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REGULATION OF BLOCKED-DSB REPAIR BY DNA-PKcs AND ATM KINASES

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Regulation of blocked-DSB repair by DNA-PKcs and ATM kinases

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-Simplemente, el que hayamos perdido cien años antes de empezar no es motivo para que no intentemos vencer- respondió Atticus.

Matar a un ruiseñor, Nelle Harper Lee

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ABSTRACT

Given the important threat to genome integrity that double strand breaks (DSBs) pose, understanding the molecular mechanisms that govern DSB repair is extremely relevant. Notably, the end structures that are present at DSBs define their complexity and are considered putative determinants for repair pathway choice and outcome. This question, however, has not been sufficiently elucidated due to the difficulty to induce homogeneous populations of DSBs with defined end structures. Taking the advantage of a recently developed genetic strategy to induce populations of DSBs that are homogeneous in end-structure, in our study we have dissected pathways required to repair TOP2-DSBs harbouring specifically clean or blocked-ends in G0/G1. For this, we have characterized factors identified in CRISPR/Cas9 genetic screens and candidates related with previous identified factors. We have found that there is an established preference for the repair of TOP2-DSBs by the unblocking activity of TDP2 instead of end-processing pathway by nucleases, which are only necessary when the ends are irreversibly blocked. This hierarchy contributes to ensure genome stability and is disrupted in the absence of DNA-PKcs. We also demonstrate that the role of ATM in blocked DSB repair is mainly related with the nucleolytic pathway, although it could also protect the ends from an excessive processing. Furthermore, we show that the established hierarchy that prioritise TDP2 activity to repair TOP2-DSBs avoids malignant transformation and cancer development.

RESUMEN

Dada la amenaza que suponen las roturas del ADN de doble cadena para la integridad del genoma, conocer los mecanismos moleculares implicados de su reparación es extremadamente relevante. En particular, las estructuras de los extremos que presentan las roturas de doble cadena definen su complejidad y son consideradas posibles determinantes de la elección de la ruta por las que van a ser reparadas y del resultado de dicha reparación. Sin embargo, esta cuestión no ha sido suficientemente esclarecida debido a la dificultad de inducir roturas homogéneas con extremos que presenten estructuras definidas. Gracias al reciente desarrollo de un método genético para inducir

roturas con extremos homogéneos, hemos diseccionado las rutas requeridas para reparar las roturas de doble cadena inducidas por la Topoisomerasa 2 cuando los extremos se encuentran específicamente limpios o bloqueados en G0/G1. Para ello, hemos caracterizado la implicación de factores identificados en escrutinios genéticos realizados con la técnica CRISPR/Cas9 y otros candidatos relacionados con los factores previamente identificados. De esta manera, hemos identificado que existe una preferencia para reparar las roturas producidas por la Topoisomerasa 2 a través de la actividad de TDP2 en vez del procesamiento llevado a cabo por las nucleasas, que sólo son necesarias cuando los extremos están bloqueados de una forma irreversible. Esta jerarquía contribuye a asegurar la estabilidad del genoma y no se mantiene en ausencia de DNA-PKcs. También demostramos que la función de ATM en la reparación de las roturas bloqueadas está principalmente relacionada con la ruta nucleolítica, aunque también podría estar implicada en la protección de los extremos frente a un excesivo procesamiento. Además, demostramos que esta jerarquía que prioriza la actividad de TDP2 impide la transformación a células malignas y el desarrollo de cáncer.

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ABBREVIATIONS

A-T	Ataxia-telangiectasia
ATM	Ataxia-telangiectasia Mutated
ATR	Ataxia telangiectasia And Rad3-Related Protein
bp	Base pair
CC	Cleavage complex
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSR	Class-switch recombination
DAPI	4'-6-diamino-2-fenilindol
DDR	DNA Damage Response
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	Double-strand break
dsDNA	double-stranded break
eGFP	**Enhanced** green fluorescent protein
G segment	Gate segment
G0	Gap 0 phase of cell cycle
G1	Gap 1 phase of cell cycle
HR	Homologous recombination
ICRF187	Dexrazoxane
Ig	Immunoglobulin
IR	Ionizing radiation
kDa	Kilodalton
LET	Linear energy transfer
LIG4	LIGASE 4
MEFs	Mouse embryonic fibroblasts
MMEJ	Microhomolgy-mediated end joining

MRN	MRE11-RAD50-NBS1 complex
NHEJ	Non-homologous end joining
nt	Nucleotide
PAM	Protospacer adjacent motif
PCR	Polymerase chain reaction
PIKK s	Phosphatidylinosytol 3-kinase-related kinases
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSS	Recombination signal sequence
SCID	Severe combined immunodeficiency
sgRNA	Single guide RNA
SSB	Single-strand break
ssDNA	Single-stranded DNA
T segment	Tranfer segment
TCR	T-cell receptor
TDP	Tyrosyl-DNA-phosphodiesterase
TDP1	Tyrosyl-DNA-phosphodiesterase 1
TDP2	Tyrosyl-DNA-phosphodiesterase 2
TOP1	Topoisomerase 1
TOP1cc	Topoisomerase 1 cleave complex
TOP2	Topoisomerase 2
TOP2cc	Topoisomerase 2 cleave complex
VP16	Etoposide

I. INTRODUCTION

I. INTRODUCTION

1. DNA damage

The X-ray diffraction image of crystallized DNA ("Photo 51") taken by Rosalind Franklin in 1952, was key for the discovery of the double helix, which immediately suggested a possible copying mechanism for genetic material. Even before the discovery of the stable structure of DNA, it was known that the exposure of exogenous agents, such as X-rays, ultraviolet (UV) light and some chemicals, can cause genetic variations that can give rise to cancer (Friedberg, 2008). It took additional 10 years after the elucidation of DNA structure to realise that DNA is also damaged by endogenous agents during normal metabolism (Lindahl, 1993; Lindahl & Nyberg, 1972). It has been estimated that each of the ~10^{13} cells in the human body receives approximately 70000 lesions per day (Lindahl & Barnes, 2000). To deal with threats posed by DNA damage, it became evident that cells must have evolved mechanisms to promote their repair and maintain genome integrity (Friedberg, 2008; Lindahl & Barnes, 2000). In 1974, Francis Crick admitted in a personal perspective "We totally missed the possible role of enzymes in DNA repair, although [...] I later came to realize that DNA is so precious that probably many distinct repair mechanisms would exist. Nowadays one could hardly discuss mutation without considering repair at the same time." (Crick, 1974). Nevertheless, errors during DNA repair may occur, and some lesions can remain unrepaired under certain circumstances (Altieri, Grillo, Maceroni, & Chichiarelli, 2008). For instance, when DNA repair machinery is impaired, or when the levels of damage are high enough to overwhelm DNA repair capacity. As a consequence of the failure to accutely repair DNA damage, mutations that compromise essential function of the cell can arise. This could lead to uncontrolled cell division, premature aging and cell death (Freitas & De Magalhães, 2011; Jackson & Loeb, 2001; Surova & Zhivotovsky, 2013; Tubbs & Nussenzweig, 2017). For this reason, DNA damage is considered a fundamental feature of human diseases such as cancer, neurodegenerative disorders and other heritable syndromes.

1.1 Types of DNA damage

DNA damage can arise from three different sources (Figure 1). First, as a consequence of chemical nature of DNA in an aqueous solution, spontaneous reactions (most of them hydrolysis) can give rise to abasic sites by depurination and deamination, causing interconversion between DNA bases (Lindahl & Nyberg, 1972). Second, cellular metabolism produces reactive oxygen and nitrogen species, lipid peroxidation products, estrogen and cholesterol metabolites, and reactive carbonyl species, all of which damage DNA. Reactive oxygen and nitrogen species alone generate several kinds of single-strand breaks (SSBs) and more than 70 oxidative base and sugar products in DNA. Third, DNA is damaged by exogenous physical and chemical agents. UV, which induces pyrimidine dimers and 6–4 photoproducts, and ionizing radiation (IR) are the main physical sources of DNA damage. IR induces oxidation of DNA bases and generates SSBs by producing radiolysis radicals that attack the sugar-phosphate backbone. Frequently, if two such nicks are present within one helical turn in complementary DNA strands, they can lead to a double-strand break (DSB). On the other hand, the major chemical sources of damage are alkylating agents, which attach alkyl groups to DNA bases, and crosslinking agents that introduce covalent links between bases of the same DNA strand (intrastrand crosslinks) or of complementary DNA strands (interstrand crosslinks or ICLs). Furthermore, other chemical agents which hamper the action of enzymes that are involved in DNA metabolism can cause different DNA lesions (Chatterjee & Walker, 2017; Ciccia & Elledge, 2010; Hoeijmakers, 2009; Mehta & Haber, 2014; Tubbs & Nussenzweig, 2017).

