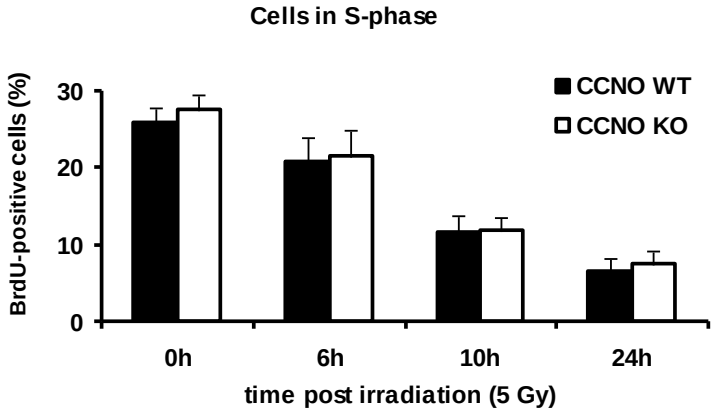


Figure.R1. – **Activation of the G1-S checkpoint in primary MEFs.** Primary MEFs were treated with 5Gy of γ-radiation, cultured and samples were harvested at different time points. 1h before harvesting, BrdU (10μM) was added to the culture. The cells were stained with an antibody against BrdU and counterstained with PI to measure the DNA-content of the cell. BrdU positive cells were quantitated by flow cytometry.

1.2. Activation of the G2/M cell cycle checkpoint after γ-radiation in Cyclin O deficient cells

The second checkpoint analyzed is the transition between the G2 and M phases of the cell cycle. This checkpoint is activated mainly by DNA damage and, then, is largely dependent on ATM. A crucial event when a cell transits from G2 to M phase is the phosphorylation of Histone H3 at S10 by Aurora B kinase (Hsu et al., 2000). This can be used as a marker of the cells that are in M phase in any moment and can be monitored by FACS.

We observed that at 1h, 2h and 4h after irradiation the percentage of cells with phosphorylated S10 HH3 is significantly higher in the

WT compared to the Cyclin O deficient cells, as we can see in Figure.R2. This indicates that the G2/M checkpoint is activated more efficiently in the KO cells compared to the WT controls. Afterwards, during the resumption of the cell cycle, we observed a progressive increase in the percentage of mitotic WT cells at 2h, 4h and 6h after irradiation, while in the KO cells this percentage is comparatively lower. This indicates a delayed exit from the G2/M checkpoint in Cyclin O deficient cells.

Altogether these results indicate a stronger and more prolonged activation of the G2/M checkpoint in Cyclin O KO cells compared to the WT controls.

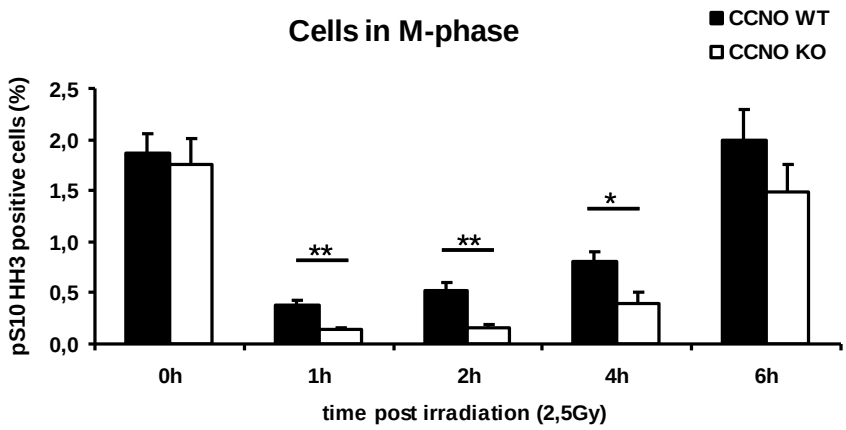


Figure.R2. – **Activation of the G2-M checkpoint in MEFs.** SV40 Tag immortalized fibroblasts were treated with 2.5Gy of γ-radiation. These cells were stained with an antibody against phosphorylated S10 HH3 and counterstained with PI to measure the DNA-content of the cell. Positive pS10 HH3 cells were quantitated by flow citometry.

To further confirm these results, we analyzed the G2-M checkpoint activation by western blotting. We treated immortalized MEFs with

2.5 Gy of γ-radiation and analyzed the HH3 S10 phosphorylation at different time points (0h, 30', 1h and 2h) (Figure.R3). In agreement with the results of the FACS experiments (Figure.R2), we detected a stronger reduction in the amount of phosphorylated S10 HH3 in Cyclin O KO cells compared to the WT controls 1 hour after gamma radiation treatment. We then observed the recovery of the basal levels of phosphorylated S10 HH3 in the Cyclin O WT cells 2h post irradiation while in the KO cells at this time point the levels are still lower than in non-irradiated cells. This indicates a delay in the exit from the G2/M checkpoint in the Cyclin O KO cells compared to the WT controls.

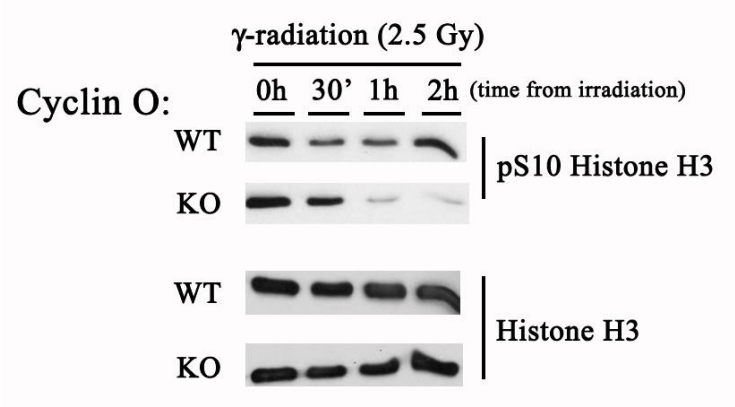


Figure.R3. – **Activation of the G2-M checkpoint in immortalized MEFs.** Phosphorylation of the S10 HH3 was analyzed at different time points after irradiation (2,5Gy) by western blotting in cell lysates from Cyclin O WT and KO SV40 Tag immortalized fibroblasts.

2. CYCLIN O AND THE DNA DAMAGE RESPONSE PATHWAYS

2.1.Activation of the DNA Damage Response in Cyclin O deficient cells

2.1.1.Activation of PIKKs and mediators of the DNA Damage Response in Cyclin O deficient cells

In the previous sections we have analyzed the effects of γ-radiation-induced DNA damage in Cyclin O deficient cells in terms o checkpoint activation. Now, we will analyze the molecular consequences of the deficiency of Cyclin O in the DNA Damage Response.

We first analyzed the activation of the kinases of the PIKK family after γ-radiation by western blotting. DNA damage transducers and sensors such as ATM and DNA-PK become activated after the recognition of the DNA damage by a sensor, (KU or the MRN complex respectively) or the 9-1-1 complex in the case of ATR. The direct consequence of the recognition of the DNA damage is their activation by autophosphorylation, which is necessary for the proper activity of these proteins. ATM, that orchestrates the organization of the DNA repair machinery mainly by HR, gets autophosphorylated at several residues including S1981. As it can be seen in Figure.R4, we observed a deficient phosphorylation of this residue in Cyclin O KO cells compared to WT controls. Moreover, DNA-PKcs, which is necessary to repair DSBs via NHEJ, is autophosphorylated at T2609 after γ-radiation, a residue

located at the ABCDE domain of the protein. In this case we also observed a deficient phosphorylation of T2609 of DNA-PKcs in Cyclin O KO cells compared to the WT controls. These results suggest that the activation of the DNA damage sensors and/or transducers in response to DSBs is compromised in Cyclin O deficient cells. Moreover, the T2609 residue of DNA-PKcs can also be phosphorylated by ATM in cells that fail to activate NHEJ (Shrivastav et al., 2008) confirming the observation that ATM is not properly activated in Cyclin O deficient cells.

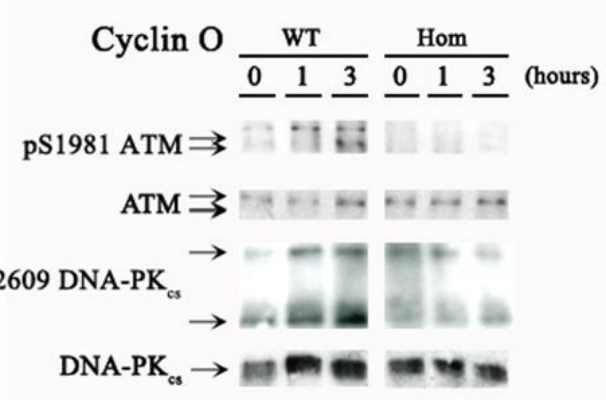


Figure.R4. – **Activation of the transducers of the DDR in response to γ-radiation in primary MEFs.** Western blot from Cyclin O WT and KO whole cell lysates treated with 5Gy of γ-radiation and harvested at different time points.

Given the deficiency in the activation of the transducers of the DNA damage we analyzed if the downstream mediator targets of ATM and/or DNA-PKcs get properly phosphorylated (Figure.R5). We observed a deficiency in the phosphorylation of H2AX in Cyclin O deficient cells. H2AX is a core histone from the HH2 family that is part of the nucleosomes and it is phosphorylated at S139 by the

PIKKs (then called ɣH2AX) in response to DNA DSBs. This phosphorylation is widely used as a marker of DNA damage and the proper formation of the IRIF, the DNA repair scaffold at the chromatin level (Bekker-Jensen & Mailand, 2010; Belyaev, 2010). Nbs1 is a member of the MRN complex necessary for the recruitment of ATM to the DSBs. ATM phosphorylates Nbs1 at S343 in response to DNA damage although its biological relevance of this modification is not known (Difilippantonio et al., 2007). By western blot we observed that the phosphorylation ot H2AX at S139 and Nbs1 at S343 were compromised in Cyclin O KO cells compared to WT after γ-radiation treatment. This, again, suggests that ATM and DNA-PK are not properly activated in Cyclin O deficient cells in response to DNA damage since their downstream targets at the level of chromatin were not properly phosphorylated.

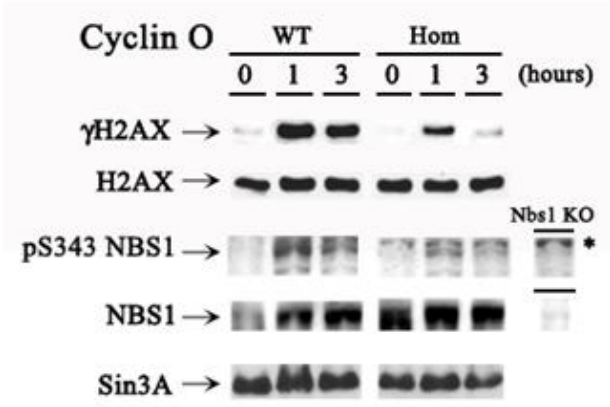


Figure.R5. – **Phosphorylation of PIKKs targets mediators of the DDR in response to ɣ-radiation in primary MEFs.** Western blot from Cyclin O WT and KO whole cell lysates treated with 5Gy of γ-radiation and harvested at different time points. Cell lysate from Nbs1 KO cells was used to identify the specific band. A non-specific band is marked by an asterisk (*).

2.1.2.Activation of the effector kinases and the p53 pathway of the DNA damage response in Cyclin O deficient cells

The next step was to analyze the activation of the effectors of the DDR downstream of ATM (Figure.R6). The first effector analyzed was CHK1 which is phosphorylated by ATR at S345, a step necessary for the activation of the G2/M checkpoint (H. U. I. Zhao & Piwnica-worms, 2001). We observed an increased phosphorylation of CHK1 at this residue in Cyclin O KO cells compared to the WT controls after treatment with γ-irradiation. We also analyzed CHK2 phosphorylation which is an ATM substrate effector. ATM phosphorylates CHK2 at T68, a prerequisite for the hyperphosphorylation of this protein necessary for its activation. This hyperphosphorylation results in a reduced mobility in gel electrophoresis that is evidenced by a shift in the western blot. We observed a robust activation of CHK2 after γ-radiation treatment in both WT and KO cells, indicating that Cyclin O is not essential for the proper activation of CHK2 in response to DNA damage.

Then we analyzed the activation of p53 in response to γ-radiation. p53 is constituvely sent to proteasomal degradation in normal cells and its levels are barely detectable by western blot. After DNA damage it gets stabilized after phosphorylation by the DNA damage PIKKs and CHK2 amongst others (Hirao, 2000; Iwakuma & Lozano, 2007), permitting its function as a transcription factor regulating the expression/repression of proteins implicated in cell cycle regulation, DNA repair and apoptosis. The phosphorylation of p53 at S15 and its stabilization were comparable in Cyclin O WT

and KO cells. Moreover, we detected similar induction of $p21^{Cip1}$ in Cyclin O WT and KO cells, which is a direct p53 target gene that gets activated in response to DNA damage. Thus, in contrast to the substrate targets of ATM/DNA-PKcs, we observed normal activation of the soluble effectors of the p53 pathway in Cyclin O deficient cells.

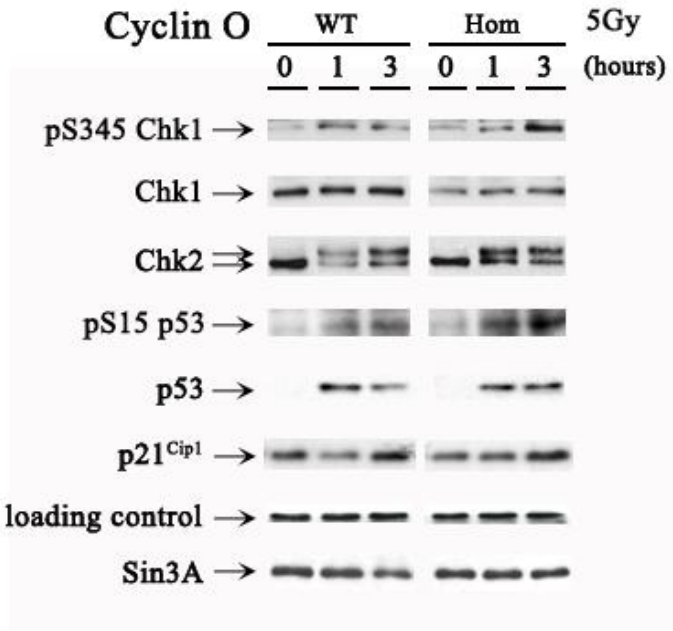


Figure.R6. – **Activation of effectors and the p53 of the DDR in response to γ-radiation in MEFs.** Western blot from Cyclin O WT and KO whole cell lysates treated with 5Gy of γ-radiation and harvested at different time points. The nuclear protein Sin3A and a non-specific band were used as loading controls.

Altogether the results shown in this section indicate that the activation of the apical transducers of the DNA damage response, ATM and DNA-PKcs, are not properly activated in Cyclin O deficient cells. As a consequence, the ATM and DNA-PKcs

substrates at the level of the chromatin (H2AX and Nbs1) are not properly phosphorylated in Cyclin O deficient cells. However, the downstream effector CHK2 and the p53 pathway responsible to activate the cellular responses to DNA damage show normal activation in Cyclin O deficient cells. These results evidence a disconnection between the activation of the PIKKs targets associated to the chromatin and the downstream effectors as a consequence of Cyclin O deficiency.

To confirm the differential activation of these substrates at the chromatin level we performed a fractionation of the cell lysates to separate the chromatin associated factors from the soluble fraction (Figure.R7). We observed that in Cyclin O KO cells CHK1 was hyperactivated in the soluble fraction whereas CHK2 activation was lower compared to the WT. We also observed that the total, non-phosphorylated, forms of both effectors are associated to the chromatin. The levels of the non phosphorylated form of CHK2 are reduced after irradiation in the chromatin fraction, indicating that the active form of CHK2 is released from the chromatin after DNA damage. However, the activation of CHK2 in the soluble fraction is lower in the Cyclin O KO cells compared to the WT controls, indicating that the phosphorylation of the ATM substrates in the chromatin does not take place properly and instead the ATR substrate CHK1 is hyperactivated. These results also suggest that ATR signaling may partially compensate for the ATM and DNA-PKcs deficiency in Cyclin O deficient cells, which will be analyzed in dept in the following sections.

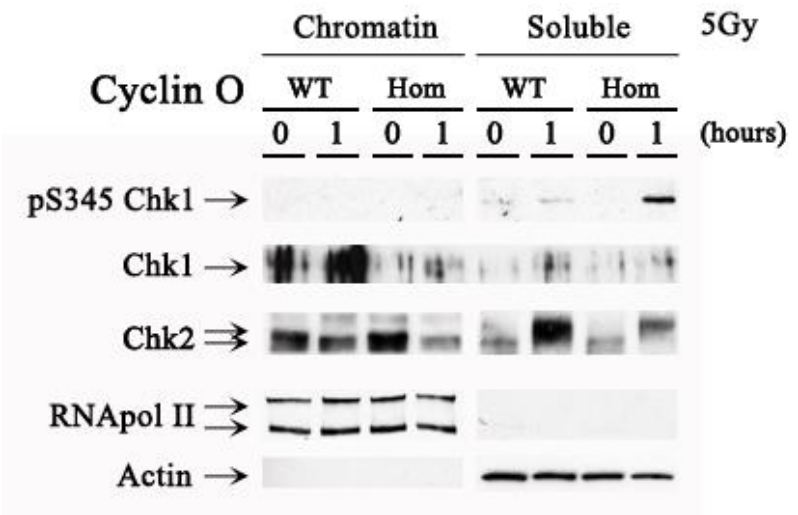


Figure.R7. – **Activation of effectors of the DDR in the chromatin and soluble fraction in response to γ-radiation in MEFs.** Western blot from Cyclin O WT and KO chromatin and soluble fractions of cells treated with 5Gy of γ-radiation and harvested at different time points. RNApol II is used as a chromatin marker. Actin is used as a soluble marker.

2.2.Formation of DNA damage-induced foci

The recruitment and modification of proteins in the vicinity of the DSBs form the IRIFs. The IRIFs can be visualized by fluorescence microscopy as discrete dots/foci in the nuclei using specific antibodies against the proteins that are recruited or PTMs that take place in the DSBs (Bekker-Jensen & Mailand, 2010; Belyaev, 2010). These foci can be quantified and used as an indication of the recruitment of proteins and the biochemical events in the DSBs necessaries for their repair. Moreover, the kinetics of the formation/disappearance of these foci is also indicative of the efficiency or speed of the DNA repair.

2.2.1.Formation of ɣH2AX and 53BP1 foci at the DSBs

In the previous section we have observed that the chromatin associated sensors/transducers of the DDR show a deficient activation when Cyclin O is not present. Next, we analyzed the formation of IRIF in response to DNA damage.

We first analyzed the phosphorylation of Histone H2AX at S139 forming ɣH2AX foci. We observed a clear induction in ɣH2AX foci formation both in WT and Cyclin O KO cells 1h after treating the cells with γ-radiation (Figure.R8). However, when we quantified the mean number of foci per nucleus we observed significantly reduced amounts in Cyclin O KO cells compared to the WT controls. This indicates that Cyclin O is necessary for the proper formation of foci early after DNA damage. However, after 3h and 6h we did not observe differences between WT and KO cells. The number of foci at 3h in Cyclin O KO cells was the same than at 1h, indicating that DNA damage persisted. In contrast, in WT cells we observed a progressive reduction in the number of ɣH2AX foci at 3h and 6h after irradiation. All these findings indicate that the capacity to resolve the DNA damage was faster in Cyclin O WT compared to KO MEFs.

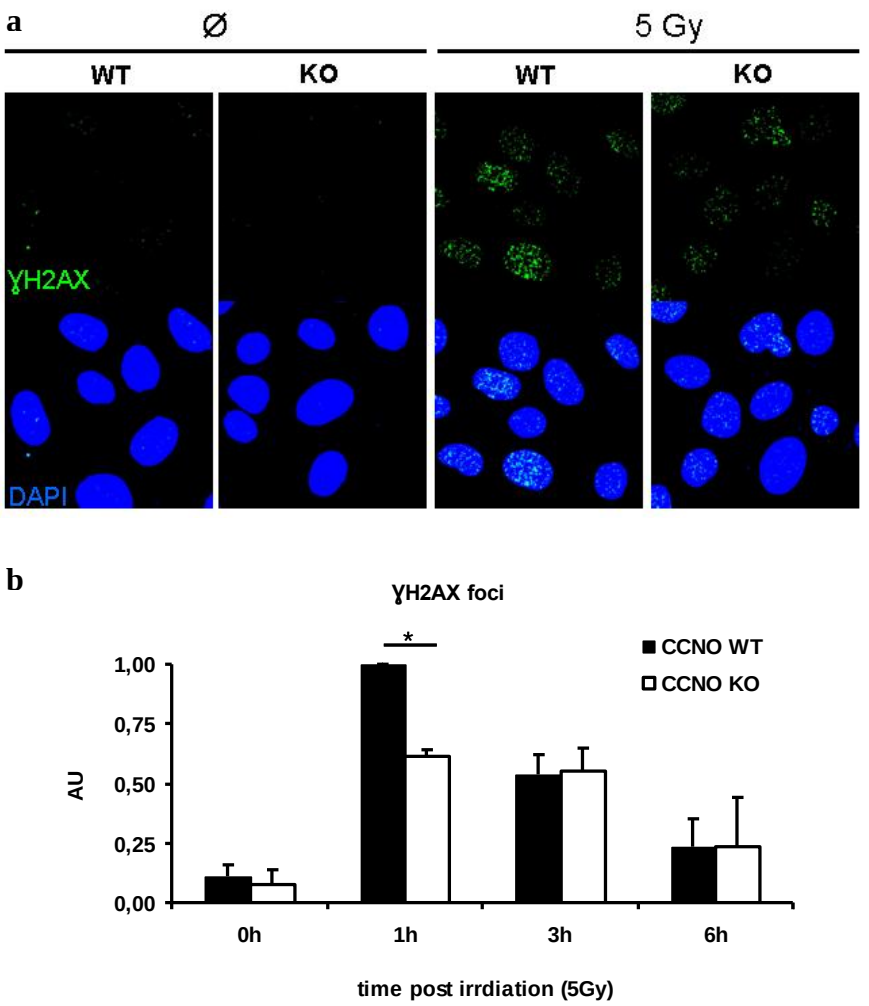
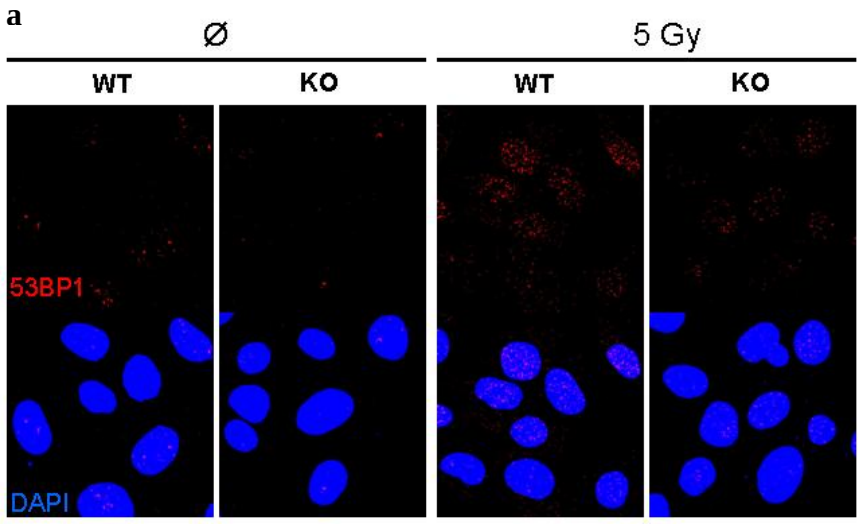


Figure.R8. – **Formation of γH2AX foci after DNA damage in Cyclin O WT and KO immortalized MEFs.** SV40 Tag immortalized Cyclin O WT and KO fibroblasts were cultured on glass coverslips. The cells were fixed at 0h, 1h, 3h and 6h after treatment with 5Gy of γ-radiation and stained using an anti-γH2AX specific antibody (green). Images were taken by confocal microscopy. Ø indicates non-irradiated cells and 5Gy corresponds to 1h after irradiation **(a)**. The number of γH2AX foci per nuclei was counted using the ImageJ software. AU indicates the ratio of the total of number of γH2AX foci per nuclei at each condition compared to the WT 1h **(b)**.

We then analyzed the formation of 53BP1 foci, a mediator protein that plays a role as a scaffold during S/G2 and it is also important for the choice between HR and NHEJ mechanisms preventing DNA resection and thus promoting NHEJ during G1 (Panier & Boulton, 2014). As in the previous figure, 53BP1 foci formation is less efficient in Cyclin O deficient cells compared to the WT controls 1h after treating the cells with 5Gy of γ-radiation (Figure.R9). However, 3h and 6h after treatment we observed a progressive reduction in the number of foci with no significant differences between the two genotypes. These results indicate a deficient recruitment of 53BP1 to the DSBs in Cyclin O deficient cells but a proper disappearance of this protein at later time points.



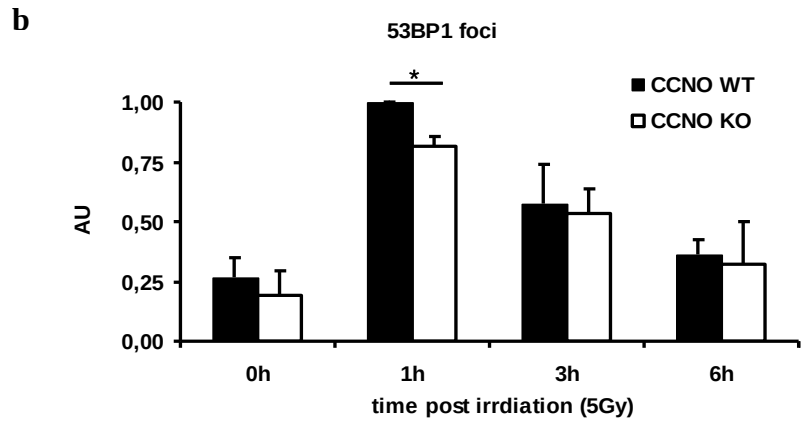


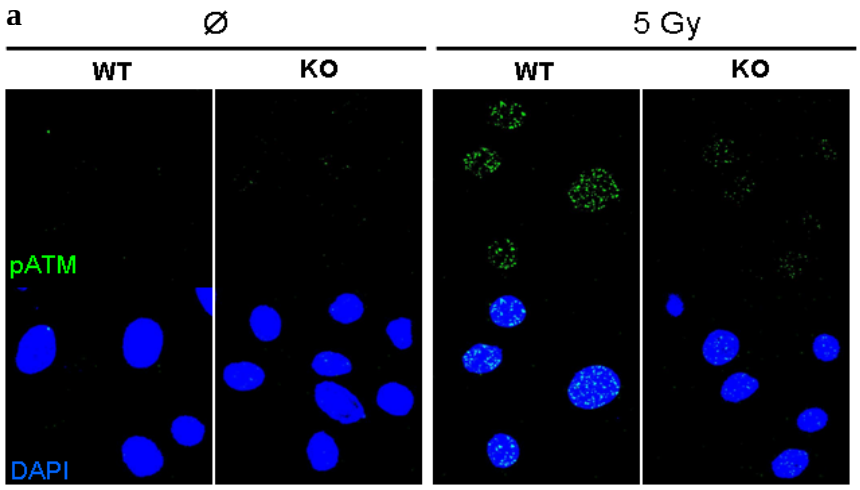
Figure.R9. – **Formation of 53BP1 foci after DNA damage in Cyclin O WT and KO immortalized MEFs.** SV40 Tag immortalized Cyclin O WT and KO fibroblasts were cultured on glass coverslips. The cells were fixed at 0h, 1h, 3h and 6h after treatment with 5Gy of γ-radiation and stained using an anti-53BP1 specific antibody (red). Images were taken by confocal microscopy. Ø indicates non-irradiated cells and 5Gy corresponds to 1h after irradiation **(a)**. The number of 53BP1 foci per nuclei was counted using the ImageJ software. AU indicates the ratio of the total of number of 53BP1 foci per nuclei at each condition compared to the WT 1h after irradiation **(b)**.

2.2.2.Recruitment of ATM to the DSBs

In the previous experiments we have shown that ATM is not properly phosphorylated at S1981 (hereforth called pS1981-ATM) and some of its chromatin associated targets are not properly phosphorylated. However, the effectors downstream of ATM were not affected. It has been described that the phosphorylation of S1981 is not essential for ATM-dependent activation of its downstream effectors and the cellular consequences after DNA damage (Daniel et al., 2008; Pellegrini et al., 2006). However, it is described that the stabilization of ATM at the DSBs depends on the

proper phosphorylation at this residue (So Sairei, Davis Anthony J., 2009).

To confirm the previous observations, we analyzed if pS1981-ATM was properly recruited to the DSBs after DNA damage. To do this we quantified the number of pS1981-ATM foci per cell in Cyclin O WT and KO cells by IF (Figure.R10). As it can be seen in the pictures and in the plots, the number of pS1981-ATM foci per cell in Cyclin O KO cells was significantly lower 1h after treatment with 5Gy when compared to the WT controls.



b

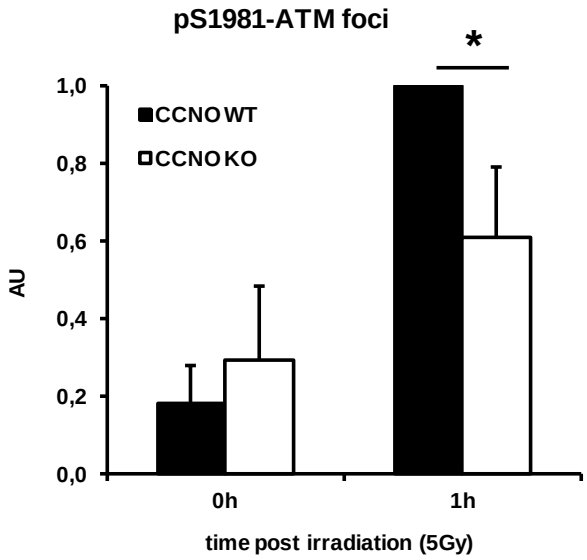


Figure.R10. – **Formation of pS1981-ATM foci at the DSBs in response to γ-radiation in immortalized MEFs.** SV40 Tag immortalized Cyclin O WT and KO fibroblasts were cultured on glass coverslips. The cells were fixed at 0h and 1h after treatment with 5Gy of γ-radiation and stained using an anti-pS1981-ATM specific antibody (green). Images were taken by confocal microscopy. Ø indicates non-irradiated cells and 5Gy corresponds to 1h after irradiation **(a)**. The number of pS1981-ATM foci per nuclei was counted using the ImageJ software. AU indicates the ratio of the total of number of pS1981-ATM foci per nuclei at each condition compared to the WT 1h **(b)**.

2.3.Cyclin O complexes are able to phosphorylate ATM *in vitro*.

One of the aims of the project was to find the molecular connections between Cyclin O and ATM signalling. Because of that, the next step was to evaluate if Cyclin O complexes were able to directly phosphorylate ATM. To do so, we performed a kinase assay incubating immunoprecipitated kinase dead ATM (HA-ATM-KD) with immunoprecipitated, active Cyclin O complexes (myc-Cyclin O). Both HA-ATM-KD and myc-Cyclin O complexes were immunoprecipitated from transiently transfected 293T cells. The ATM-KD sequence contains point mutations on the aa 2870 (changing D to A (D2870A)) and 2875 (changing N to K (N2875K)) in the catalytic loop which inactivates its kinase activity and then preventing its autophosphorylation (Canman, 1998).

Active Cyclin O complexes were able to phosphorylate immunoprecipitated ATM-KD (Figure.R11). We performed the appropriate controls to check for the activity of the myc-Cyclin O complexes which are known to phosphorylate Histone H1 and Cyclon O itself (Roig et al., 2009). Moreover, immunoprecipitation with irrelevant antibodies or using lysates from mock transfected cells did not yield any kinase associated activity. This confirms that the phosphorylation of HA-ATM KD was specific of myc-Cyclin O complexes. This result indicates that ATM, a central protein in the DDR, is a substrate of Cyclin O *in vitro*.

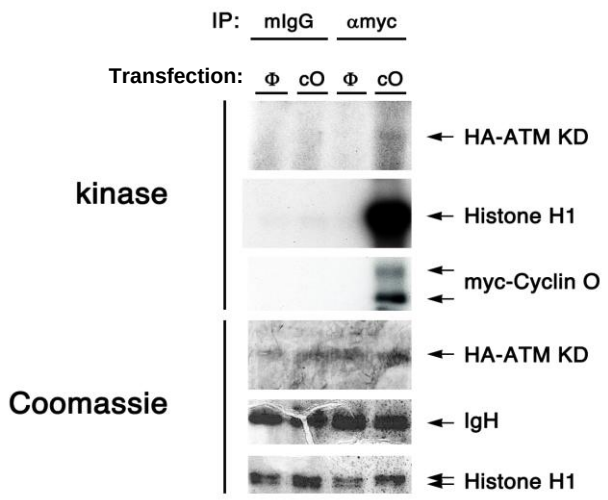


Figure.R11. – **Cyclin O-CDK1/2 complexes are able to phosphorylate ATM *in vitro*.** Protein extracts from 293T cells mock transfected (Ø) or transfected with a myc-Cyclin O expression vector were immunoprecipitated with either mIgG or anti-myc SV40 Tag (αmyc) antibodies. Extracts from 293T cells transitively transfected with a HA-ATM KD expression vector were immunoprecipitated with anti-HA antibody and used as a substrate for the kinase assay. HH1 and the immunoprecipitated Cyclin O were used as positive control substrates for the activity of the Cyclin O complex. Total HA-ATM KD, IgH and Histone H1 protein were detected by Coomassie staining.

However, to study the biological implication of the phosphorylation of ATM by Cyclin O-CDK complexes, experiments to find out which is/are the residue/s phosphorylated and their relevance for the kinase activity or protein-protein interactions are required.