The majority of lesions (75%) are SSBs, which can also be converted to DSBs during replication. The type of damage that occurs is important for the repair outcome. DSBs, although much less frequent than SSBs, are one of the most cytotoxic forms of lesion as they interrupt the continuity of DNA molecule and threat genome integrity due to the lack of an intact template to copy the information lost. Because of this reason, DSBs strongly promote chromosome rearrangements which, in turn, can lead to malignant

transformation. Indeed, a single unrepaired DSB is enough to trigger permanent growth arrest and cell death (Panier & Boulton, 2014).

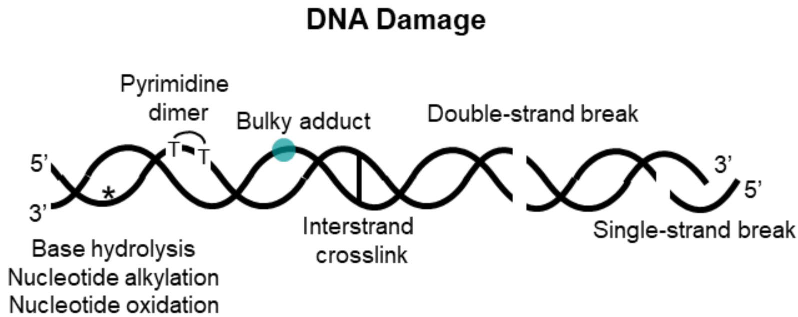

Figure 1: Types of DNA damage. Different types of lesions can alter DNA structre. Alkylation and oxidation of nucleotides, hydrolysis of bases or interstrand crosslink and pyrimidine dimers are commonly found. Chemicals can also interact with the DNA molecule forming bulky adducts altering DNA structure. Single-strand breaks (SSBs) and double-strand breaks (DSBs) interrupt continuity of the DNA strands.

1.2 DSB repair in mammalian cells

Mammalian cells rely on two major pathways to repair DSBs that can be largely divided between those that use extensive homology from a sister chromatid or another homologous sequence elsewhere in the genome and those that do not use an homologous template: homologous recombination (HR), which needs a several hundred base pairs of homologous undamaged template to restore any sequence information lost at the DSB site, and non-homologous end joining (NHEJ) (Figure 2), which detects and directly tethers the ends without any homologous template (Lieber, 2008; Pannunzio, Watanabe, & Lieber, 2018; San Filippo, Sung, & Klein, 2008). Although mammalian cells are diploid, HR rarely uses the homologous chromosome as a template for DSB repair (Johnson, 2000). Consequently, HR is usually restricted to late S/G2 phase when a sister chromatid is available, whereas NHEJ can operate in any phase of the cell cycle. HR is always initiated by 5′ resection which generates 3′ protuding ends that invade the homologous template (Figure 2).

Moreover, there is an alternative NHEJ pathway, termed microhomology-mediated end-joining (MMEJ). While in NHEJ there are up to 4 bp of microhomology between the two broken ends, MMEJ occurs when end-resection exposes micro-homologies of 5–25 bp that enable DNA single strands to anneal before joining. This pathway leads to deletions of the sequence flanking the DSB and is associated to chromosomal rearrangements. However, there is still some debate about to which extent MMEJ pathway physiologically contributes to DNA repair and its relevance (Sfeir & Symington, 2015).

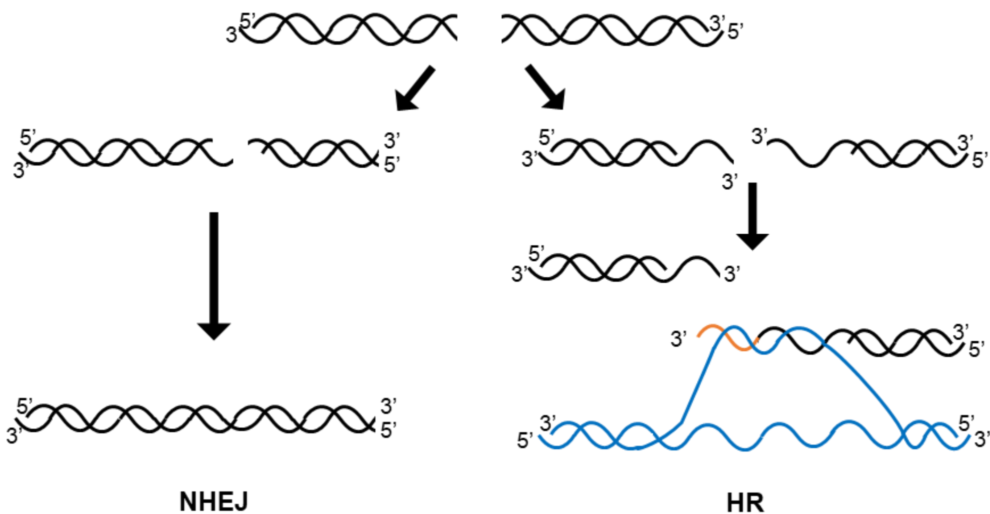

Figure 2: Pathways for the repair of DSBs in mammalian cells. DSBs are repaired by non-homologous end joining (NHEJ) pathway by direct ligation of DNA ends (left). In the homologous recombination (HR) pathway (right), DNA ends are resected by 5’ strand degradation and newly formed 3’ protruding ends invade a homologous sequence, which is used as template for repair.

1.3 DNA Damage Response

To counteract potential threats, DNA damage is sensed by a signal transduction pathway that is called the DNA damage response (DDR) (Figure 3), which coordinates DNA repair with checkpoint activation, chromatin reorganization and changes in gene expression. After damage recognition, the DDR is primarily mediated by kinases from

the phosphatidylinositol 3-kinase-like protein kinase (PIKKs) family: ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3–related (ATR) or DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Harper & Elledge, 2007). Specifically, they are serine/threonine-directed kinases and have preference for SQ/TQ motifs (Kim, Lim, Canman, & Kastan, 1999). These kinases share partially overlapping phosphorylation substrates in DNA repair and embryonic development (Jiang et al., 2015). In particular, after the induction of a DSB, ATM and DNA-PKcs are robustly activated, while ATR is activated by the generation of ssDNA (B. Shiotani & Zou, 2009).

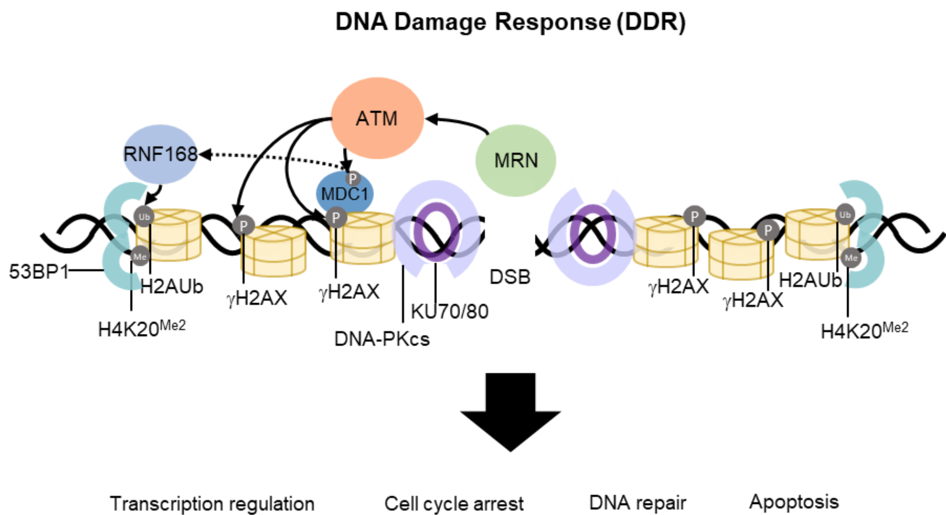

Figure 3: DNA damage response (DDR). DSBs are detected by KU70/80 heterodumer and the MRN complex. KU70/80 associated with DNA ends recruits DNA-PK catalytic subunit (DNA-PKcs) forming the DNA-PK homoenzyme. This, in collaboration with ATM activation, triggers the DDR signalling cascade. MRN collaborates to tether DSB ends and to maintain ATM active in the vicinity of DSBs. This leads to H2AX phosphorylation in Ser139 (γH2AX), promoting the recruitment of downstream factors such MDC1, RNF168 and 53BP1. The DDR modulates important cellular functions such as transcriptional regulation, cell cycle arrest, repair of the lesion, and apoptosis.