2.4.Cyclin O is necessary for the regulation of CtIP activity

2.4.1.Identification of CtIP as an indirect target of Cyclin O

We used phosphoproteomics in order to identify putative substrates of the Cyclin O complexes. To do this we infected Cyclin O KO MEFs with lentiviral particles carrying expression constructs for Cyclin O. These construcs encoded the WT form and a point mutant that results in the replacement of the lysine in the aa in the position 190 by an alanine (mutation K190A). This mutation affects a residue in the cyclin box critical for the binding with the CDKs. The Cyclin O K190A mutant has been described as a CDK binding deficient form of Cyclin O (A.Balsiger and G.Gil-Gomez, unpuiblished). After the expression of these two forms of Cyclin O in a KO background we analyzed the lysates for differentially expressed phosphoproteins. The first step was to digest the proteins from the lysates with trypsin. Then, we enriched the phosphopeptides from the cell lysates using a metalchelate chromotagraphy on TiO_2 columns and processed the eluates by Mass Spectometry. Trypsin digested lysates were also analyzed by Mass Spectometry in order to identify the changes in the total abundance of proteins as a result of the expression of both WT and K190A variants of Cyclin O. A scheme of the procedure followed is shown in Figure.R12.

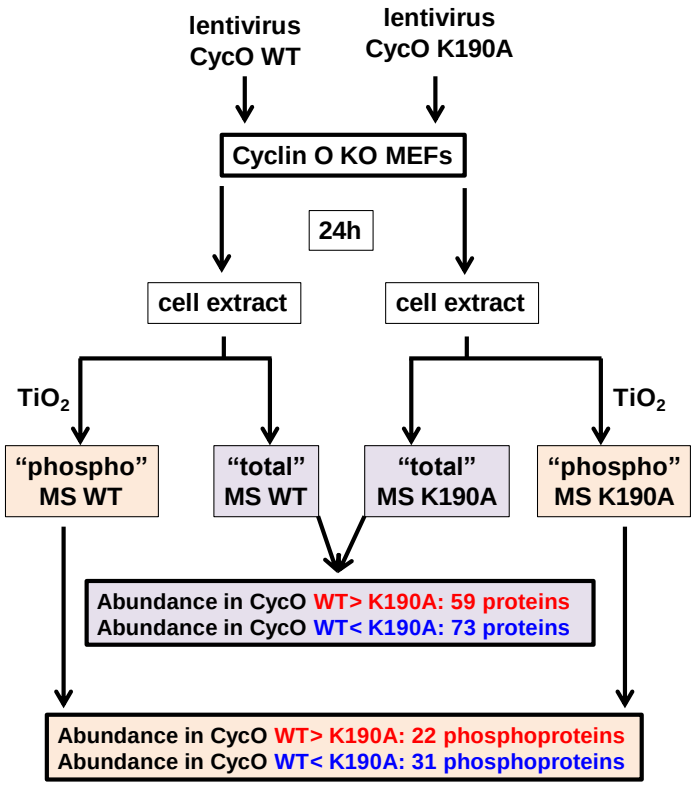


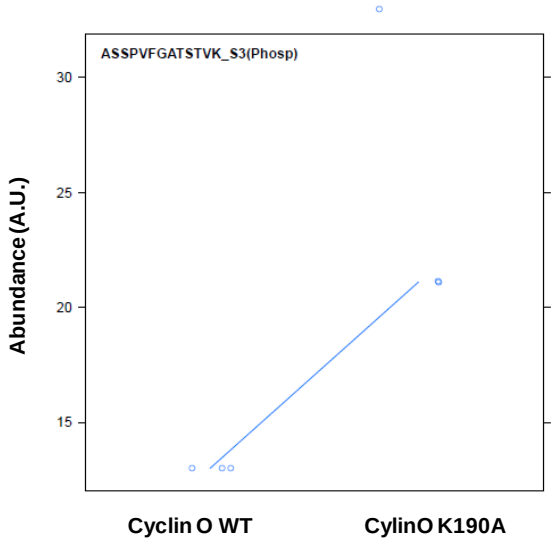
Figure.R12. – **Scheme of the procedure for the identification of Cyclin O target proteins.** MS: Mass Spectrometry. K190A: CDK2 binding-deficient form of Cyclin O (Lysin (K) in the position 190 of the Cyclin O aa sequence substituted by an Alanine (A))

Among other phosphopeptides, it was identified a fragment of the RBBP8/CtIP protein. The fragment identified contained a phosphorylated serine corresponding to S327 in the human CtIP protein (mouse S326). In the next figure (Figure.R13a) we can see the circles in each condition representing the relative abundance of phosphopeptide in the three independent experiments performed. In the table under the plot is indicated the estimated relative abundance of the phosphopeptide and the statistical significance

(p-value). The amount of this phosphopeptide was higher in the lysates of cells expressing the mutant Cyclin O K190A compared to the WT protein. The estimate value was of -8.098 meaning that the amount of the phosphopetide was $2^{8.098}$ times more abundant in the Cyclin O K190A infected cells than in the WT controls. This indicates that the levels of the peptide (and, hence, the levels of CtIP phosphorylated in S327) were higher in the absence of Cyclin O-CDK complexes, indicating that the formation of Cyclin O-CDK complexes impairs the phosphorylation of this residue.

This residue has been described as a CDK2 target phosphorylated in response to DNA damage and responsible of the interaction of CtIP with BRCA1, regulating DNA resection and thus promoting HR (Figure.R13b) (Yu & Chen, 2004). We can then conclude that the phosphorylation of the S327 of CtIP was impaired by the Cyclin O-CDK complexes.

a



sequence identified	Estimate Log2(WT) – Log2(K190A)	p value
ASSPVFGATSTVK_S3(Phosp)	-8,0982	0,003

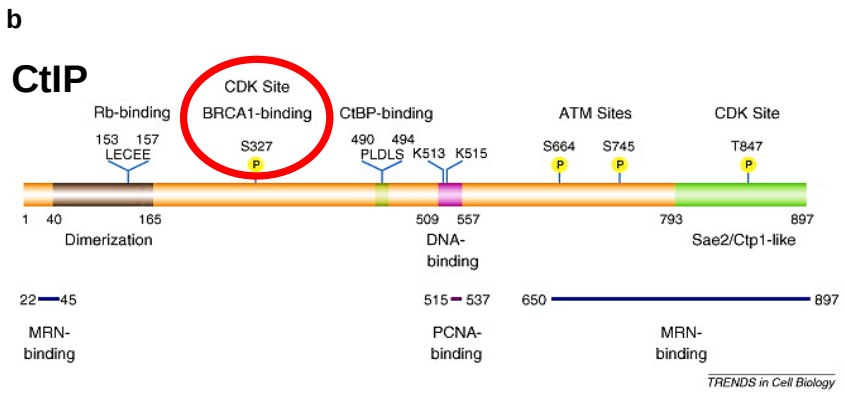


Figure.R13. – **Identification of CtIP as an indirect target of Cyclin O. (a)** Sequence identified by MS and relative abundance (circles) of the three experiments in the lysates of Cyclin O KO MEFs infected with Cyclin O-WT vs Cyclin O-K190A lentivirus. The table shows the sequence (with the residue identified in red font), the estimate (Log2 (WT) – Log2 (K190A)) and the significance (p-value). **(b)** Scheme of the human CtIP protein and PTMs. The red circle indicates the localization of the residue identified, S327. Adapted from (You & Bailis, 2010).

2.4.2.Cyclin O regulates the formation of resected DNA after DNA damage

CtIP is a protein crucial for the initial resection of DSBs in the process of DNA repair by HR. Resection of DNA depends on the exonuclease activity of this protein which is enhanced by its phosphorylation by CDK2 and its interaction with BRCA1 and the MRN complex. Resected DNA ends are ssDNA fibers that are rapidly covered by RPA avoiding the formation of secondary structures that would inhibit HR. RPA is phosphorylated at S4 and S8 residues in response to DNA damage by ATM, ATR and DNA-PK (Dou, Huang, Singh, Carpenter, & Yeh, 2010). The formation of phosphorylated S4/S8 RPA (hereforth phospho-RPA) foci can also be used as a readout of the resection activity and, thus, of the

activity of CtIP. According to the results obtained by phosphoproteomics, we expected to see more phospho-RPA foci in Cyclin O KO cells compared to WT controls, since cells expressing a CDK binding deficient form of Cyclin O showed increased phosphorylation of CtIP at S327 (mouse S326).

We performed immunofluorescence experiments to quantify phospho-RPA foci in primary MEFs. We observed more phospho-RPA foci 1h after irradiation in Cyclin O KO cells compared to WT controls (Figure.R14). Unexpectedly; we also found a high number of phospho-RPA foci in non-treated primary MEFs of both genotypes. In the case of the WT cells, we observed a reduction to the half in the number of foci 1h after a treatment of 5Gy of γ-radiation. In contrast, the amount of phospho-RPA foci was similar before and after irradiation in Cyclin O deficient cells. We could explain this result taking into account the fact that RPA is also able to bind ssDNA fibres formed during replication in the S-phase. A higher percentage of cells in S-phase in primary MEFs compared to other cell types (specially immortalized cell lines) could be the responsible of these high levels of phospho-RPA foci in non treated primary MEFs (Callén et al., 2009; K. a Cimprich & Cortez, 2008). Thus, after iradiation the cells stop the cell cycle and DNA replication, so the replication rate and the ssDNA fibres associated to replication decrease progressively. On the other side, the amount of ssDNA formed as a consequence of DNA repair after radiation (resected ends) increase. Hence, the total amount of phospho-RPA foci after radiation correspond to the balance between the reduction of ssDNA fibers associated to replication and the increase of the fibers associated with DNA resection.

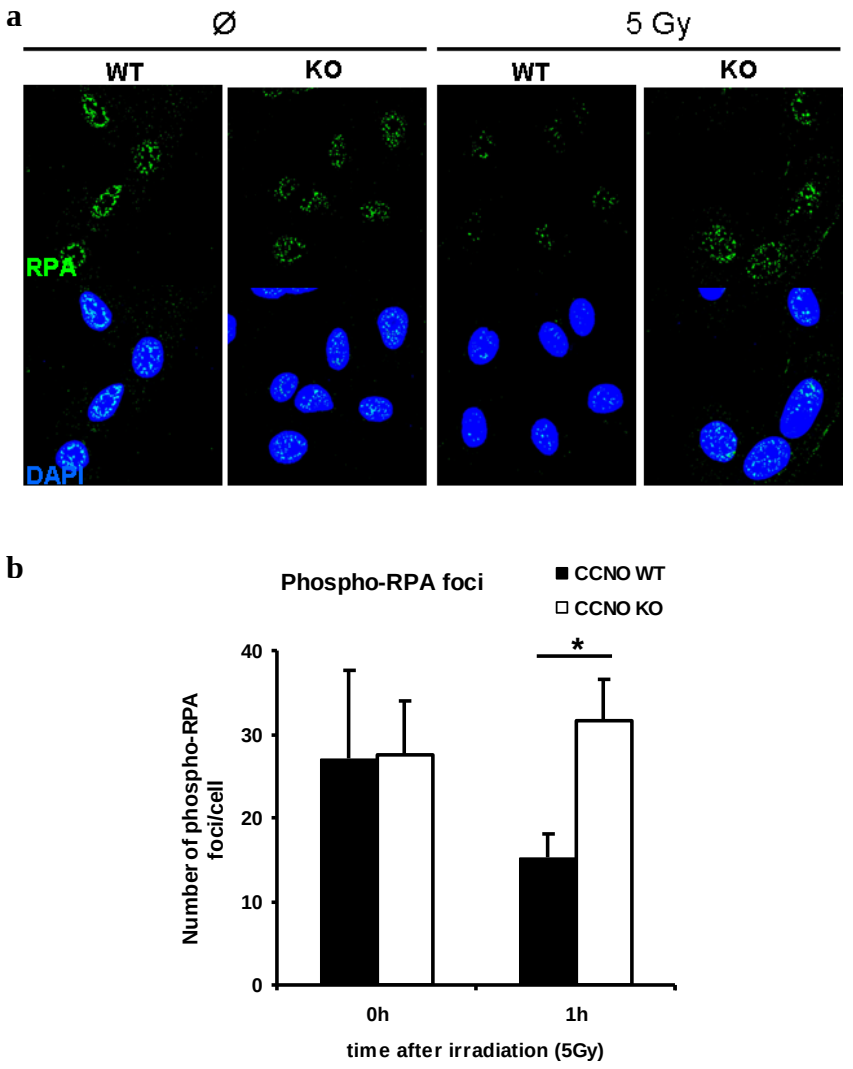
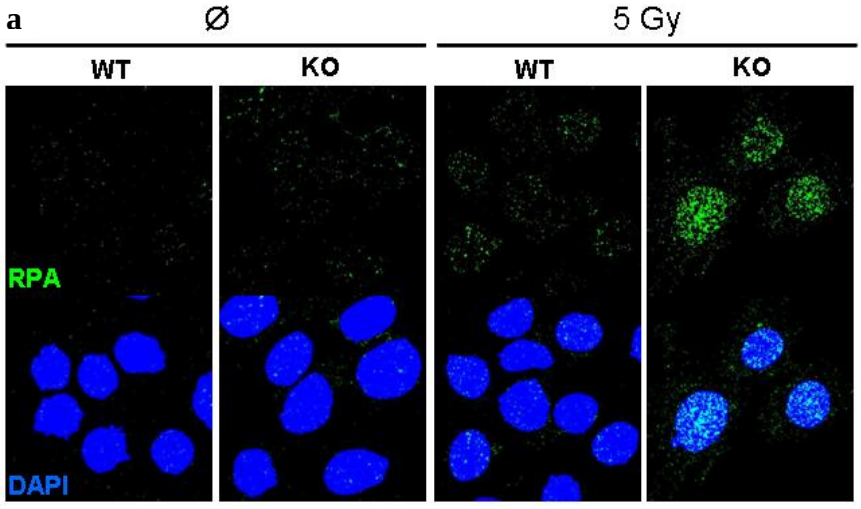


Figure.R14. – **Formation of phospho-RPA foci in Cyclin O deficient primary MEFs.** SV40 Tag immortalized Cyclin O WT and KO fibroblasts were cultured on glass coverslips. The cells were harvested at 0h and 1h after treatment with 5Gy of γ-radiation and stained using an anti-phospho-RPA specific antibody (green). Images were taken by confocal microscopy. Ø indicates non-irradiated cells and 5Gy corresponds to 1h after irradiation **(a)**. The number of phospho-RPA foci per nuclei was counted using the ImageJ software **(b)**.

The problem of the high number of cells showing phospho-RPA foci without irradiation gets solved by using SV40 Tag immortalized MEFs. In this case we observed that the number of phospho-RPA foci in non treated cells were lower than in cells 1h after being treated to 5Gy of γ-radiation (Figure.R15). Moreover, we detected an increase in the number of phospho-RPA foci between non treated WT cells and irradiated indicating a proper induction of DNA resection after generating DNA damage. In addition to this, one hour after treatment we counted significantly higher levels of phospho-RPA foci in Cyclin O KO cells compared to the WT controls. This confirms and reinforces the observations made in the experiments performed with primary MEFs



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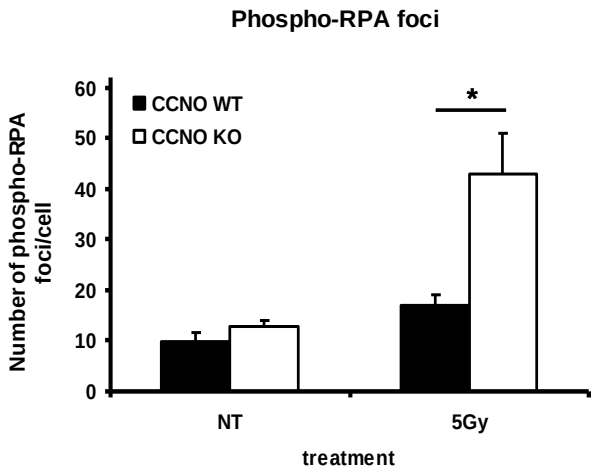
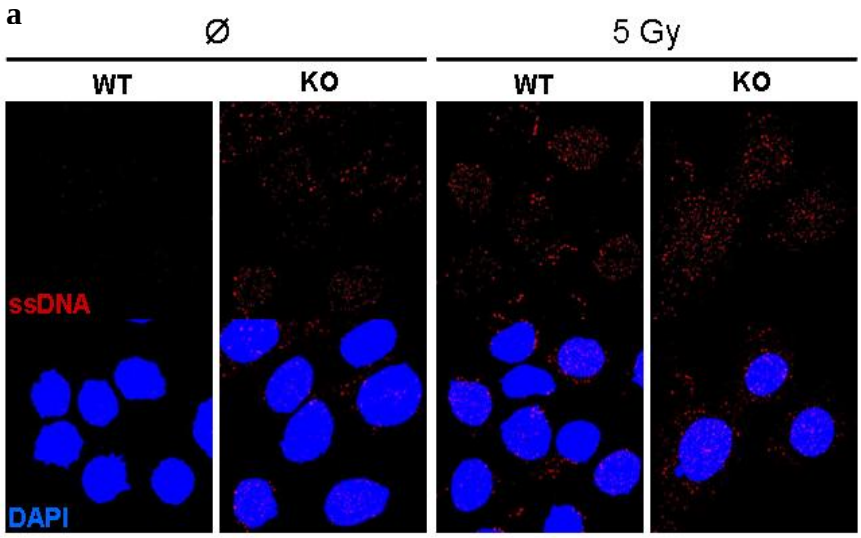


Figure.R15. – **Formation of phospho-RPA foci in Cyclin O deficient immortalized MEFs.** SV40 Tag immortalized Cyclin O WT and KO fibroblasts were cultured on glass coverslips. The cells were harvested at 0h and 1h after treatment with 5Gy of γ-radiation and stained using an anti-phospho-RPA specific antibody (green). Images were taken by confocal microscopy. Ø indicates non-irradiated cells and 5Gy corresponds to 1h after irradiation **(a)**. The number of phospho-RPA foci per nuclei was counted using the ImageJ software **(b)**.

In order to accumulate more evidences that prove that CtIP is hyperactivated in Cyclin O deficient cells, we performed BrdU ssDNA direct labeling experiments. BrdU is an analogue of thymidine that is incorporated to the DNA during replication in S-phase. When γ-radiation generates DSBs and CtIP promotes DNA resection and the formation of ssDNA, we can detect DNA-incorporated BrdU in these fibers by immunofluorescence using specific antibodies in non denaturing conditions. We first incubated the immortalized MEFs with 1μM of BrdU during 24h to get the DNA of the cell homogenously labeled with BrdU. After this, cells were exposed to 5Gy of γ-radiation. One hour after irradiation they

were fixed and the BrdU present in ssDNA fragments (that are exposed unlike the dsDNA protected by chromatin) detected by IF. We observe that, as in the case of the phospho-RPA foci, a significantly higher number of ssDNA fibers in the Cyclin O deficient cells compared to the WT were formed (Figure.R16). Moreover, in non-treated cells, we already observed a significant increase of ssDNA fibers in the case of Cyclin O deficient cells, indicating that in cells without induced DNA damage there is increased DNA resection.



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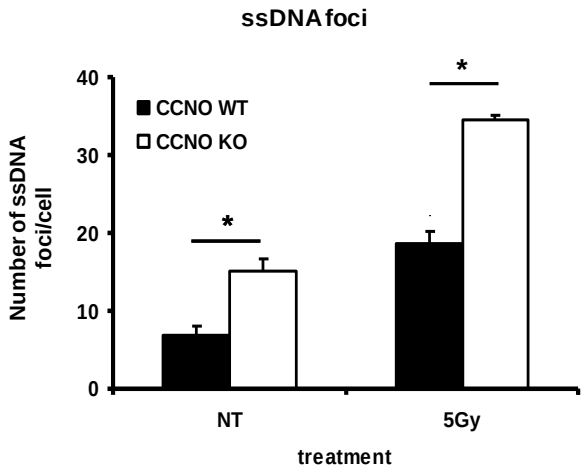


Figure.R16. – **Formation of ssDNA foci in Cyclin O deficient immortalized MEFs.** SV40 Tag immortalized Cyclin O WT and KO fibroblasts were cultured on glass coverslips. 10µM of BrdU was added to the culture 24h before irradiation. The cells were harvested at 0h and 1h after treatment with 5Gy of γ-radiation and stained using an anti-phospho-RPA specific antibody (green). Images were taken by confocal microscopy. Ø indicates non-irradiated cells and 5Gy corresponds to 1h after irradiation **(a)**. The number of phospho-RPA foci per nuclei was counted using the ImageJ software **(b)**.

All these results are indirect evidences that CtIP may be hyperactivated in the absence of Cyclin O as it is deduced from the phosphoproteomics results. A direct evidence of this fact would require evaluating the phosphorylation status of CtIP but it has not been possible because of antibodies that specifically recognize the phosphorylated form of the S327 of CtIP are not available yet.

3.REPAIR OF DSBs INDUCED BY ɣ-RADIATION IN CYCLIN O DEFICIENT CELLS

The observations that Cyclin O deficient cells get arrested at the G2/M checkpoint longer than the WT cells led us to wonder whether this has consequences in the efficiency of DSB repair. In addition to this, we observed an increased DNA resection and reduced ATM and DNA-PK signaling indicating that probably Cyclin O KO cells are skewed towards HR in order to repair the DSBs. This made us to think that perhaps Cyclin O KO cells need more time to repair the DBSs but they repair them more efficiently.

To analyze this possibility we measured the number of ɣH2AX foci (indicative of the number of DSBs) in mitotic cells (phosphorylated S10 HH3 positive) 6h after γ-radiation by immunofluorescence (Figure.R17). In the case of the WT cells we observed an increase from 2 to 15 ɣH2AX foci per cell after irradiation indicating that not all the DSBs are repaired during the arrest at the G2/M checkpoint. We counted significantly less ɣH2AX foci in cells deficient for Cyclin O and positive for phosphorylated S10 HH3 after irradiation. These experiments confirm that Cyclin O deficient cells repair the DSBs more efficiently during the G2/M checkpoint arrest triggered by irradiation than the WT controls.

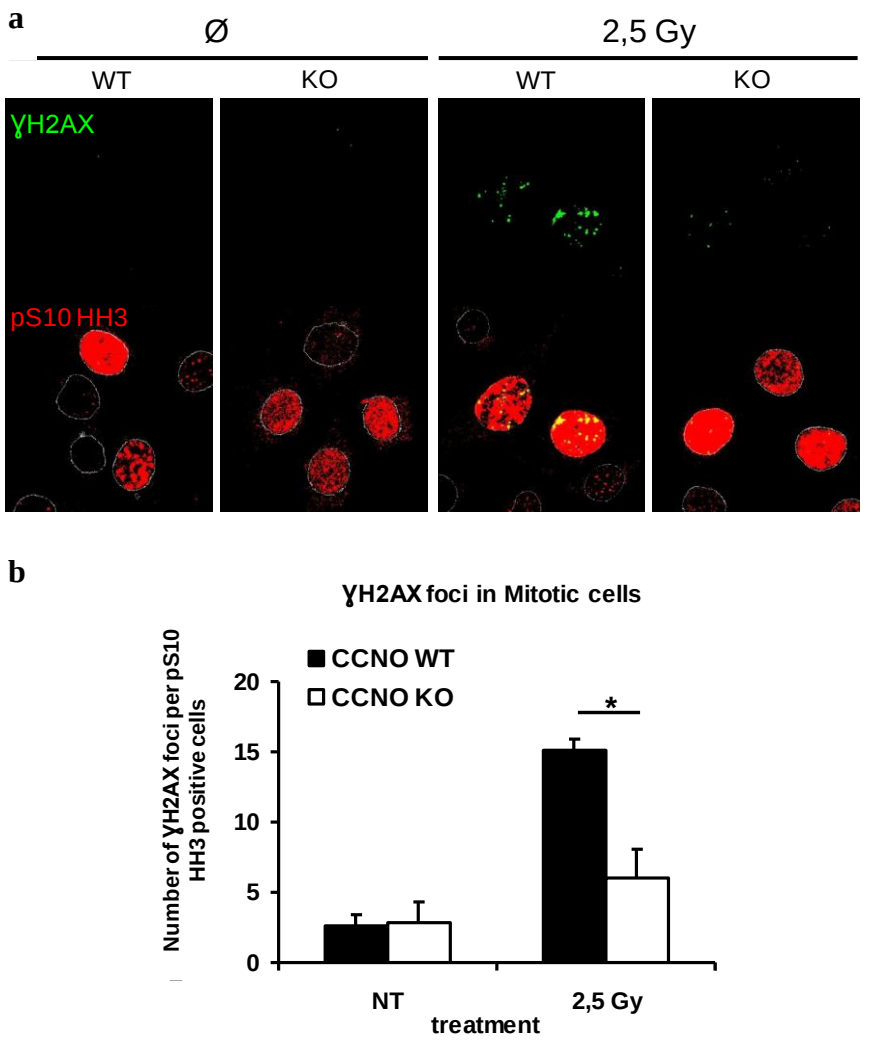


Figure.R17. – **DNA repair efficiency in Mitotic cells.** SV40 Tag immortalized Cyclin O WT and KO fibroblasts were cultured on glass coverslips. The cells were fixed 6h after treatment with 2,5Gy of γ-irradiation and stained using anti-γH2AX (green) and anti-pS10 HH3 (red) specific antibodies. Images were taken by confocal microscopy. Ø indicates non-irradiated cells. Dashed lines indicate the nuclei perimeter. **(a)**. The number of γH2AX foci per pS10 HH3 positive cell was counted using the ImageJ software. Only the cells positive for pS10 HH3 were scored **(b)**.

4.CYCLIN O AND DNA DAMAGE-INDUCED APOPTOSIS

This part of the results deals with the analysis of the effects of the lack of the Cyclin O gene in DNA damage-induced apoptosis and cell survival. To do so, we used different cellular models obtained from Cyclin O knock-out (KO) mice. It is important to note that previously our lab had demonstrated that the downregulation of Cyclin O in a lymphoid cell line protects the cells against DNA damage-induced apoptosis (Roig et al., 2009). Now, we aim to confirm and further characterize these observations using cells coming from mice lacking the Cyclin O gene.

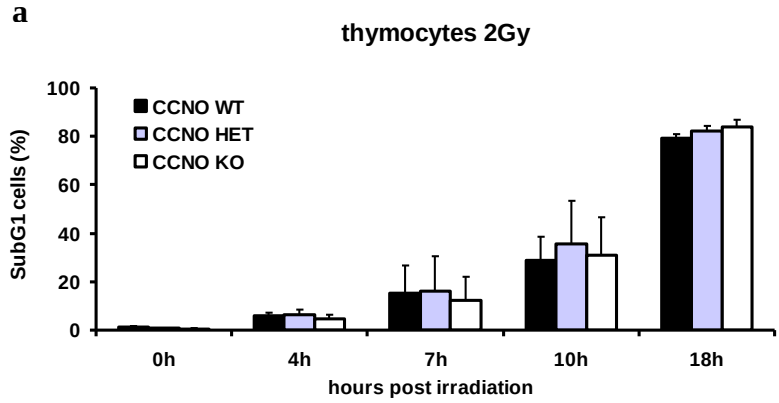
4.1.DNA damage-induced apoptosis in Cyclin O deficient thymocytes

To analyze the implication of the Cyclin O in DNA damage-induced apoptosis, we first analyzed the implications of the loss of the Cyclin O gene in quiescent thymocytes. Previously our lab had demonstrated that Cyclin O expression and its associated kinase activity appeared after γ-radiation in quiescent thymocytes (Roig et al., 2009), presumably associated with CDK2 (Granes, F., Roig, M.B., Brady, H.J. & Gil-Gomez, 2004). Now, we checked whether Cyclin O was necessary for DNA damage-induced apoptosis in Cyclin O deficient thymocytes.

We isolated thymocytes from young siblings from mice WT, HET and KO for the Cyclin O gene. We exposed the cells to 2 Gy or 5 Gy of γ-radiation using a ^{137}Cs irradiator, and incubated them at 37ºC. At different time points (4h, 7h, 10h and 18h) samples were

taken and the DNA content of the cells measured by FACS (Figure.R18). Non-irradiated cells were also cultured in the same conditions and used as a negative control.

We observed a time-dependent increase in the subG1 (DNA content < 2n) population after γ-radiation compared to the non-treated group. The increase in the subG1 population was faster in the cells irradiated with 5Gy compared to those irradiated with 2Gy. However, we did not observe any difference amongst cells from the three genotypes at any time point. This indicates that Cyclin O is not necessary for DNA damage induced apoptosis in thymocytes.



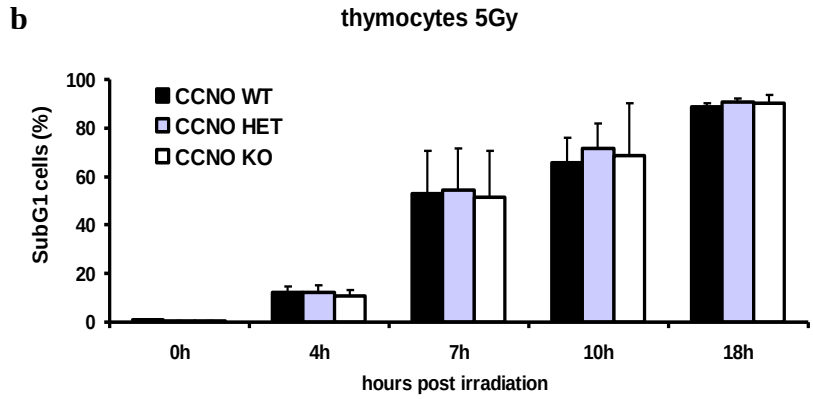


Figure.R18. - **DNA damage induced apoptosis in Cyclin O deficient thymocytes.** Thymocytes from Cyclin O WT, HET and KO mice were treated with 2Gy **(a)** and 5Gy **(b)** of γ-radiation. Cells were harvested and stained with PI at the indicated time points. The SubG1 population was measured by flow cytometry.

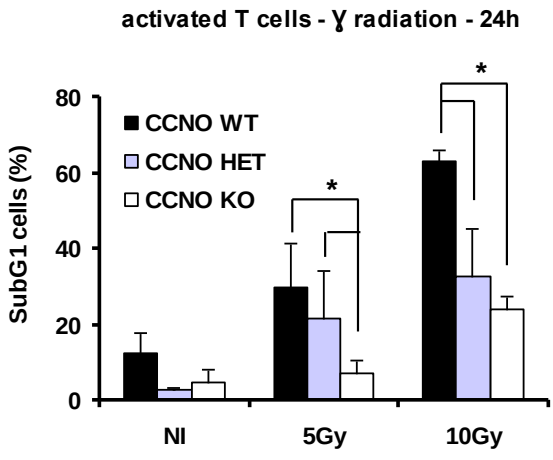
4.2.DNA damage induced apoptosis in activated, Cyclin O deficient, T-cell blasts

We then analyzed the implications of the loss of the Cyclin O gene in a population of proliferating T-cells in terms of apoptosis induced by γ-radiation. To obtain proliferating T-cells, we isolated splenocytes from Cyclin O WT, HET, and HOM young mice (around 3 months after birth) and cultured them with IL2 + Concanavalin A (2µg/mL) during 3 days. This treatment stimulates the proliferation and enriches the T-cell population respect to other cell types. After this period, we performed a Percoll gradient to eliminate the debris and we cultured the cells 2 more days with IL2 to expand the T-cell blast population. We then treated the cells with 5Gy and 10Gy of γ-radiation and collected samples at 24h and 48h

after treatment to measure the percentage of apoptotic cells by FACS in four independent experiments (Figure.R19).

In the case of the WT cells we observed an increase of the SubG1 population in a time and dose dependent manner. When we analyzed HET and KO cells treated with the highest dose (10 Gy) the induction of apoptosis was significantly lower compared to the WT cells. However, when we used a lower dose of 5Gy, only the SubG1 population of the KO cells was significantly lower compared to the WT controls but not in the HETs. We also want to remark that there are not significant differences in the SubG1 population between 24h and 48h after treatment with γ-radiation for the same genotype. This suggests that after 24h the cells have repaired the lesions. From this we conclude that the loss of only one allele of the Cyclin O gene confers partial protection against DNA damage-induced apoptosis indicating that Cyclin O sensitizes the activated T-cells to γ-radiation in a dose dependent manner.

a



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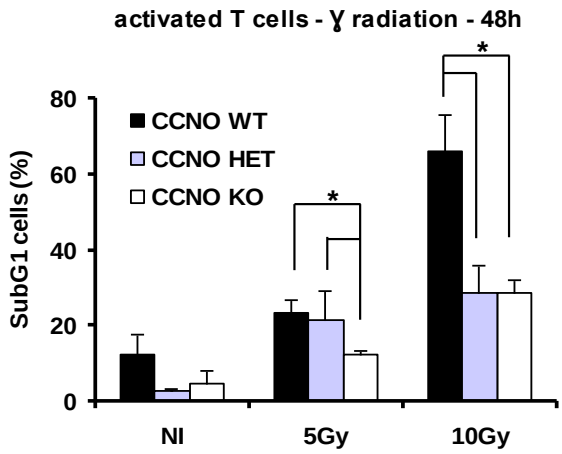
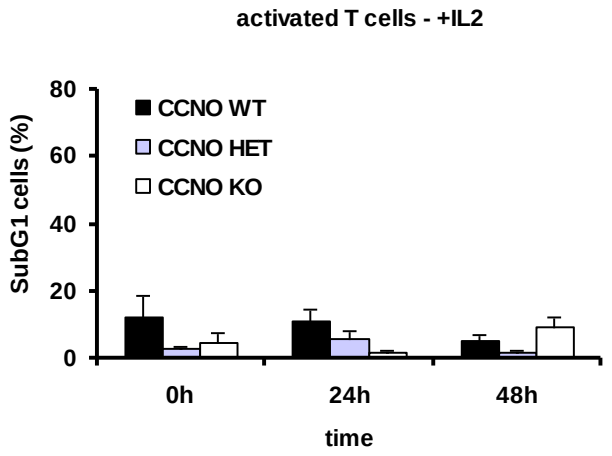


Figure.R19. - **DNA damage induced apoptosis in activated Cyclin O deficient T-cells.** Splenocytes from WT, HET and KO mice were cultured for 3 days with IL2 and Concanavalin A (2µg/ml). Cell debris was removed by Percoll gradient centrifugation and the T-Cell blasts were cultured 3 more days with IL2. Cells were treated with 5 Gy or 10Gy of γ-radiation and harvested at 24h **(a)** and 48h **(b)**, and stained with PI at different time points. SubG1 population was analyzed by flow cytometry. NI: non-irradiated.