Therefore, ATR is mainly activated upon replicative stress during S-phase, sensing damage at replication forks (Cimprich & Cortez, 2008; I. M. Ward & Chen, 2001). However, ATR can be also activated when DSBs are resected and a ssDNA region is produced (Bunsyo Shiotani & Zou, 2009). Additionally, once activated, ATM or ATR also regulate the role of effector proteins such as CHK2, CHK1 and the tumour

suppresor p53, which are essential for cell cycle arrest (Rouse & Jackson, 2002). Thus, in the presence of DNA damage, cellular proliferation is avoided. Finally, if the DNA lesion cannot be repaired, DDR participates in triggering apoptosis (Roos & Kaina, 2013). This mechanism is crucial to get rid of potentially dangerous cells when the repair capacity is overwhelmed.

Specifically, after the generation of a DSB, the MRN complex acts as a sensor and recruits ATM to the break. MRN complex is formed by MRE11, which is an exo and endonuclease, RAD50, an ATPase in the ABC transporter family, which also contains long, intramolecular coiled coils that are similar to cohesins and condensins, and NBS1, that regulates both MRE11 and RAD50 activities and is only found in eukaryotes. ATM exists as a catalytically inactive noncovalent homodimer that, when recruited to a DSB, dissociates and autophosphorylates at serine (S)1981 in order to become active. In addition, ATM autophosphorylation also depends on Tip60/KAT5-dependent acetylation on lysine (K)3016, which also takes place immediately upon DNA damage (Paull, 2015). The MRN complex is also phosphorylated by ATM, and this event is relevant for downstream signalling on the recruitment of additional ATM substrates (Harper & Elledge, 2007).

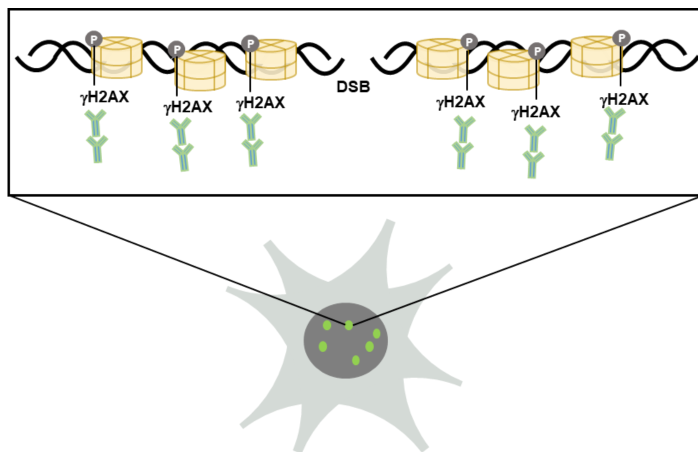

Figure 4 : γH2AX foci. Post-translational phosphorylation in Ser139 of histones H2AX, known as γH2AX, largely expands along megabases flanking DSBs. By performing immunofluorescence using γH2AX specific primary antibody and fluorochrome-conjugated secondary antibody, discrete foci can be observed under the microscope.

Once activated, an early substrate of ATM in response to DSBs is the so-called histone H2AX, a variant form of the histone H2A, which is phosphorylated at S139. This modification, designated γH2AX (Rogakou, Pilch, Orr, Ivanova, & Bonner, 1998), spreads away from the DSB into adjacent chromatin up to several megabases in mammals, leading to the formation of discrete foci that can be detected by immunofluorescence using phospho-specific antibodies (Figure 4) (Polo & Jackson, 2011; Stiff et al., 2004). Although H2AX is predominantly phosphorylated by ATM (Takahashi et al., 2010), this phosphorylation can be carried out by DNA-PKcs and ATR (Firsanov, Solovjeva, & Svetlova, 2011). This phosphorylation directs the assembly of downstream DDR components. First, MDC1 binds to γH2AX and also interacts with ATM and NBS1. MDC1 is also phosphorylated by ATM, this event promotes oligomerization of MDC1 and may facilitate the spreading of MDC1 and ATM on chromatin (Jungmichel et al., 2012; Liu et al., 2012; Luo, Yuans, & Lous, 2011; Matsuoka et al., 2007), which contributes to the generation of a positive feedback loop that promotes spreading of γH2AX. MDC1 then recruits the E3 ubiquitin ligase complex RNF8/HERC2/UBC1 and the ubiquitin-activating enzyme UBA1, which eventually allows RNF168 to ubiquitinate H2A and H2AX. The ubiquitination of H2A is thought to provide a recruitment platform for additional mediator factors such as BRCA1 or 53BP1 (J. M. Daley & Sung, 2014). Although not endowed with enzymatic activities, the recruitment of these factors and its irradiation-induced foci formation are critical for DSB repair, as they can dictate the repair pathway that will handle the lesion (J. R. Chapman, Sossick, Boulton, & Jackson, 2012; Panier & Boulton, 2014). Consistently, numerous knouckout mouse models of DDR factors and cells derived from patients show increased genome instability, hypersensitivity to DSB inducing agents, infertility and immunodeficiency (Barlow et al., 1996; C. H. Bassing et al., 2002; Celeste et al., 2002; Franco, Alt, & Manis, 2006; Lou et al., 2006). These features are strongly related with defects in the efficiency and accuracy of DSB repair. Therefore, these data support the tight coordination and interplay between DSB repair and the DDR to ensure an appropriate outcome for the cell.

1.4 Non-homologous end joining (NHEJ)

During the initiation of NHEJ (Figure 5), the KU70/80 heterodimer recognizes and binds to double stranded DNA ends in an extraordinary efficient and rapid manner due to its strong avidity for end-binding and its high abundance. DNA-bound KU recruits DNA-PKcs to form the DNA-PK holoenzyme. In the presence of DNA-PKcs, KU translocates inward and DNA-PKcs has direct contacts with ~ 10 bp at the termini of DNA ends. The two molecules of DNA-PKcs bound to opposing sides of a DSB can interact, contributing to synapsis of broken DNA ends. How DNA-PK activates and de-activates its kinase activity has been investigated for decades. Although it is clear that DNA binding is an absolute requisite for activation and that autophosphorylation induces dissociation of DNA-PKcs from KU bound DNA, the precise mechanistic basis for either of these phenomena is unknown. Moreover, although a number of DNA-PKcs substrates involved in NHEJ have been identified, these phosphorylation events do not seem relevant for successful NHEJ, being the autophosphorylation of the catalytic subunit itself the only physiologically target required (Neal & Meek, 2011). DNA-PK predominantly regulates NHEJ and facilitates recruitment of DNA ligase IV (LIG4) and accessory factors such as X-ray cross complementing Group 4 (XRCC4), XRCC4-like factor/Cernunnos (XLF) and Paralog of XRCC4 and XLF (PAXX), which contribute to end-pairing and perform the ligation of the DSB (Conlin et al., 2017; Kakarougkas & Jeggo, 2014; Ochi et al., 2015). In vertebrates, NHEJ further evolved an end-processing capacity that allows for the repair of complex ends (i.e., hairpins). This is, in part, regulated by DNA-PKcs. In this context, DNA termini that contain non-ligatable end groups are processed by nucleases prior to DNA ligation. End-processing can lead to the creation of DNA gaps that require the action of DNA polymerases for their repair. Members of the DNA pol X family of DNA polymerases, pol μ, pol λ and terminal deoxyribonucleotidyltransferase (TdT), have all been implicated in NHEJ (Mahaney, Meek, & Lees-Miller, 2009). Interestingly, defects in NHEJ entail human health diseases such as microcephaly, immunodeficiency, premature aging and cancer, which highlight the relevance of this process in mammals (Jiang et al., 2015; Lieber, 2010).