We then set cultures of purified T-cell blasts without IL2 and harvest samples at the same time points. We did so in order to check whether this protection against apoptosis of Cyclin O deficient cells was specific for γ-radiation or it was a general anit-apoptotic effect. IL2 is essential for the proliferation and survival of activated T-cells and in its absence they undergo apoptosis. In this case, we observed a big increase in the subG1 population 24h and 48h after removing IL2 from the culture, with no significant differences amongst the three genotypes. We also included a control incubating the cells with IL2 and collecting samples at the same time points and we did not detected significant induction of

apoptosis in any of the three genotypes (Figure.R20). It is also important to note that the values of 0h in the Figure.R20 and the NI values in the Figure.R19 are the same because they correspond to the SubG1 population of non-treated T-cell blasts at the initial time point (t=0h).

a



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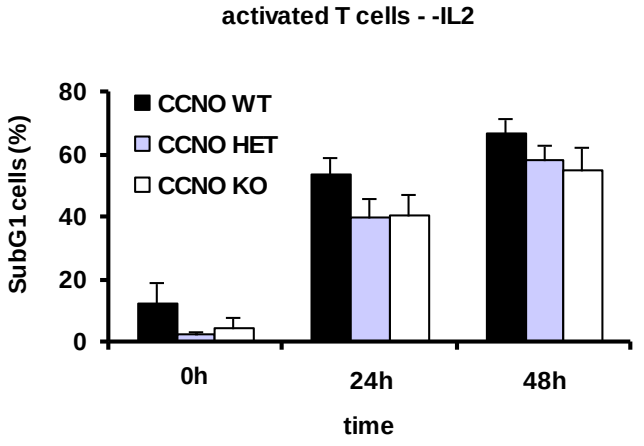


Figure.R20. - **Apoptosis in activated Cyclin O deficient T-cells.** Splenocytes from WT, HET and KO mice were cultured 3 days with IL2 and Concanavalin A (2µg/ml). Cell debris was removed after Percoll gradient centrifugation and the T-Cell blasts were cultured 3 more days with **(a)** or without **(b)** IL2. Samples were harvested at 24h and 48h, and stained with PI. SubG1 population was analyzed by flow cytometry.

4.3.Survival of Cyclin O deficient MEFs after γ-radiation

Another model to evaluate the importance of Cyclin O in the response to DNA damage is measuring the capacity of the cells to survive and resume cell growth after receiving DNA damage (clonogenic assay). To do so, we measured the efficiency of T-antigen immortalized MEFs to form colonies after being treated with γ-radiation at different doses. We irradiated T-antigen immortalized MEFs from the three genotypes and seeded 500 cells per Petri dish (in the case of 0, 2 and 4Gy) or 1000cells/plate (in the case of 6Gy). We let them grow for 13 days after which we stained the colonies with crystal violet and counted them to calculate the survival ratio.

We observed a decrease in cell survival when we increased the dose of radiation (Figure.R21). However, we observed a significantly higher number of surviving colonies in HET and KO MEFs when we irradiated them with 4Gy and 6Gy of γ-radiation. This shows that Cyclin O deficient (HET and KO) cells had a higher survival capacity after being treated with γ-radiation. These results suggest a higher survival and/or DNA repair capacity in the HET and KO cells compared to the WT controls as they recover better

after being treated with higher doses of γ-radiation, where more DNA repair is needed.

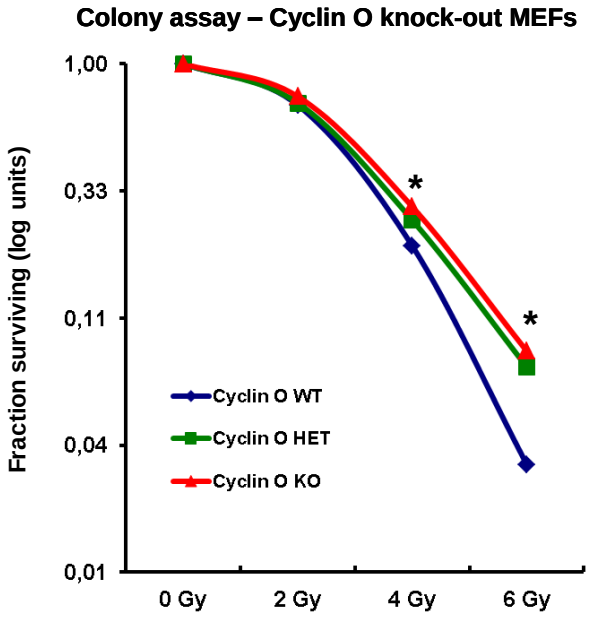


Figure.R21. – **Clonogenic assay of Cyclin O deficient MEF after γ-radiation.** SV40 Tag immortalized fibroblasts were treated with at 0, 2, 4 and 6 Gy of γ-radiation, seeded onto a 10cm plate in triplicate and cultured for 13 days. Then, the plates were fixed and stained with Cristal Violet and the number of colonies counted.

5. PHENOTYPE OF CYCLIN O DEFICIENT MICE

In order to study the *in vivo* role of Cyclin O we generated knock-out (KO) mice. The Cyclin O gene was targeted by homologous recombination (HR) in ES cells by the KOMP (Knock-Out Mouse Project). The Cyclin O ORF (including introns 1 and 2) was replaced by the LacZ coding sequence preserving the Cyclin O regulatory elements of the promoter. The inserted sequence also includes a floxed neomycin resistance cassette that could be removed later on by interbreeding with transgenic mice expressing the Cre recombinase (Figure.R22).

We worked mainly with the mice containing the Neo cassette in the targeted locus. However, we also confirmed some of the phenotypes found with mice carrying the Neo-deleted allele.

5.1.Survival and growth of Cyclin O deficient mice

Cyclin O KO mice were viable and born at the predicted Mendelian frequencies (Figure.R23), indicating that the complete loss of Cyclin O does not lead to embryonic lethality. However, Cyclin O KO females were sterile and the males subfertile (producing a lower number of pups per litter and some even no offspring) with evidences of hypogonadism and defects in gametogenesis (data not shown). Cyclin O KO mice were maintained on a C57Bl/6J genetic background, although the hydrocephalus has been confirmed using outbreed mice (CD1 x C57Bl/6J) to rule out an effect of the genetic background selected. The C57Bl/6J genetic background develops spontaneously hydrocephalus at a frequency

between 0.029% (According to JAX® NOTES Issue 490, Summer 2003- Hydrocephalus in Laboratory mice).

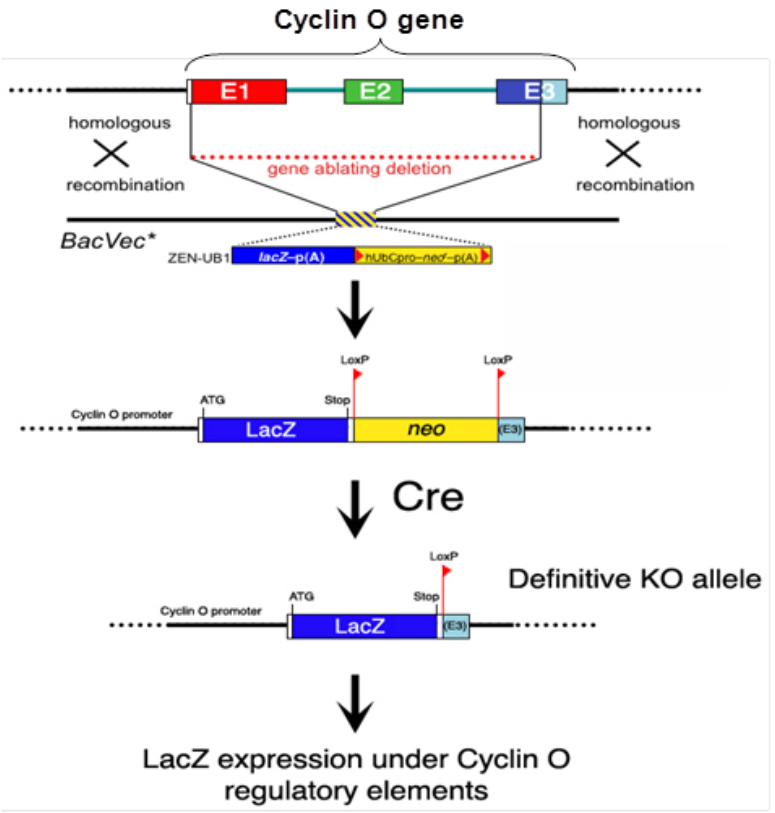


Figure.R22. – **Targeting strategy of the Cyclin O gene in ES cells.** The 1st, 2nd and part of the 3rd exons of the Cyclin O gene were replaced by a LacZ-Neomycin cassette by HR in ES cells. The targeted gene preserves the ATG and promoter of the Cyclin O gene permitting the expression of LacZ under the regulatory elements of the Cyclin O promoter. The floxed neomycin cassette contains a promoter that allows to the constitutive expression of the selectable marker.

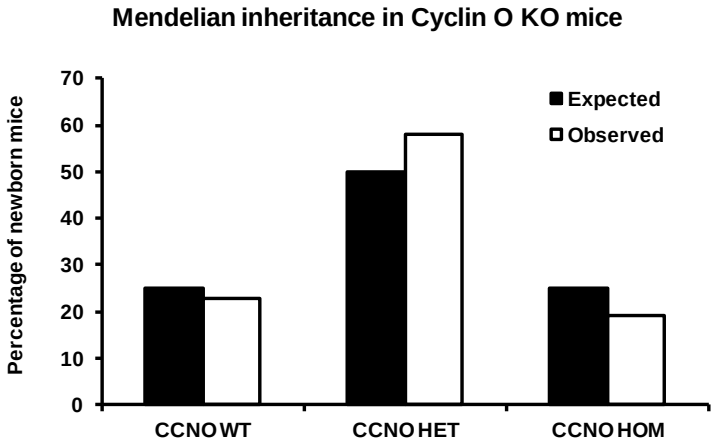


Figure.R23. – **Mendelian ratios from a total of 444 pups born from interbreeding Cyclin O heterozygous mice.** The plot shows the distribution of Cyclin O WT, HET and KO genotypes in the cohort. The table shows the total counts and percentages of the three genotypes.

We also evaluated the lifespan and the incidence of pathologies of the Cyclin O KO mice (Figure.R24). Around 80% of Cyclin O KO mice die or become moribund because of the development of hydrocephalus within the first month of postnatal life. We also observed the progressive dead of Cyclin O HET mice during the first year of life because of an increased incidence of ulcerative idiopathic dermatitis most likely as a consequence of an autoimmune reaction. The C57Bl/6J genetic background develops dermatitis at a lower frequency than observed in Cyclin O HET mice (Csiza CK, 1976). Cyclin O HET mice show a trend towards a shorter half life, although it did not reach statistical significance.

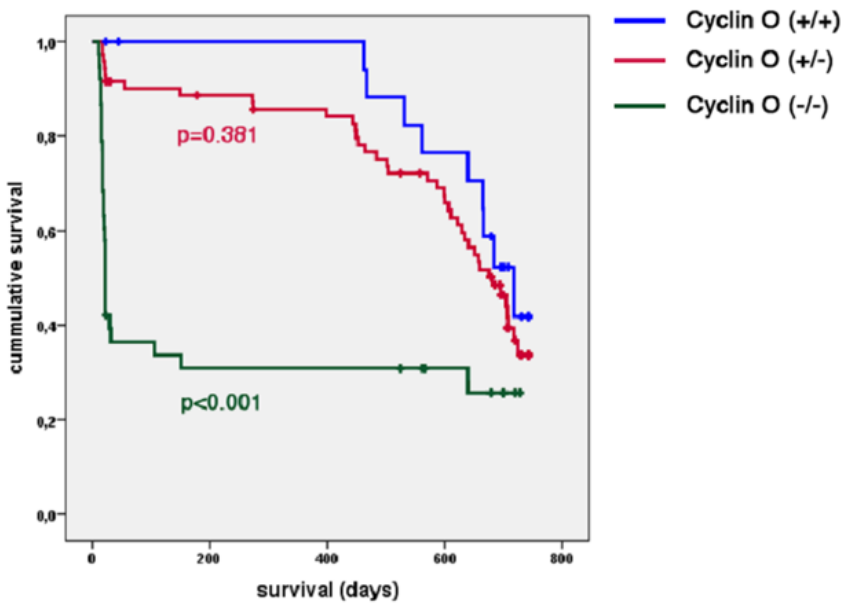
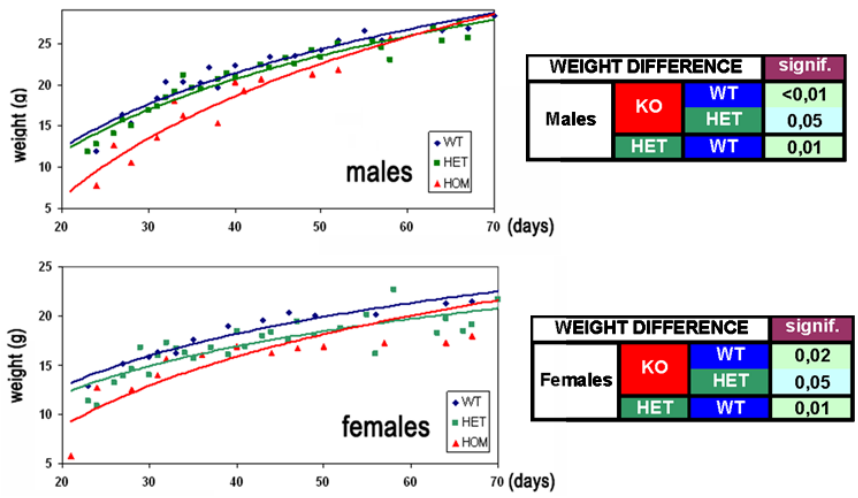


Figure.R24. – **Kaplan-Meier survival curve of Cyclin O WT, HET and KO mice.**

In order to evaluate the progress of the postnatal mouse development, we weighted them during the first 70 days of life. We found a significantly retarded growth of Cyclin O KO mice compared to the WT controls both in males and females (Figure.R25). Again, the Cyclin O HET mice showed an intermediate growth rate. However, the difference in the weight amongst the three genotypes was more evident during the first postnatal days and became less evident when the mice got older and disappearing in the adulthood. This difference is probably due to the development of hydrocephalus during the first month after birth, since the affected mice show deficient mobility and probably impaired access to the food. However we cannot rule out the role of other developmental defects like abnormalities in the intestinal

epithelium (K.Künnemann and G.Gil-Gomez, unpublished work). After the first month of life, most of the Cyclin O KO mice have already died of hydrocephalus and only Cyclin O WT and healthy Cyclin O HET and HOM mice survive with no difference in weight or size.



WEIGHT DIFFERENCE			signif.
Males	KO	WT	<0,01
		HET	0,05
	HET	WT	0,01

WEIGHT DIFFERENCE			signif.
Females	KO	WT	0,02
		HET	0,05
	HET	WT	0,01

Figure.R25. – **Growth retardation in Cyclin O deficient mice.** Cyclin O WT, HET and KO mice were weighted every 3 days from P21 until P70.

5.2.Causes of death of Cyclin O deficient mice

We evaluate the health status and the cause of dead of the cohort during the 586 days of the observation period and we performed the necropsy of the surviving mice (Figure.R26).

We observed a significant increase in the incidence of hydrocephalus in Cyclin O KO and HET mice compared to the WT. The Cyclin O KO mice that survived to this period do not die prematurely because of other pathologies. Cyclin O HET mice also

developed hydrocephalus with lower penetrance. The fact that the heterozygous mice develop an intermediate phenotype is indicative of haploinsufficiency indicating that only one allele of the gene is not sufficient to maintain a WT phenotype. This is in agreement with most of the biochemical data shown in the preceding section.

The incidence of tumours appearing from 400 days on is higher in the Cyclin O WT mice compared to the HET and KO. Moreover, in the HET mice we observed an intermediate incidence of tumours. This is of special relevance because indicates that the deficiency in Cyclin O confers a protection against tumour development proportional to the dosage of the Cyclin O gene. Most of the tumours were of lymphoid origin, although no histological analysis has been done yet.

Finally, some Cyclin O WT and HET mice developed spontaneous dermatitis starting from 4 months after birth as it has been described for mice of the C57Bl/6J genetic background. This incidence was much lower in Cyclin O KO mice.

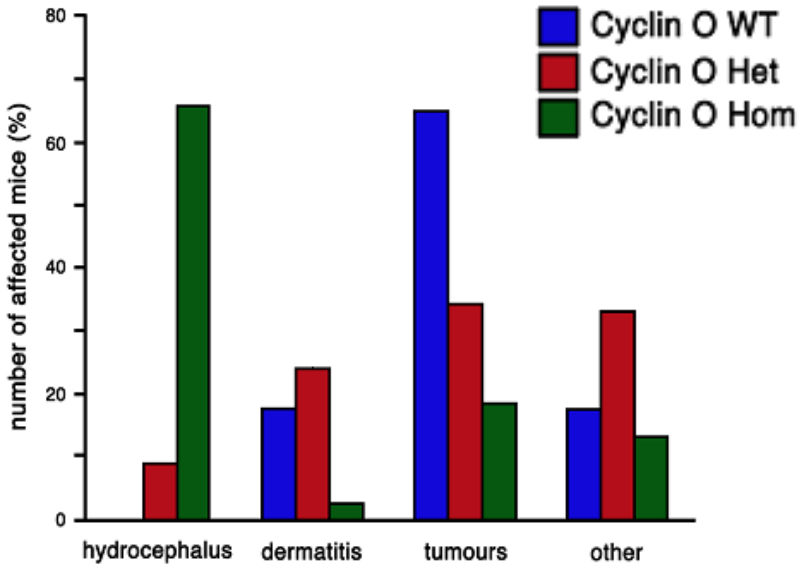


Figure.R26. – **Incidence of different pathologies in Cyclin O KO mice.** The cause of dead of Cyclin O WT, HET and KO was analyzed after necropsy. Three main groups of diseases were established ("hydrocephalus", "dermatitis" and "tumours"). Non-determined or other causes of dead are grouped under the category "other".

5.3.Hydrocephalus and brain damage in Cyclin O deficient mice

Although Cyclin O loss does not lead to embryonic lethality, almost 80% of Cyclin O KO mice become moribund during the first weeks after birth because of the development of hydrocephalus. The mice that developed this conditions show evident growth retardation, mobility problems and a swollen skull. When we dissected the head we observed an accumulation of CSF in the brain, probably being the cause of the deformation of the skull giving rise to the characteristic swollen head (Figure.R27 upper panels). The accumulation of CSF may cause high intracranial pressure and be

the cause of hemorrhages and brain herniation. Moreover, the volume of Cyclin O KO mice brain was also bigger compared to the WT, healthy littermates, indicating an accumulation of CSF in the brain parenchyma (Figure.R27 lower panels).

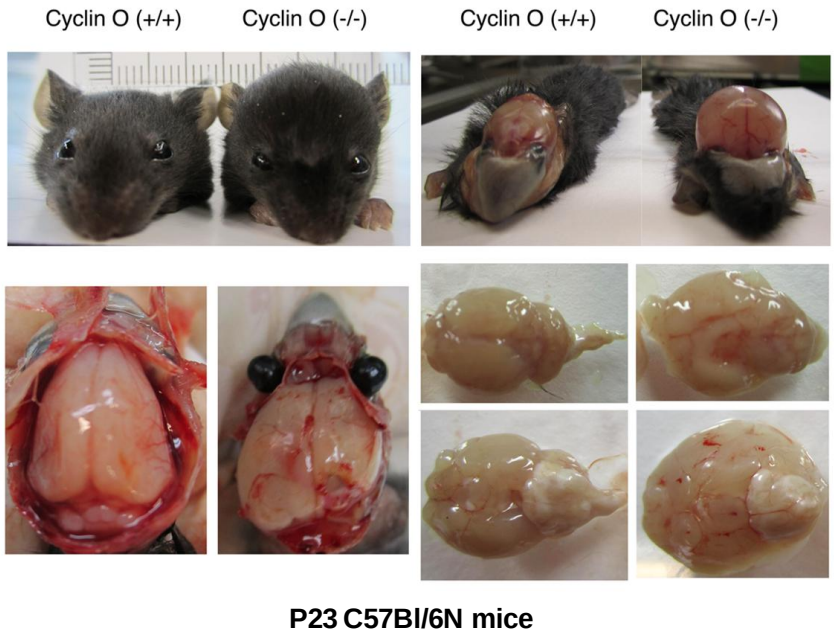


Figure.R27. – **Hydrocephalus in P23 Cyclin O KO mice.** Pictures from hydrocephalic Cyclin O KO P23 mice heads, skull and brains were taken and compared to the WT.

As explained before, the Cyclin O KO mice that survive beyond the first month without developing hydrocephalus live as long as the Cyclin O WT littermates without developing any other apparent pathology. Also around 10% of the heterozygous mice developed hydrocephalus with features indistinguishable from the Cyclin O KO mice.

We analyzed the morphology of the CNS by MRI of Cyclin O KO mice that overcome the perinatal period (38 and 190 days old) without an evident phenotype. In Figure.R28 we can observe the accumulation of cerebrospinal fluid between the 1st and 3rd ventricle in Cyclin O KO and HETs (white areas in the ventricles). This observation leads us to think that Cyclin O KO mice developed Normal Pressure Hydrocephalus due to the accumulation of the CSF inside the skull.

Moreover, the enlargement of the 1st, 2nd and 3rd ventricles was higher in young compared to the old mice, indicating that the hydrocephalus worsens with age. We also observed this accumulation of fluid in the case of the HETs, but the hydrocephalus observed was not so severe. This again indicates that the loss of only one allele of the Cyclin O gene leads to an intermediate phenotype, confirming the haploinsufficiency for the Cyclin O gene.

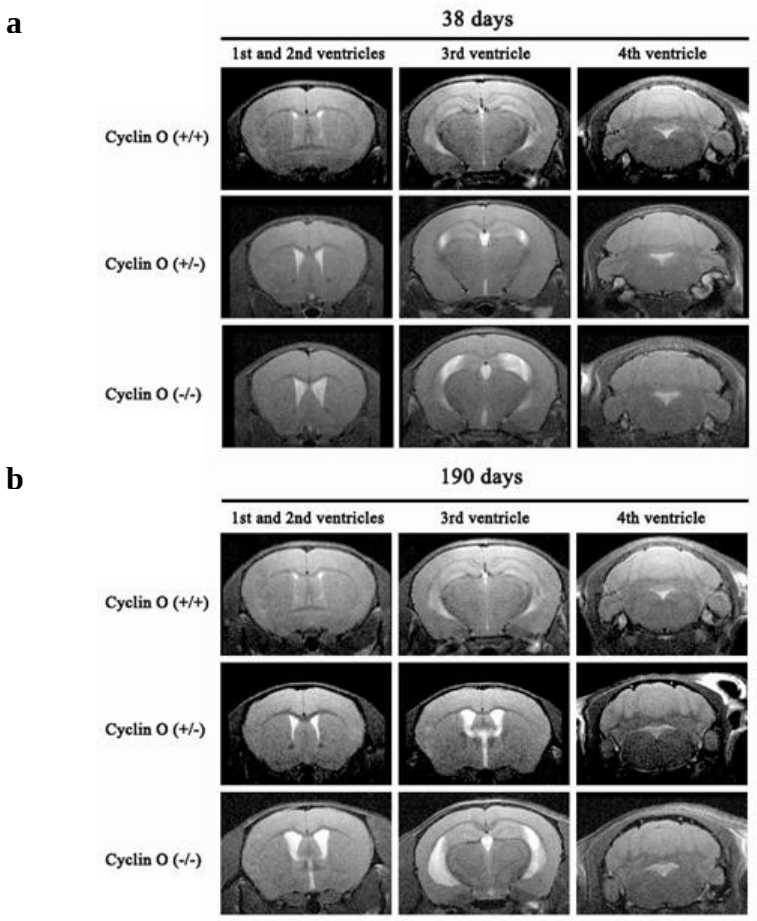


Figure.R28. – **Hydrocephalus in adult Cyclin O deficient mice.** We analysed by Magnetic Resonance Imaging (MRI) Cyclin O WT, HETs and HOM mice either at a young age (38 days, panel **(a)**) or aged (190 days, panel **(b)**) which did not show any apparent symptom of hydrocephalus. The T2 weighted MRI images allow to the detection of aqueous fluids (CSF in this case) as white areas.

Preliminary histological analysis of the brain of young Cyclin O KO mice shows a disorganization of the layers of the brain cortex and degenerative changes (such as vacuolization of the nucleus) (Figure.R29).

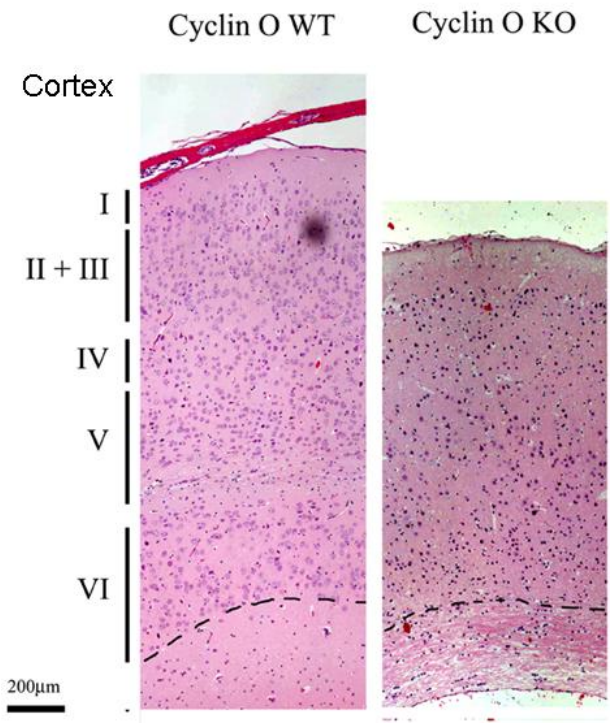


Figure.R29. – **Disorganization of brain cortex and degenerative changes in the nucleus of Cyclin O KO mice.** Histological section from Cyclin O WT and KO mice cerebral cortex stained with H&E. The layers of the normal brain are shown. Dashed lines indicate the frontier between cortex and nucleus.

We also observed bleeding and vacuolization in the cortex parenchyma probably due to the high intracranial pressure consequence of the hydrocephalus. Moreover, we observed other signs of neuronal damage such as pyknotic neurons and dead astrocytes (Figure.R30 upper panel). Finally, we detected degenerative changes of hippocampus (vacuolization, Figure.R30 middle panel) and acidophilic pyramidal neurons (Figure.R30 lower panel). All these observations evidence severe brain damage in hydrocephalic Cyclin O KO mice.

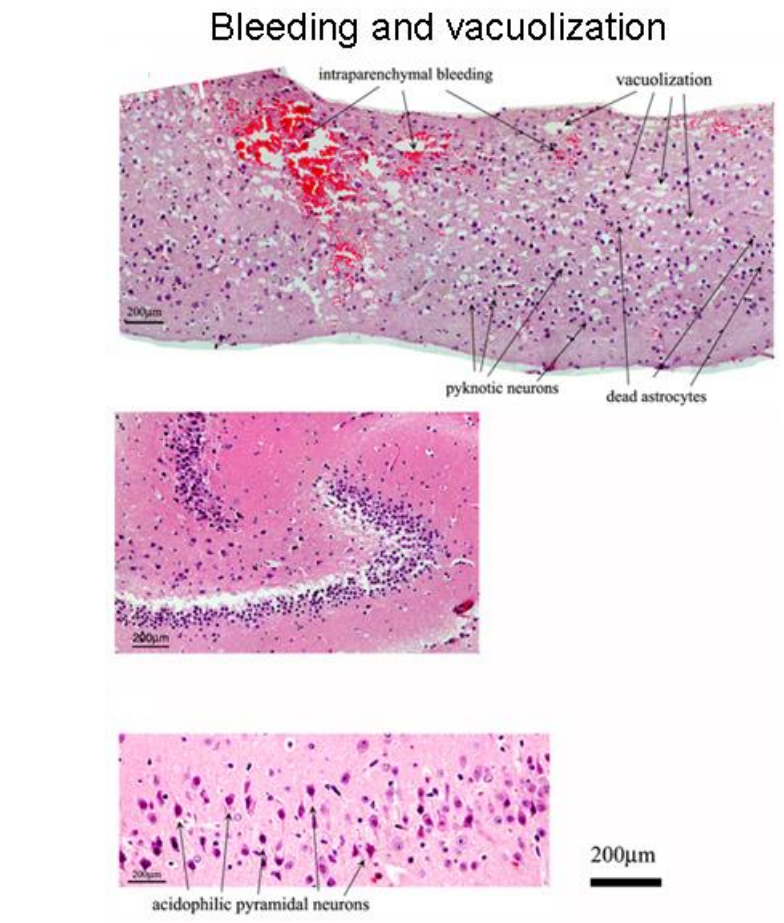


Figure.R30. – **Evidences of brain damage in Cyclin O KO mice.** Histological sections of Cyclin O KO mice brains stained with H&E. Arrows indicate different types of neuronal damage.

In hydrocephalic and non-hydrocephalic Cyclin O HET and KO mice, we have detected hyppocampal malformations. We can observe defective folding, severe neuron loss and disorganization of the neuronal nuclei, more evident in the CA1 and CA3 regions (Figure.R31).

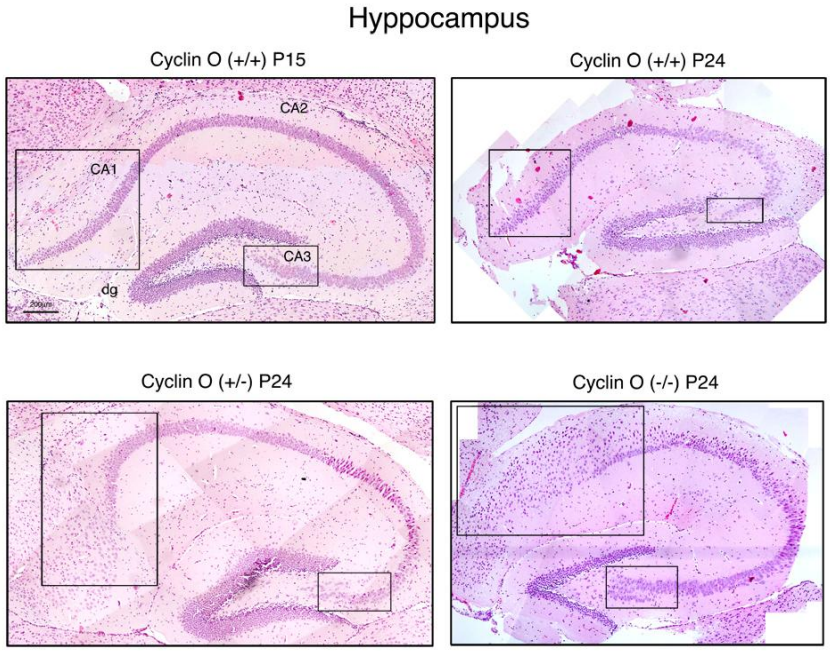


Figure.R31. – **Hyppocampal malformations in Cyclin O KO mice.** Histological sections from Cyclin O WT (P15 and P24), HET and KO (P24) hyppocampus stained with H&E. Squares indicate affected parts of the CA1 and CA3 regions.

However, no histological analysis has been performed yet in brain samples of adult Cyclin O KO mice.

DISCUSSION

1. APOPTOSIS, SURVIVAL AND DNA REPAIR

1.1.Regulation of cell cycle checkpoints by Cyclin O

Cyclin O has been previously described to be necessary for the signalling of the intrinsic apoptotic pathways such as apoptosis induced by DNA damaging agents and glucocorticoids (Roig et al., 2009).

In previous observations, it was shown that CDK2 kinase activity was increased in thymocytes after exposure to γ-radiation in a mechanism independent of Cyclin A and Cyclin E, the two partners of CDK2 during the cell cycle. Treatment of thymocytes with roscovitine (a CDK inhibitor) inhibited DNA damage and glucocorticoid-induced apoptosis (Granes, F., Roig, M.B., Brady, H.J. & Gil-Gomez, 2004). Moreover, CDK2 kinase induction in response to apoptotic stimuli correlated with increases in the expression of Cyclin O and with the activation of Caspase 3 in thymocytes. The interaction of Cyclin O with CDK2 in response to DNA damage and the requirement of the activation of this complex for the induction of the intrinsic apoptotic pathway was demonstrated in a lymphoid cell line (WEHI 7.2 cells) (Roig et al., 2009).

In spite of the correlation observed, Cyclin O is not essential for DNA damage-induced apoptosis in thymocytes as we have found using Cyclin O KO thymocytes (Figure.R18). This is in contrast with what it is observed in activated T-cells (Figure.R19 and R20), where Cyclin O deficient cells (HET and KO) show reduced DNA damage-induced apoptosis. These observations suggest that

Cyclin O is necessary for DNA damage-induced apoptosis in proliferating cells (activated T-cells and WEHI 7.2) but not in quiescent cells (thymocytes).

Thymocytes are a cell model suitable to study the role of the CDKs in cellular processes independently of their cell cycle functions since around 90% of the population is quiescent (Gil-Gómez et al., 1998). It is likely that the function of Cyclin O in thymocytes in response to γ-radiation can be replaced by other cyclins or CDK2-activating proteins in Cyclin O KO thymocytes (gene compensation). We have not performed measuremens of CDK2 kinase activity in Cyclin O KO thymocytes yet, but preliminary results not shown in this thesis indicate that roscovitine is still able to block DNA damage-induced apoptosis in Cyclin O KO thymocytes. This would demonstrate that CDK2 is important for the regulation of DNA damage-induced apoptosis in thymocytes in the abscence of Cyclin O in collaboration with another protein.