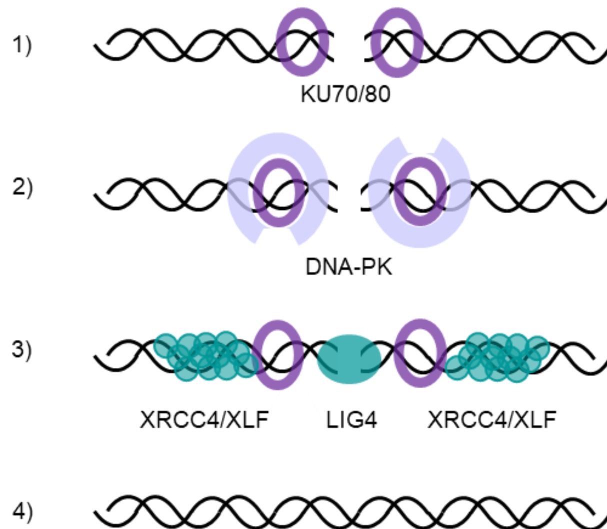

Figure 5: The Non-homologous end joining (NHEJ) pathway. First, DSBs are recognized by the ring-shaped KU70/80 heterodimer. DNA-PK catalytic subunit (DNA-PKcs) is recruited and activated, translocating KU70/80 ring inward the break. This forms the DNA-PK holoenzyme that phosphorylates several downstream factors. Finally, DNA-PK undergoes conformational changes allowing XRCC4/XLF filament to form and recruitment of DNA ligase IV (LIG4), which ligates DNA ends.

1.5 Pathway choice

Given the ability of HR to restore sequence information lost at DSBs using an undamaged homologous template, and its abundant usage in lower organisms, it was widely assumed that this pathway is preferentially used in S/G2 phases. Nevertheless, some observations support that NHEJ functions prior to HR (Beucher et al., 2009), making a first attempt to repair DSBs and if ligation is impeded or delayed, then resection and repair by HR takes over. One factor that influences and enhances the switch from NHEJ to HR is the complexity of damage, regarding the type of structure at DNA ends and at chromatin surrounding the damage (Shibata et al., 2011). It is interesting to understand how complex DSBs are repaired in G1 phase, during which HR does not take place. It has been proposed that they can be repaired with a slow

kinetics mechanism that involves a resection mediated NHEJ, although further work is required to precisely define this process (Kakarougkas & Jeggo, 2014).

As aforementioned, the step that commits to repair by HR is the 5′ resection of DNA ends. Resection can be subdivided into an early step carried out by MRE11 endonuclease and stimulated by CtIP, which proceeds by an initial single-strand (ss) nick followed by a 3′-5′ resection, and a second process of elongation that extends the length of resected DNA by 5′-3′ exonuclease activity of EXO1 and/or DNA2-BLM. The switch from NHEJ to HR is regulated by the antagonistic relationship between 53BP1 and BRCA1. In response to DSB damage, 53BP1 is recruited to act as a barrier that restricts DNA nuclease activities (presumably, 5′-3′ extension of resection, because 53BP1 foci do not interfere with the initiation of resection by MRE11-CtIP) to prevent excessive end-resection while the DSB is repaired through the NHEJ pathway. Microscopy-high-resolution analyses demonstrated that 53BP1 relocates from the foci center to the periphery in a BRCA1-dependent manner over time during the switch from NHEJ to HR (Shibata, 2017).

1. 6 Relevance of end-structure in DSB repair

The only essential step in NHEJ is the ligation of, at least, one strand of the DSB (Waters et al., 2014). During this process, lysine K273 from the catalytic site of LIG4 is adenylated. Secondly, this adenyl group is transferred to the strand break 5′ phosphate terminus, which is attacked by 3′ hydroxyl terminus of the strand break, completing the ligation step (Ellenberger & Tomkinson, 2008). Thus, LIG4 activity requires compatible ends harbouring canonical 5′ phosphate and 3′ hydroxyl termini. However, DSBs often have complex ends with structures that do not allow straight-forward joining of the termini. These chemical variations can be sensed by LIG4 though the disruption of its catalytic cycle (Reid et al., 2017). Therefore, when DSBs harbour different chemical structures at the ends they must be restored to conventional 5′ phosphate and 3′ hydroxyl termini for gap filling and DNA ligation to occur. There are two conceptually different ways in which ends can be restored to their canonical chemical structures

(Figure 6). First, cells provide a large number of enzymes available to directly convert these structures into ligatable substrates. Considering that this process does not involve sequence modification, it can be called "unblocking". On the other hand, under certain circumstances, such as the presence of complex lesions, unblocking activities may be compromised or overwhelmed, resulting in breaks that require "end-processing" by the action of nucleases that cleave DNA sequence from the ends to remove the chemical modifications. Regarding unblocking, there is a large number of factors with different enzymatic activities that are available for this process during DSB repair (Figure 7), such as TDP1, TDP2, PNKP, Aprataxin, and even KU. This, in turn, reflects the variety of damaged termini that can arise, as each of these factors removes specific chemical modifications at DNA ends (Andres, 2015; Povirk, 2012). These activities, and unblocking in general, are specially relevant for the NHEJ pathway, as they can support accurate religation, while processing may involve nucleotide loss or gain, and hence sequence modification. On the contrary, as HR involves extensive 5′ resection followed by invasion of a homologous undamaged template by the exposed 3′ protruding end (San Filippo et al., 2008), chemical modifications do not necessarily impact on the repair outcome, regardless of blockage being at 5′ or 3′ terminus (Povirk, 2012).

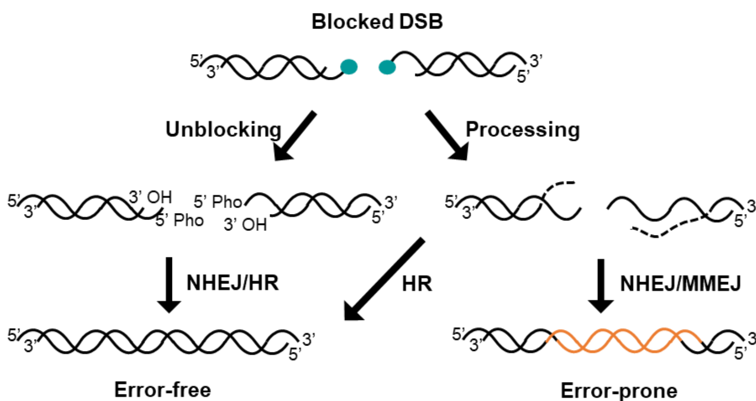

Figure 6: Unblocking and processing of DSBs. Unblocking pathways directly convert ends into 5'phospahte and 3' hydroxyl but the nucleotide sequence remains intact, promoting error-free repair (left). Processing can also facilitate blocked DSBs repair removing aberrant structures from DNA ends by nucleotide trimming (right). This pathway can lead to error-prone repair when non-templated repair pathways such as NHEJ or MMEJ are used.

Furthermore, incompatibility of DNA ends for repair by NHEJ can also be due to non-complementarity of the ends. It has been demonstrated that LIG4 can ligate across short gaps or rejoin several incompatible DNA end configurations that do not share even 1 bp of terminal microhomology (Gu et al., 2007). For this, NHEJ also employs several processing enzymes that can modify the ends until they become ligatable substrates (Strande, Waters, & Ramsden, 2012). Therefore, single-stranded overhangs and blunt ends can be trimmed by nucleases such as Artemis and the resulting gaps can be filled in by X family polymerases (Polymerases μ and λ) (Lieber, 2010). It is worth noting that non-complementary DNA ends are indeed the most likely result of end processing at chemically modified structures.

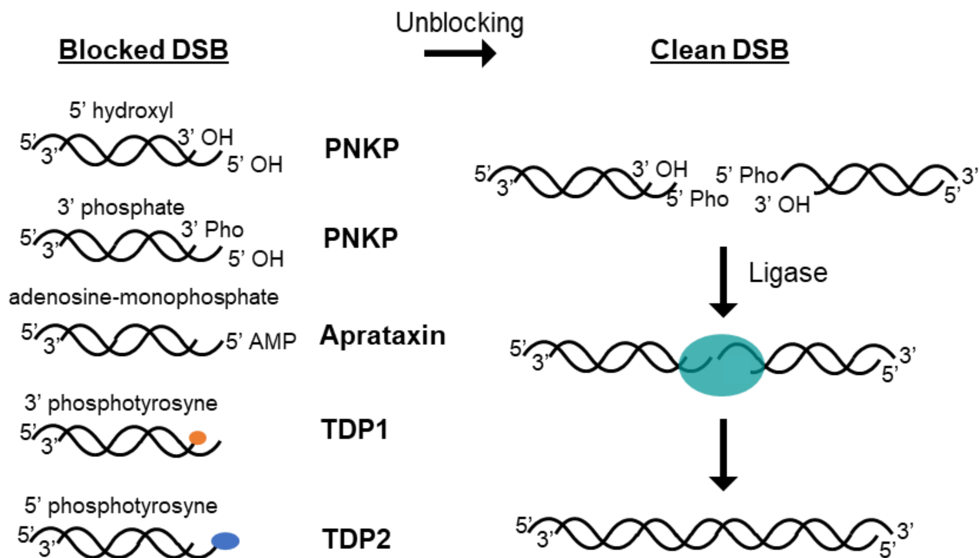

Figure 7: Structure of DNA ends and unblocking enzymes. DSB termini can be blocked by numerous chemical structures *in vivo*. Several unblocking enzymes are present in mammalian cells and efficiently convert these structures in clean 5' phosphate and 3'hydroxyl DSB termini (left). These clean DSBs can be in theory, directly repaired with the only enzymatic activity of a ligase (right).

2. Sources of DNA double-strand breaks

Every day approximately ten DSBs are generated in each human cell. Although this incidence is much less frequent than other types of damage, understanding how DSBs arise and get repaired is a key question given the important threat to genome integrity that accidental DSBs pose. Furthermore, several physiologically and developmentally important processes, such as gametogenesis and lymphocyte development, require the generation of programmed site-specific DSBs and their subsequent repair by various pathways (Bednarski & Sleckman, 2012; Lam & Keeney, 2015; Stavnezer, Guikema, & Schrader, 2008).

2.1 Programmed DNA double-strand breaks

During gametogenesis, haploid cells are generated from diploid progenitors. For this, meiotic homologous recombination events between homologous chromosomes increase genetic variation in populations and support a proper chromosome segregation at the first meiotic division. This is performed by a highly regulated pathway involving the induction of programmed DSBs that are catalyzed by the evolutionarily conserved SPO11 protein (Borde & de Massy, 2013). These DSBs are not induced at random sites along chromosomes, as shown in several studies and recent high resolution genome-wide DSB maps in yeasts and mammals (Choi & Henderson, 2015; Martín-Castellanos, Fowler, & Smith, 2013; Pan et al., 2011; Smagulova et al., 2011). SPO11-induced DSBs harbour blocked ends, since the protein remains covalently linked to the 5′ termini of the DNA, which are repaired by HR (Lam & Keeney, 2015). Consistent with this, human syndromes linked to defects in HR and polymorphisms in these DSB repair genes, can be associated with infertility (Cooke & Saunders, 2002; Ji et al., 2013).

On the other hand, programmed DSBs are induced in developing and mature lymphocytes at specific genome sites as they are required for the physiological DNA rearrangements associated to antigen receptor and immunoglobulin (Ig) gene assembly

by V(D)J recombination, as well as for immunoglobulin class switch recombination (CSR) (Bednarski & Sleckman, 2019). Therefore, both V(D)J and CSR recombination induce programmed DSBs and require NHEJ pathway for their completion (Craig H. Bassing & Alt, 2004). T-cell receptor (TCR) or Ig genes comprise an array conformed by several different coding variable (V), diversity (D) and joining (J) segments, which are flanked by recombination signal sequences (RSS). During early steps of lymphocyte maduration, V(D)J recombination rearranges a single combination of V, J, and in some cases, D segments randomly. At a molecular level, this mechanism is initiated by the

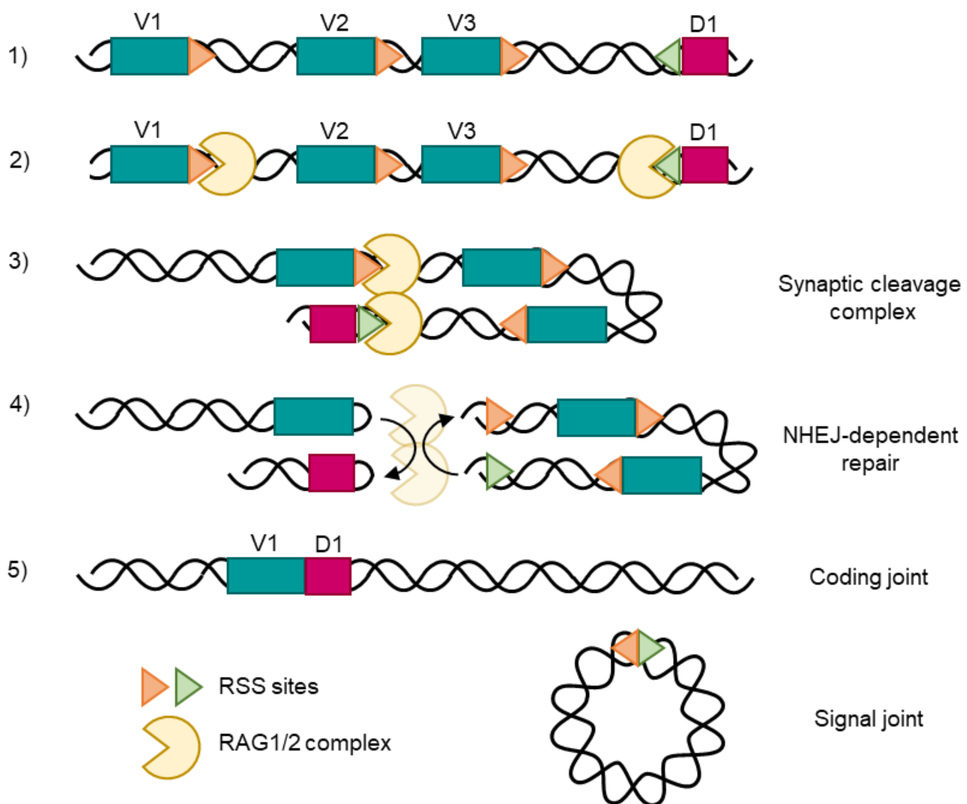

Figure 8: V(D)J recombination during lymphocyte maduration. 1) T-cell receptor and Immunoglobulin genes contain arrays of different V, D and J coding segments, flanked by recombination signal sequences (RSS). **2)** RAG1/2 recombinase recognizes two different RSS sequences amongst the V, D or J segments. **3)** It brings genomic regions together forming the synaptic cleavage complex. **4)** It promotes DNA cleavage in each RSS, forming two DSBs. Each of them is formed by a hairpin structure in the coding end, and blunt-end in the signal end. **5)** NHEJ pathway joins the two blunt-ends forming a circular extrachromosomal signal joint. Artemis endonuclease catalyses the opening of the hairpin-ends, licensing them for NHEJ repair. This results in a lymphocyte precursor with a specific combination of single V, D and J regions rearranged together.