The activation of DNA damage-induced apoptosis in thymocytes is largely depending on the tumour suppressor gene p53 (Hirao, 2000; Iwakuma & Lozano, 2007). On the other hand, in proliferating cells DNA damage-induced apoptosis can also be regulated by mechanisms independent of p53, as it is the case of activated T-cells and in the WEHI 7.2 cell line (Jackson & Bartek, 2009). As we show in the Figure.R6, the deficiency of Cyclin O in primary MEFs does not affect p53 activation, stabilization and expression of downstream targets in response to DNA damage. This supports the hypothesis that Cyclin O is absolutely required for p53-dependent apoptosis.

Cyclin O deficient T-cell blasts are radioresistant. However, when we remove the IL2 from the culture medium, we observe the time-dependent appearance of comparable levels of apoptosis independently of the genotype. This indicates that the Cyclin O is involved in regulating DNA damage-induced apoptosis, but it is not a general apoptosis regulator. However, we have identified other pro-apoptotic factors which fail in inducing apoptosis in Cyclin O deficient T-cells blasts, such as thapsigargin and dexamethasone (data not shown).

It will be interesting in the future to further investigate the selective regulation of the intrinsic apoptosis pathway by Cyclin O. It is also important to note that T-cell blasts from Cyclin O HET mice have an intermediate phenotype, indicating the haploinsufficiency for the Cyclin O gene.

To study the role of Cyclin O in the recovery of fibroblasts after suffering DNA damage, we performed colony assays after exposing them to different doses of γ-radiation. The colony assay evaluates the capacity of the cells to overcome DNA damage, survive and resume growing (Figure.R.21). Regardless of the dose of γ-radiation, we obtained a higher number of colonies in Cyclin O deficient cells indicating a better capacity to repair the DNA damage and proliferate than the WT controls. This supports the observations made with the other cellular models where we found that proliferating Cyclin O deficient cells are less sensitive to γ-radiation in terms of apoptosis induction. However, the capacity to form colonies after γ-radiation treatment can not be attributed only to the deficient induction of apoptosis as observed in WEHI 7.2 cells and T-cells blasts. Other factors such as checkpoint

deregulation or the capacity to repair DNA damage also contribute to this effect.

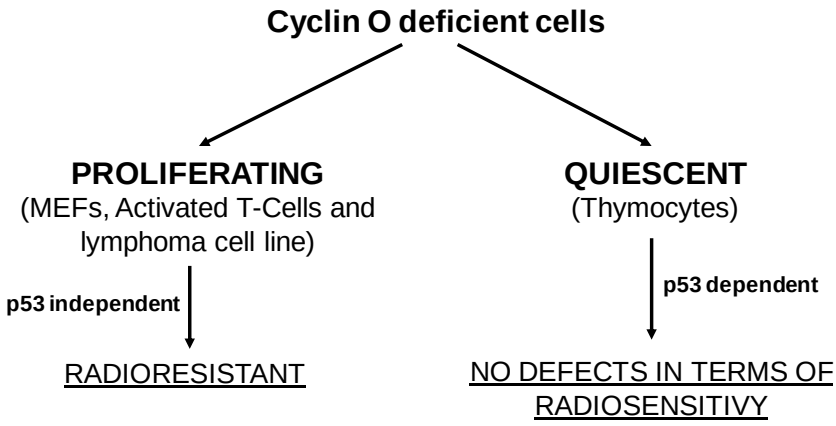


Figure.D1. – **Cyclin O mediates DNA damage-induced apoptosis in proliferating cells**

1.2.Cyclin O regulates G2/M checkpoint activation, resolution and the DNA repair efficiency

As explained before, the capacity of Cyclin O deficient MEFs to survive better to increasing doses of γ-radiation suggests a better capacity of these cells to repair DSBs. This is in agreement with the fact that Cyclin O deficient cells show a lower amount of γH2AX foci in mitotic cells 6h after irradiation (Figure.R.17). Moreover, the exit from the G2-M checkpoint triggerd by γ-radiation in Cyclin O KO cells is slower compared to the WT controls.

Altogether these results indicate that Cyclin O deficient cells take more time to repair the DSBs generated by γ-radiation but the efficiency of repair is higher. The DSBs that have not been properly

repaired before resuming cell cycle progression and entering mitosis could induce apoptosis in the following replication round or generate genomic instability (Hoffelder et al., 2004; C. Lukas et al., 2011; Petersen, Hasvold, Lukas, Bartek, & Syljuåsen, 2010; Wahl GM1, Linke SP, Paulson TG, 1997). However, the DNA repair is not 100% efficient and the checkpoints are inactivated before all the DSBs are repaired in normal cells in a phenomenon called DNA damage adaptation (Deckbar, Jeggo, & Löbrich, 2011; Syljuåsen, 2007). This permits the resolution of the checkpoint before all the DNA damage is repaired and the cell cycle resumed. One way to quantify the presence of DBS is by counting the number of ɣH2AX foci per cell. It has been calculated that a cell is able to reactivate the cell cycle progression and enter into mitosis with 10-20 ɣH2AX foci per cell (Deckbar et al., 2011). This coincides with our observations in the WT controls. In Cyclin O KO cells 6h after irradiation the mean number of ɣH2AX foci was 5 while the percentage of cells that exit the G2-M checkpoint is comparable to the WT controls.

This may also indicate that the tolerance to the non-repaired DSBs in Cyclin O deficient cells is lower than in WT control cells. There is not a consensus about the evolutionary significance of this mechanism in multicellular organisms, as it can be the cause of generating cancer. A possible explanation is that adaptation may occur to allow proper repair of the damage in other cycle cell phases (P. J. L. and K. A. Cimprich, 2004; Galgoczy & Toczyski, 2001). However, from a developmental point of view, it has been proposed that a balance between the permission for cell proliferation (in the presence of DNA damage) and the prevention

of genomic instability is needed to permit the proper development of the organism (Deckbar et al., 2011).

The mechanisms that regulate the G2/M DNA damage adaptation are the same that regulate the G2/M checkpoint maintenance and termination (H. Y. Yoo, Kumagai, Shevchenko, Shevchenko, & Dunphy, 2004). Then, ATR signalling and Plk1 play an important role in this sense and a lower level of DNA damage in the M-phase could be a consequence of the fact that the cells take more time to repair the DNA damage using a more efficient mechanism. In our case, Cyclin O KO cells show a delayed exit from the G2-M checkpoint compared to the WT controls. This may indicate that Cyclin O deficient cells use a slower mechanism of DNA repair. NHEJ is a fast mechanism of DSB repair that acts during all the cell cycle but can generate chromosomic translocations and genomic instability. On the other hand, HR is a slower mechanism that preserves the genomic information avoiding mutations that could generate cell transformation and cancer (Shibata et al., 2011). Our observations suggest that Cyclin O KO cells preferentially use HR instead of NHEJ as it is a slower but more efficient mechanism to repair DSBs. The biochemical analysis of the DDR and the deficient signal transduction at the level of the PIKKs supports this hypothesis. In this sense, Cyclin O deficient cells show a reduced activation of ATM, DNA-PKcs and phosphorylation of some ATM targets in the context of the chromatin but an increased DNA resection and ATR activity. All these observations support the hypothesis that in Cyclin O deficient cells the balance between both repair mechanisms favours HR instead of NHEJ. Finally, the fact that Cyclin O HET and KO mice develop fewer spontaneous tumours compared to WT mice reinforces the idea that Cyclin O

deficiency prevents genomic instability and the transformation of normal to cancer cells.

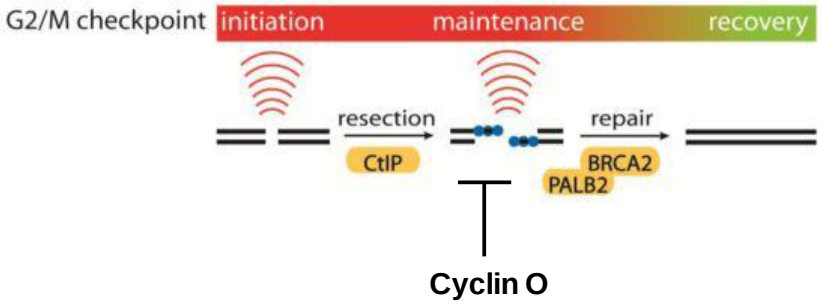


Figure.D2. – **Deficiency of the Cyclin O delays the exit of the G2/M checkpoint and permits the repair of more DSBs.**

2.DDR SIGNALLING PATHWAYS

2.1.Cyclin O limits DNA resection

The DDR response pathways involve a sequence of molecular events that range from the sensing of the DNA damage to the execution of mechanisms aimed to stop the cell cycle in order to repair the DNA and drive the cells to apoptosis if it is necessary. The repair of the DSBs occurs via two main mechanisms, HR and NHEJ (Ciccia & Elledge, 2010). The decision of which mechanism is used to repair the DSBs has been the focus of several publications in the last years. NHEJ is active throughout the cell cycle and relies on rejoining free DNA ends without the need for sequence homology (Mahaney et al., 2009). On the other hand, HR uses sister chromatids that facilitate the searching of homologous sequences restricting the HR to the postreplicational (G2/M) cell cycle phases (Moynahan & Jasin, 2010) and to the repair of a

subset of DSBs mapping to heterocromatic regions (Beucher et al., 2009). Thus, the molecular events that regulate the pathway choice are of special interest in those cell cycle phases where both mechanisms are active. Previous work in our lab showed that the CDK activity associated to Cyclin O was higher in G2/M when compared to G1 or S cell cycle phases (MB Roig and G Gil-Gomez, unpublished results). This observation and the fact that Cyclin O deficient cells show defects in the G2/M checkpoint suggests that Cyclin O could be implicated in the regulation of DNA repair during the G2 phase of the cell cycle.

Several publications have described that DNA resection is a crucial event that regulates this balance. In particular, CtIP is a protein necessary for the initiation of short range DNA resection in collaboration with BRCA1 and the MRN complex (Buis et al., 2012; L. Chen et al., 2008; Gomez-Cabello, Jimeno, Fernández-Ávila, & Huertas, 2013; Huertas, 2010). We identified CtIP as a putative indirect Cyclin O target using a phosphoproteomics approach. We observed increased phosphorylation of S327 (murine S326) of CtIP in fibroblasts expressing a CDK binding deficient mutant of Cyclin O when compared to the expression of the WT form. The phosphorylation of the residue S327 of CtIP by CDK2 in response to DNA damage is necessary for its interaction with BRCA1 and enhances DNA resection (Yu & Chen, 2004; Yu et al., 2006). This suggests that CtIP-BRCA1 interaction and DNA resection would be increased in Cyclin O KO cells compared to the WT controls.

It has been proposed a mechanism that determines the repair of DNA damage by either HR or NHEJ through the competition of BRCA1 with and 53BP1 at the DSBs. This mechanism suggests

that BRCA1-CtIP prevents the chromatin association of the 53BP1-RIF1 complex. RIF1 has been descrived to be important in the telomere protection and recently it has been shown to be important in the regulation of NHEJ (Chapman et al., 2013; Escribano-Díaz et al., 2013). The release the 53BP1-RIF1 from the DSBs permits the activation of CtIP by deacetylase sirtuin 6 (SIRT6) and the end resection associated activity (Panier & Boulton, 2014). However, some publications suggest that the phosphorylation of S327 of CtIP and its interaction with BRCA1 are not essential for DNA resection and tumour development (Reczek et al., 2013). One plausible explanation that could match with all these observations is that the phosphorylation of S327 of CtIP by CDK2 and the interaction of BRCA1 with CtIP favour DNA resection and HR by preventing the recruitment of 53BP1 to the DSBs, but this interaction would not be essential for the nuclease activity of Mre11 and the initiation of the short range DNA resection. On the other side, the phosphorylation of CtIP at T847 by CDK2 seems to be necessary for the recruitment of CtIP to the DSBs and the enhancement of DNA resection (Huertas & Jackson, 2009). Recent reviews compilate the publications that demonstrate an implication of the CDKs in DNA repair and in particular in DNA resection (Ferretti et al., 2013; Trovesi, Manfrini, Falcettoni, & Longhese, 2013).

With all this background in mind we hypothesized that the deficiency the Cyclin O leads to an increased phosphorylation of S327 of CtIP and to an increased interaction of CtIP with BRCA1 leading to an enhanced resection activity after DNA damage. We confirmed this hypothesis by accumulating several indirect evidences such as the increased assembly of phosphorylated RPA fibers and the increased generation of ssDNA after γ-radiation in

Cyclin O KO cells. Moreover, we observed an increased phosphorylation of CHK1 at S345 in Cyclin O KO cells after γ-radiation, indicative of the hyperactivation of ATR. The consequences of the increased DNA resection and ATR activity are consistent with the higher DNA repair efficiency, the delayed exit from the G2/M checkpoint and with the radioresistance observed in Cyclin O deficient, proliferating cells respect to WT controls. Our observations match with the results reported in different publications where impairing the activity of CtIP has consequences on the DNA repair efficiency, the activation of the G2/M checkpoint arrest and the capacity to survive after DNA damage (L. Chen et al., 2008; Huertas et al., 2008; Huertas & Jackson, 2009; A. N. Kousholt et al., 2012).

All these data suggest that Cyclin O limits DNA resection in the process of repair by HR of the DSB created by exposure to γ-radiation.

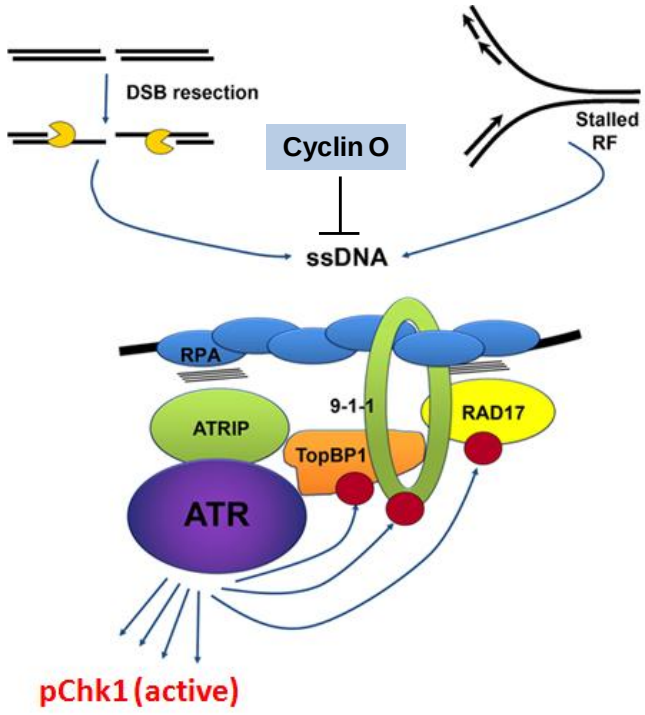


Figure.D3. – **Cyclin O limits the DNA resection and the activation of the ATR signaling.**

2.2.Cyclin O is necessary for a proper activation of ATM and DNA-PKcs at the DSBs

The firsts steps of the DDR after sensing the DBSs consist in the autophosphorylation of the PIKKs ATM and DNA-PKcs. ATM forms inactive dimmers in the chromatin and their activation and monomerization depends on the MRN complex (Bakkenist & Kastan, 2003). Active ATM gets autophosphorylated at the S1981 amongst other sites. Although this phosphorylation has been widely

used as a marker of ATM activation, it is not essential for its complete activation. Activated ATM has been largely related to the early steps of HR in response to the detection of DSBs, although it can also regulate NHEJ during G1, depending on the cell type and context (Callén et al., 2009; B.-S. Lee et al., 2013). ATM phosphorylates and propagates the signal to several substrates with functions at the level of the chromatin necessary for a proper assembling of IRIF (such as γH2AX, Nbs1 or CtIP). ATM also phosphorylates soluble proteins that transduce and amplify the signal (CHK2) or effector proteins that regulate the cellular response to DNA damage (p53). On the other hand, DNA-PKcs gets autophosphorylated in the T2609 cluster (also known as the ABCDE cluster) after DSBs sensing by Ku. Activated DNA-PKcs commits the cell to NHEJ after stabilizing DSBs and protecting them from DNA resection. As ATM, DNA-PKcs also phosphorylates γH2AX, CHK2 and p53 (Ciccia & Elledge, 2010).

We have observed a deficient autophosphorylation of ATM and DNA-PKcs in Cyclin O deficient cells. We have also seen deficient activation of the PIKK downstream targets at the level of the chromatin such as γH2AX and Nbs1. However, the downstream targets of ATM and DNA-PKcs CHK2 and p53 were properly activated. This suggests that the activation of the chromatin associated factors and solubles is independent, what has already been observed by other groups (Daniel et al., 2008; Pellegrini et al., 2006). These studies show that mice expressing a mutated form of ATM that cannot be phosphorylated at the S1981 do not develop the characteristics of A-T such as defects in the CNS development, genomic instability or defects on the immune system although show a deficient phosphorylation of the chromatin

associated factors. Furthermore, another report indicates that the phosphorylation of ATM at S1981 was necessary for the stabilization of ATM the DSBs (So Sairei, Davis Anthony J., 2009). The deficient S1981 ATM phosphorylation in Cyclin O KO cells in response to γ-radiation lead us to speculate that the deficient phosphorylation of the chromatin associated factors of the DDR was due to a deficient stabilization of ATM at the chromatin. To confirm this hypothesis we evaluated the formation of foci of ATM phosphorylated at the S1981 as readout of the stabilization of this PIKK at the IRIF. As it is shown in Figure.R10, the formation of these foci was deficient in Cyclin O KO cells.

Together with the observations of the deficient activation of ATM and DNA-PKcs, we have observed an increased phosphorylation of CHK1 at S345 in response to γ-radiation of Cyclin O KO cells respect to the WT controls. This suggests that ATR is hyperactivated in Cyclin O deficient cells in response to γ-radiation. This is supported also by the hyperloading of RPA at the DSBs and the increased number of ssDNA foci indicative of an increased DNA resection. During HR, ATM is the first PIKK activated, however, when DNA resection starts there is an ATM-to-ATR switch in the regulation of HR (Shiotani & Zou, 2009). Also, ATM is released from the DSB due to extended DNA resection (Geuting et al., 2013). The fact that Cyclin O KO cells show an increased ATR activation and DNA resection activity (via CtIP) after γ-radiation will provide another possible explanation of why ATM and its targets are not properly activated at the DSBs. As a consequence of the increased ATR activity and DNA resection, the release of ATM from the DSBs occurs more efficiently and faster in Cyclin O KO cells.

The analysis of total cell extracts of Cyclin O KO cells show that the activation of CHK2 is not affected after γ-radiation. However, when we fractionated the cell extracts in chromatin bound and soluble proteins, we observed that the total non phosphorylated form of CHK2 protein is bound to the chromatin. After γ-radiation treatment CHK2 is activated and released from the chromatin. We have observed lower levels of hyperphosphorylated CHK2 in the soluble fraction of KO cells respect to WT controls indicating that this protein is not properly released from the chromatin. On the contrary, in the case of CHK1 protein, the phosphorylation at S345 was higher in the soluble fraction of Cyclin O KO cells. The increased CHK1 phosphorylation, also observed in total cell extracts, suggests that ATR signalling is compensating the deficient ATM signalling. This would permit the proper activation of p53 by CHK1 in Cyclin O KO cells. Moreover, we have observed a more efficient instauration of the G2/M checkpoint and a delayed exit in Cyclin O KO cells. Some studies show that the activation of the G2/M checkpoint relays more in the CHK1 than in CHK2 activity (J. Jin, Ang, Ye, Livingstone, & Harper, 2008; Sanchez, 1997; Sørensen et al., 2003; H. Zhao et al., 2002). Our observations that the more efficient switch from ATM to ATR that leads to hyperactivation of CHK1 in Cyclin O KO cells and the delayed exit from the G2/M checkpoint support this hypothesis.

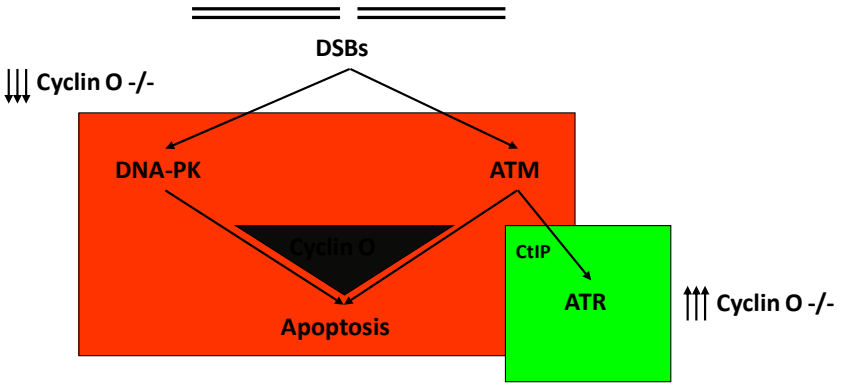


Figure.D4. – **Cyclin O deficient cells show a deficient activation of ATM and DNA-PK and hyperactivation of ATR.**

3.PHENOTYPE OF THE CYCLIN O DEFICIENT MICE

Another focus of this work has been to characterize the Cyclin O KO mice. The most remarkable phenotype observed is the hydrocephalus that affected the Cyclin O KO mice. Other signs of CNS defects appeared such as neuronal damage or disorganization and hippocampus malformation, although are only preliminary observations. The hydrocephalus was only externally detectable in the 80% of mice that become moribund during the first month after birth. However, MRI analysis shows that it was also affecting the rest of the Cyclin O KO adult mice that survived this period. The difference between old mice and the mice that become moribund before day 30 is probably in the fusion of the skull bones. Mice that are able to properly fuse the skull bones after birth before developing hydrocephalus, they do not develop external features of hydrocephalus. However, they still accumulate cerebrospinal fluid between the first and third ventricle of the brain

developing normotensive hydrocephalus. The mice that develop hydrocephalus early before the skull bones are fused show external features as head deformation and growth retardation becoming moribund as is the case of around 80% of Cyclin O KO.

The malformation of the CNS and neurological alterations is a common feature of the KO or mutant animals for proteins implicated in the DNA damage response, such as microcephaly, ataxia, neurodamylation, etc (McKinnon, 2009; Stracker & Petrini, 2011). Also hydrocephalus is developed in certain KO mice such as Rad50 (Roset et al., 2014) and p73 (a Yang et al., 2000).

From a histological point of view, several models hydrocephalus in mice are developed by ependymal denudation in the ventricles. Ependymal are epithelial-like cells that line the CSF-filled ventricles in the brain and the central canal of the spinal cord. These cells are ciliated simple cuboidal epithelium-like cells. Their apical surfaces are also covered with microvilli, which absorb CSF (McAllister, 2012). Moreover, mouse models with impaired cilia function have provided insight into the mechanisms involved in hydrocephalus (Davis et al., 2007; Ibañez-Tallon et al., 2004; Nathalie Spassky; Florian T. Merkle; Nuria Flames; Anthony D. Tramontin; Jose Manuel Garcıa-Verdugo; Alvarez-Buylla, 2005; Tissir et al., 2010). On the other hand, a very recent paper came out where it is shown that Cyclin O mutations in chronic destructive lung disease patients is the cause of deficient ciliated cells formation (Wallmeier et al., 2014). All this background makes us to think that probably Cyclin O deficient mice has a problem in the generation of the ependymal-ciliated cells being the cause of the hydrocephalus. However, a

more profound histological analysis has to be done in order to know the anatomical causes of hydrocephalus.

Finally, other phenotypes observed in the Cyclin O KO mice that could be related to defects in he DNA damage response, are the hypogonadism and sterility (in females) or subfertility (in males). Meiotic recombination is known to be regulated by HR in ATM dependent manner (Keeney, Giroux, & Kleckner, 1997). Thus, as we observed a deficient activation of the ATM pathway in Cyclin O KO cells, it is probably that this imparing gametogenesis and hypogonadism. However, the gametogenesis has to be analyzed in detail in the gonads of those mice. Also, Cyclin O KO are anemic (data not shown). Anemia is also a common feature in disorders caused by deficiency in DDR proteins such as Fanconi Anemia.

4.CYCLIN O AND THE CHOICE OF THE DNA REPAIR MECHANISM

During the last years it has been of special interest the characterization of the mechanisms that regulate the balance between HR and NHEJ in the repair of the DSBs. In this sense, it has been demonstrated that the depletion of the factors that promote HR enhances NHEJ and, the other way around, the depletion of NHEJ factors enhances HR (Gomez-Cabello et al., 2013).

Initiation of the DNA end resection has been postulated as a point of no return in the commitment to the repair via HR. Several publications strongly support this hypothesis and postulate several models and mechanisms of regulation. One model is the mutual exclusion of BRCA1 and 53BP1 from the DSBs as explained

before (Panier & Boulton, 2014). Other mechanisms involve the activity of the CDK2 in the formation of a complex that mediate the initiation of the DNA resection formed by MRN-CtIP-BRCA1 (L. Chen et al., 2008; Ferretti et al., 2013; Huertas, 2010). Also, a recent study propose the regulation of the turnover of CtIP by PIN1 and CDK2 acting as a negative regulator of DNA resection and HR (Steger et al., 2013).

During this thesis we have accumulated several indirect evidences that the Cyclin O deficient cells have an increased rate of DNA resection, a hyperactivation of the the ATR pathway and a deficient activation of ATM and DNA-PKcs. Moreover, we also show several indirect evidences that NHEJ is not working properly in Cyclin O KO cells, such as the reduced number of 53BP1 foci at the DSBs and the deficient activation of the DNA-PKcs. Also, results obtained in the lab but not shown in this thesis reinforce this hypothesis. These results show a deficient development of B-cells in Cyclin O KO mice, a process dependent on V(D)J recombination regulated and NHEJ. We also detected an increased titer of IgMs in the serum of young Cyclin O KO mice compared to other Igs. CSR is the mechanism that regulates the generation of different Ig isotypes in a process dependent on NHEJ (Bothmer et al., 2011). All these observations are indirect observations that Cyclin O deficient cells have a deficient NHEJ and a proficient HR. However, more specific experiments, such as measuring the efficiencies of HR and NHEJ using reporter systems have to be done to confirm this hypothesis. A more efficient repair by HR would explain the fact that the Cyclin O KO mice develop less spontaneous tumours, as the NHEJ is prone to generate genomic instability and tumour development.

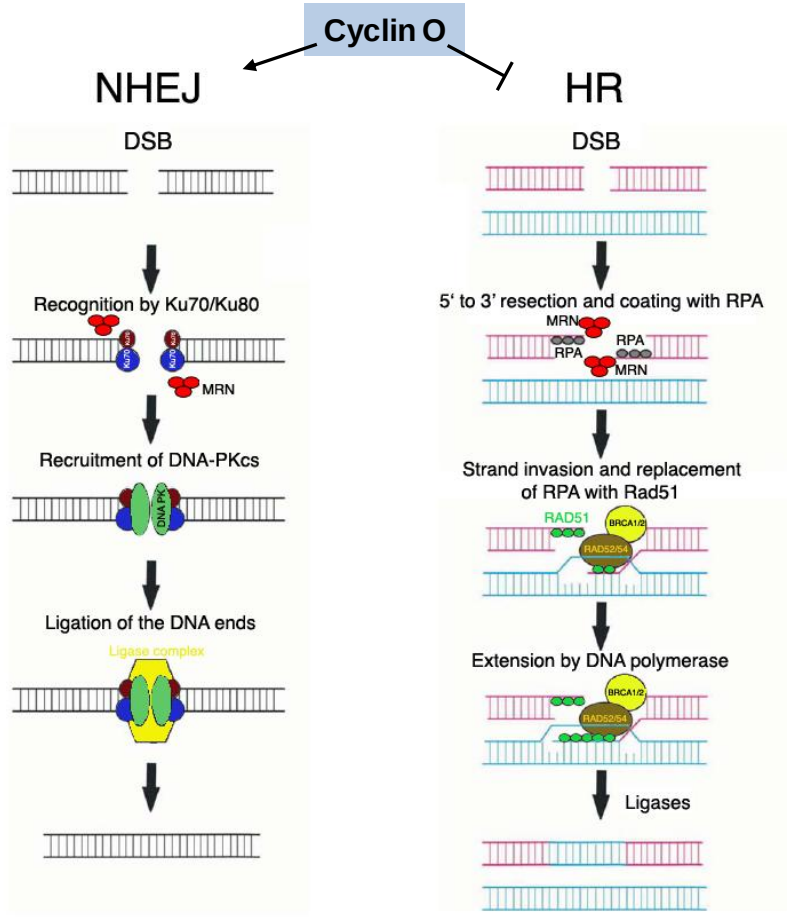


Figure.D5. – **Cyclin O drives the cell to repair the DNA damage through NHEJ.**

With all these evidences we propose that Cyclin O is involved in the regulation of NHEJ and its deficiency results in an increased DNA resection and ATR activity in response to the DBS and confers radioresistance in proliferating cells. Moreover, these observations confirm the hypothesis that CDK2 phosphorylate specific substrates in response to proapoptotic stimuli different than those phosphorylated during normal cell cycle progression. In fact,

Cyclin O limits the phosphorylation of CtIP at S327, a bona-fide target of CDK2 during DNA repair. One possible mechanism to explain this could be the competition of Cyclin A and E with Cyclin O to bind CDK2 and the phosphorylation of specific substrates depending on which is the cyclin bound to the CDK2. It is likely that the phosphorylation of this residue takes place when CDK2 binds Cyclin A and E but the efficiency of this phosphorylation decreases when the levels Cyclin O increase after DNA damage. The Cyclin O-CDK2 complexes would then be targeted towards other substrates, i.e. proteins involved in the activation of ATM and DNA-PK. It is important to note that previous experiments in our lab have shown that the expression of Cyclin O in response to DNA damage depends on ATM and p53 and that its half life is short. Thus, it is likely that the intensity and the duration of the insult determine the Cyclin O protein levels. However, studies of competition and affinity of CDK2 for the different cyclins have to be done to study this possibility.

Another important and paradoxical issue in the DDR signalling by the CDKs is the activation of the mechanisms that inhibit the CDK activity (i.ex. p21, degradation or nuclear export of CDC25 proteins, inactivation of Wee1 by phosphorylation, etc...). Thus, the activity of the CDKs is important for the DDR signalling that results in its own inhibition. One possible explanation is that the activity of the CDKs is only necessary to prime the activation of certain proteins such as CtIP and that the stabilization of its activity corresponds to additional mechanisms. Another possible explanation is that the activities of the targets of the CDKs during the DNA repair and, in particular, DNA resection is the short range resection that takes place before extensive DNA resection happens. However, recent

work indicate that the activity of the nucleases that regulate the long range resection are also regulated by CDKs (Ferretti et al., 2013; Tomimatsu et al., 2014). Finally, it is also possible, that the activity of CDK1/2 bound to Cyclin O is not affected by some of the classical mechanisms of CDK inhibition, allowing the CDK to phosphorylate specific substrates involved in DNA repair or apoptosis.

Moreover, we have found that Cyclin O – CDK complexes are able to phosphorylate ATM *in vitro*. We are now trying to identify which residue/s is/are phosphorylated and their biochemical importance. ATM has been identified to be a target of CDK5 in brain tissue at the T794 and this phosphorylation has been related to apoptosis (Tian et al., 2009). Thus, it is necessary to identify the specific substrates of Cyclin O after γ-radiation to better understand its importance in the DNA repair.

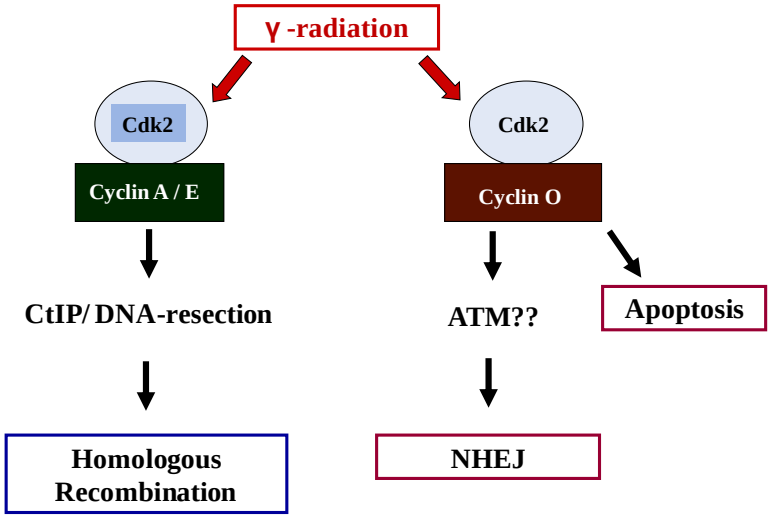


Figure.D6. – **CDK2 partners determine the response of the cell to DNA damage.**

CONCLUSIONS

1. Cyclin O is not essential for thymocyte apoptosis in response to γ-radiation.

2. Cyclin O is necessary for the γ-radiation induced apoptosis of T-cell blasts.

3. Cyclin O deficient fibroblasts survive better to DNA damage-induced by γ-radiation.

4. Cyclin O deficient fibroblasts show a more efficient instauration and a slower resolution of the G2/M checkpoint in response to γ-radiation.

5. Cyclin O deficient cells repair more efficiently the Double Strand Breaks in response to γ-radiation during the G2/M checkpoint arrest.

6. Autophosphorylation of ATM and DNA-PKcs and phosphorylation of some chromatin associated targets are deficient in Cyclin O KO fibroblasts.

7. Cyclin O complexes are able to phosphorylate ATM *in vitro*.

8. Cyclin O deficients cells show an increased phosphorylation of the ATR target CHK1 at the S345.

9. Cyclin O limitates the phosphosporylation of S327 of CtIP and limits DNA resection in response to γ-radiation.

10. Cyclin O deficient mice develop hydrocephalus and develop fewer tumours.

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