RAG endonuclease that recognizes, pairs and cleavages RSSs (Figure 8), resulting in two kinds of DNA ends: blunt, signal ends (SEs) and hairpin-sealed coding ends (CEs). The two SEs are directly ligated via NHEJ to form the signal joint (SJ). The two hairpin-sealed CEs must first be opened by the ARTEMIS endonuclease activity, which is recruited by DNA-PKcs, before they are ligated to form the coding joint (CJ). In this regard, this process has been widely used to distinguish between end-processing and end-ligation steps of NHEJ. CJs encode the variable region exon of antigen receptor genes necessary for lymphocyte development, therefore, defects in any component involved in end-processing and end-ligation during NHEJ, such as deficiencies in ARTEMIS or DNA-PKcs abolish V(D)J recombination and lymphocyte development, entailing a severe combined immunodeficiency in patients and animal models (Franco et al., 2006; Lieber, 2010).

2.2 Incidental DNA double-strand breaks

Apart from programmed DSBs that arise during physiological processes, there are a wide variety of manners in which DSBs can be generated (Mills, Ferguson, & Alt, 2003), either by exogenous or endogenous sources.

2.2.1 Exogenous sources of incidental DSBs

Ionizing radiation (IR) can be considered one of the most relevant exogenous source of DSBs due to its importance in cancer treatment. IR is a type of high-energy radiation that is able to release electrons from atoms and molecules generating ions which can break covalent bonds. It induces a plethora of different types of DNA damage, of which the main are chemically identical to those formed by ROS. Within DNA damage induced by IR, DSBs are a relatively small proportion (<5%) whereas DNA single-strand breaks (SSBs) and DNA base damage predominate. All these lesions can lead to oncogenic mutations and cell death (Carter et al., 2018; Lomax, Folkes, & O'Neill, 2013). IR can be classified into low Linear Energy Transfer (LET) which comprises X- or γ-rays, that are commonly used in radiotherapy for cancer treatment, and high LET, including

α-particles (a component in space radiation) and high-energy ions. It is known that IR generates DSBs that can harbour ends with a high diversity in structure. The complexity of these structures correlates with increasing LET. Consistently, the impact from α-particles is much greater than that of γ-rays, as α-particles typically induce a high number of complex and clustered DNA damage, which cause a higher cyto and genotoxicity increasing its oncogenic potential (Asaithamby, Hu, & Chen, 2011; Goodhead, 1994; Hada & Georgakilas, 2008).

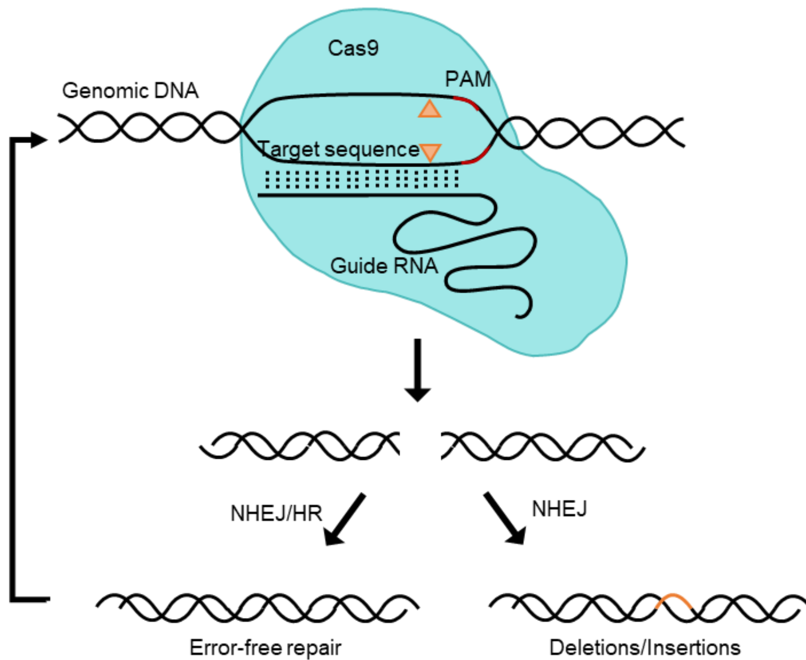

Figure 9: CRISPR-Cas9 system. First, recognition of DNA target requires both base pairing to the sgRNA sequence and the presence of a 5′-NGG-3′ consensus sequence (PAM) immediately downstream of the target site. DNA double-strand breaks (DSB) produced by CRISPR-Cas9 can be repaired by HR or NHEJ pathway. Cas9 cleaves once the DSB is repaired, increasing the probability that a mutation arises due to insertion and/or deletion during NHEJ repair.

Additionally, there are other relevant exogenous agents that can induce DSBs, such as the exposure to chemicals that either directly interact with DNA or with proteins harbouring endonuclease activity. Furthermore, the direct effect of restriction enzymes

and genome editing nucleases can also be considered. Finally, sources that induce SSB can generate a DSB if two lesions are generated randomly in close proximity.

Regarding genome editing nucleases, bacteria and archaea have evolved RNA-mediated adaptive immunity called CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) that protect them from viruses and plasmids. The CRISPR associated protein Cas9 is an endonuclease that uses a guide sequence within an RNA duplex, tracrRNA:crRNA, that is complementary to a DNA target sequence. This enables Cas9 to introduce a site-specific double-strand break in the genome (Gasiunas, Barrangou, Horvath, & Siksnys, 2012; Jinek et al., 2012). The dual tracrRNA:crRNA was engineered as a single guide RNA (sgRNA) that retains two critical features: a sequence at the 5′ side that determines the DNA target site by Watson-Crick base-pairing and a duplex RNA structure at the 3′ side that binds to Cas9. This finding created a simple two-component system in which changes in the guide sequence of the sgRNA program Cas9 to target any DNA sequence of interest (Figure 9). DNA target recognition requires both base pairing to the sgRNA sequence and the presence of a 5′-NGG-3′ consensus sequence (so called PAM "protospacer adjacent motif" sequence) immediately downstream of the target site (Doudna & Charpentier, 2014). Recently, it was found that DSBs induced by Cas9 endonuclease harbour 1-bp staggered ends rather than generally assumed blunt ends (Zuo & Liu, 2016). These DSBs can be repaired either by HR in the presence of the corresponding homologous template or by NHEJ, which is, as aforementioned, quantitatively the major DSB repair pathway in higher eukaryotes. NHEJ is perfectly suited for managing these lesions, reconstituting the original sequence. However, Cas9 recurrently cleaves once the DSB is repaired, selecting for mutations due to insertion and/or deletion (the so-called indels) during NHEJ repair. Therefore, CRISPR-Cas9 DNA cleavage system can be used as a tool to generate frameshift mutations that disrupt a targeted gene through NHEJ (Su et al., 2016). One of the most important advantages of the CRISPR-Cas9 system is that it does not rely on protein engineering (as happened with previous systems such as ZFNs, meganucleases and TALE nucleases), but on the simplicity of

complementarity between the sgRNA and the target DNA to be recognized. Moreover, CRISPR-Cas9 system is easy to use due to the only requirement of 20 nt RNA synthesis to target a new locus and the expression of Cas9 nuclease. This technology also avoids RNAi (interference RNA) disadvantages, such as the incomplete loss of function, the difficulty to predict off-targets and the temporary inhibition. Because of its versatility and simplicity, CRISPR-Cas9 system is currently the most widely used tool for genome targeting experiments and is being used in high-throughput approaches. In this sense, genome-scale guide RNA libraries which enable *in vitro* and *in vivo* genome-wide screening approaches have been synthesised (Martinez-Lage, Torres-Ruiz, & Rodriguez-Perales, 2017). This provides an opportunity for systematic classification of human genetic elements into functional categories and biological processes. In this context, CRISPR-Cas9 screens has been carried out to identify genes whose loss of function enables drug and toxin resistance (Dev et al., 2018; Koike-Yusa, Li, Tan, Velasco-Herrera, & Yusa, 2014; Shalem et al., 2014; T. Wang, Wei, Sabatini, & Lander, 2012), accelerates metastasis (S. Chen et al., 2015), or influences the immune response (Parnas et al., 2015). This confirms the huge possibilities that the CRISPR-Cas9 technology provides for mammalian genetic screens.

2.2.2 Endogenous sources of incidental DSBs

Endogenous processes also contribute to the generation of incidental DSBs. For instance, the oxidative attack of reactive oxygen species (ROS) introduces DNA base or sugar damage that leads to SSB formation. These SSB can be converted to DSBs when replication proceeds through them, following their encounter with the transcription machinery or when they arise in close proximity (Cortés-Ledesma & Aguilera, 2006; Kuzminov, 2002; Woodbine, Brunton, Goodarzi, Shibata, & Jeggo, 2011). It is estimated that 1% of SSBs convert to DSBs per cell cycle. Taking into account that the majority of these conversions takes place during S phase, non-dividing cells are expected to produce a negligible amount of DSBs from SSBs (Vilenchik & Knudson, 2003). Furthermore, DSBs can arise directly by the abortive action of enzymatic activities

required during DNA metabolism. For instance, the catalytic action of Topoisomerase 2 (TOP2) requires the cleavage of both strands of the double helix to modulate DNA topology. Under normal circumstances, this cleavage is a transient intermediate that is quickly resealed. Nonetheless, in certain conditions, such us the exposure to specific chemical agents or the presence of DNA lesions in close proximity, the cleavage intermediate can be stabilized, which increases the possibility of the generation of a DSB (Deweese & Osheroff, 2009). As repair of DSBs induced by the aberrant action of TOP2 is a main focus of this thesis, this process will be further explained below. Interestingly, recent reports suggest the existence of programmed DSBs induced by non-canonical functions of topoisomerases promote transcription. Specifically, upon different kinds of stimulation such as heat shock, serum induction, insulin, androgen, estrogen, and various neuronal stimuli, transcription seems to require DNA damage induced by topoisomerases, which is recognized by ATM, DNA-PKcs and PARP1 (Bunch et al., 2015; Haffner et al., 2010; Ju et al., 2006; Lin et al., 2009; Madabhushi et al., 2015; Perillo et al., 2008; Wong et al., 2009). For instance, postmitotic neurons activated with NMDA promote γH2AX phosphorylation in transcribed regions of early-response genes (Madabhushi et al., 2015). Furthermore, DNA damage signalling was proposed to be required for the release of paused RNA polymerase at promoters or for conformational changes that promote interactions between promoters and enhancers (Calderwood, 2016).

3. DNA topoisomerases.

The three-dimensional organization of the genome in the space of the cell nucleus is complex. DNA enclosed in higher eukaryotic cells usually measures ~2 m in length, which must be packed into a nucleus of ~10 μm in diameter. Thus, the safe and accurate DNA propagation during replication and cell division, as well as allowing accessibility of regulatory factors at the right moment and place, entail a challenge for cells. To ensure DNA functionality and accommodate its immense length into the nucleus, the genetic material is wrapped into higher-order chromatin fibers that finally are

organized into chromosome territories. Indeed, despite its extraordinary length, DNA only takes up an estimated 15% of the nuclear volume (Dekker & Misteli, 2015). Besides the issue of DNA organization in three-dimensional space, two aspects of DNA topology significantly affect nuclear processes. First, the double stranded nature of DNA creates a special set of problems. During replication and transcription strand unwinding is required and implies topological problems due to overwinding-compensations (Nitiss, 2009). Apart from supercoiling, a second topological aspect is the relationship between separate DNA segments. Intramolecular knots (formed within the same DNA molecule) can be generated, as well as intermolecular tangles (formed between sister chromatids), that are produced as a consequence of DNA replication (Deweese & Osheroff, 2009).

DNA topoisomerases are enzymes that solve these topological problems by introducing transient breaks in DNA. All topoisomerases initiate DNA cleavage by the nucleophilic attack of a catalytic tyrosine residue on the phosphate of the nucleic acid backbone. This transesterification reaction results in the formation of a covalent tyrosine-nucleic acid bond that links the protein to a newly generated terminus of the DNA (Deweese & Osheroff, 2009). These catalytic intermediates are denominated cleavage complexes. The reverse religation reactions are carried out by attacks of the deoxyribose hydroxyl ends towards the tyrosylphosphodiester bond (Pommier et al., 2014).

There are two major types of toposiomerases, type I and type II, and each of them can be subdivided in subfamilies A and B. Main differences between both types are the number of DNA strands that are cleaved and the mechanism of action. Type I topoisomerases are monomeric and do not require high-energy cofactor. They change topology by generating single-stranded breaks, following by passage of the opposite intact strand through the break (type IA) or by controlled rotation of the helix around the break (type IB). Type IA topoisomerases require divalent metal ions for cleaving and are covalently bound to the 5′ phosphate end. On the other hand, type IB topoisomerases do not need divalent metal ions and covalently attach to the 3′ end. Due to this single-stranded cleavage mechanism, type I topoisomerases are able to modulate

under and overwinding, however they cannot deal with knot or tangles in duplex DNA. In contrast, type II topoisomerases act as homodimers and need divalent metal ions and ATP for their function. They covalently attach to 5′ ends and generate a transient DSB to carry out the passage of another duplex DNA. Because of their reaction mechanism, they are able to modulate DNA supercoiling as well as to remove DNA knots and tangles (Deweese & Osheroff, 2009).

Human cells express six different topoisomerases that have redundant and specific functions involved in replication, transcription, chromosomal segregation and DNA repair: TOP1 and TOP1mt (type IB), the two isoforms of type IIA topoisomerases, α and β (termed TOP2α and TOP2β), TOP3α and TOP3β (type IA). Additionally, the meiotic recombination protein SPO11 is a specialized type IIB topoisomerase (Pommier, Sun, Huang, & Nitiss, 2016). Studies in transgenic mouse models of topoisomerase dysfunction have highlighted the biological relevance of these enzymes. Deletions in *Top1*, *Top2α* or *Top3α* in mice confers embryonic lethality, and *Top2β* or *Top3β*-mutant mice die shortly after birth (Akimitsu et al., 2003; Kwan & Wang, 2001; W. Li, Wang, & Wang, 1998; Morham, Kluckman, Voulomanos, & Smithies, 1996). Consistent with its specific role in meiosis, SPO11 loss results in viable, but infertile mice (Keeney, 2008).

Although isoforms TOP2α and TOP2β share 77% of identity (Linka et al., 2007), they show different patterns of expression and distinct cellular functions. TOP2α has a role in DNA replication and chromosome segregation. Consistently, it is essential for the survival of proliferating cells, and is highly expressed during G2/M phases of cell cycle. In contrast, TOP2β expression can be found throughout the cell cycle, although it is predominantly expressed in post mitotic cells (Deweese & Osheroff, 2009). TOP2β is generally involved in managing DNA supercoiling that accumulates ahead and behind the transcription machinery (J. L. Nitiss, 2009a) but also is specifically implied in the transcriptional regulation of genes that respond to hormones (Ju et al., 2006), that are more long than 200 kb (King et al., 2013), and in neural development and function (Isik et al., 2015) (Madabhushi et al., 2015). Strikingly, as mentioned above, in some of these studies it was proposed that DSBs generated by TOP2 trigger transcriptional activation.

3.1 Topoisomerase 2 and DSBs

Despite the fact that TOP2 functions are essential, their action is intrinsically dangerous because of the potential generation of DSBs. Therefore, TOP2 activity is a double-edged sword that requires a carefully controlled regulation to maintain genome integrity (Deweese & Osheroff, 2009).

Although TOP2 acts globally along the genome, there are significant preferred sites for cleavage. However, these consensus sequences are not completely successful at predicting new cleavage sites and many sites of action do not conform to it (Capranico & Binaschi, 1998). Most likely, the specificity of topoisomerase II-mediated cleavage is determined by the local structure, flexibility, or malleability of the DNA that accompanies the sequence, instead of by a direct recognition of the bases that comprise that sequence (Vélez-Cruz et al., 2005).

During the catalytic cycle of TOP2 (Figure 10), first the TOP2 homodimer binds to an intact segment, the G (gate) segment, and then captures another DNA region, the T (transported) segment. Upon ATP binding, TOP2 undergoes a conformational change from open to a closed clamp form. In the presence of Mg^{2+}, a tyrosine from each TOP2 monomer attacks a DNA phosphodiester bond four base apart on opposite strand of the G duplex and becomes covalently linked to the 5′ end of the broken DNA. It also generates a 3′ hydroxyl moiety on the opposite terminus of the cleaved strand, resulting in the cleavage complex intermediate. The T segment is then transferred through the gap generated. After this, the T segment is released from the clamp and the broken ends of the G segment are resealed. Finally, the G segment is released upon ATP hydrolysis that converts the complex back to its open clamp form, leaving the DNA product chemically unchanged from the initial form. Only the topological properties of the double helix are altered by the action of topoisomerase II (Deweese & Osheroff, 2009; J. L. Nitiss, 2009a; Pommier, Leo, Zhang, & Marchand, 2010).

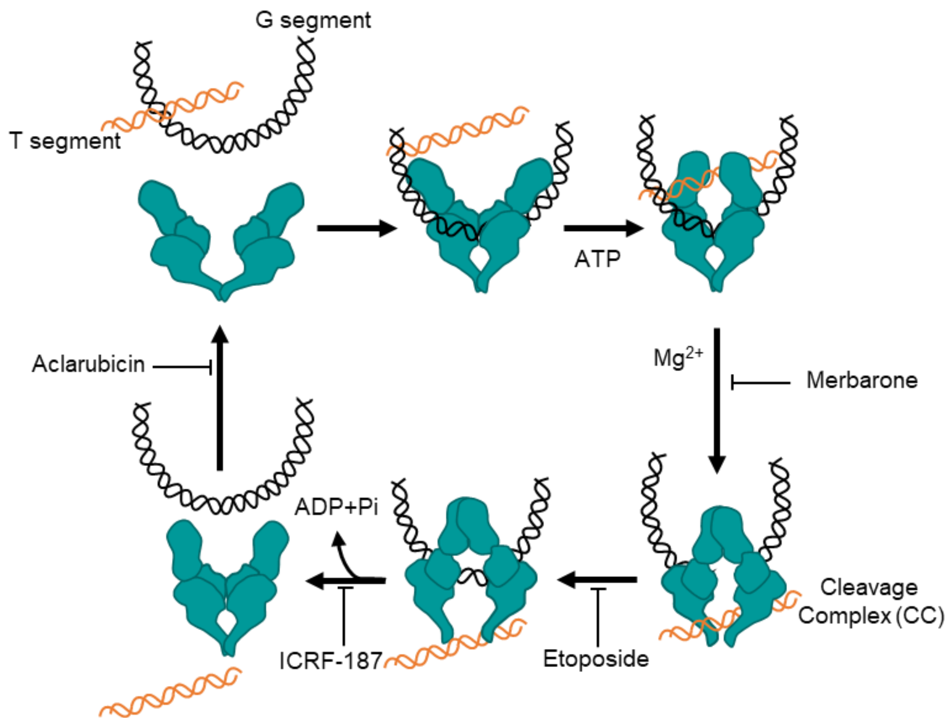

Figure 10: Catalytic cycle of Topoisomerase 2. The TOP2 homodimer binds to the G segment (black), bending it. In the open clamp conformation, it interacts with a second DNA segment (T segment, in orange). When the T segment enters the N-gate, the clamp closes and the G segment is cleaved, forming the TOP2 cleavage complex (TOP2cc) in which a phosphotyrosine bond links each 5' strand and a tyrosine of each TOP2 subunit. Then the DNA-gate is opened and the T segment passes through to the central cavity. Then, the DNA-gate closes and the G segment is religated. The exit C-gate opens to release the T segment and the G segment can either be released, or undergo an additional catalytic cycle. TOP2 can be inhibited or poisoned at several different points in the enzyme reaction cycle. First, aclarubicin prevents the binding of TOP2 to DNA. Merbarone inhibits DNA cleavage by TOP2, while etoposide prevents the resealing of DNA, stabilizing TOP2cc. Finally, ICRF-187 inhibits both ATP hydrolysis and maintains the TOP2 structure as a closed clamp.

TOP2 cleavage complexes (TOP2cc) are key intermediate that normally are short-lived and easily reversible, the DNA cleavage/ligation equilibrium of the enzyme highly favouring rejoining. Nevertheless, TOP2cc can be stabilized due to the presence of nearby lesions in DNA or the exposure to chemicals termed TOP2-poisons (Deweese & Osheroff, 2009; J. L. Nitiss, 2009b). Stable TOP2cc act as impediments for elongating RNA and DNA polymerases. This collision between TOP2cc and replication or transcription machinery triggers the degradation of TOP2cc by the 26S proteasome (Figure 11) (Mao, Desai, Ting, Hwang, & Liu, 2001; A. Zhang et al., 2006). Due to TOP2

degradation, an irreversible DSB is generated, which is characterized by a residual peptide adduct that remains covalently bound to the 5'end of the DNA through the tyrosyl-phosphodiester bond. Thus, these DSBs are blocked, and they require to be unblocked or processed to allow repair. Regardless of end structure, TOP2-DSBs rapidly elicit DNA damage responses such as phosphorylation of ATM and activation of downstream damage responses in an identical way compared to DSBs generated by IR. Therefore, γH2AX modification spreads away into chromatin adjacent to the DSB and all downstream events are also common. (J. L. Nitiss, 2009b; Sunter, Cowell, Willmore, Watters, & Austin, 2010).

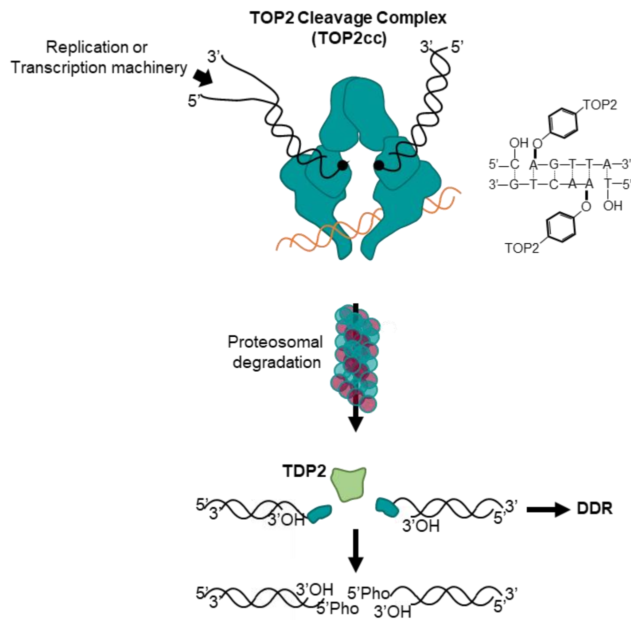

Figure 11: Induction of DSBs by the abortive activity of Topoisomerase 2. If ongoing replication or transcription encounters a TOP2cc, TOP2 homodimer is signalled and degraded by the 26 proteasome. Degradation of TOP2cc leads to the formation of an irreversible DSB characterized by peptide adducts covalently bound to 5' DNA ends through a phosphotyrosine bond, leading to the activation of the DNA damage response. TDP2 promotes the unblocking of 5' phosphotyrosines, converting them into clean 5' phosphate and 3' hydroxyl DSBs.

As TOP2 activity levels are greater in proliferating cells than in their quiescent counterparts (Heck, Hittelman, & Earnshaw, 1988), TOP2-targeting agents have been

commonly used as antineoplastic drugs in the treatment of a broad range of tumours (J. L. Delgado, Hsieh, Chan, & Hiasa, 2018). There are two classes of TOP2-targeting agents. The first class comprises compounds termed TOP2 poisons, which are most of the clinically active agents and operate by increasing the levels of TOP2cc. These agents poison TOP2 by two different mechanisms. Etoposide (VP-16), teniposide (VM-26), and the DNA intercalators doxorubicin, daunorubicin, amsacrine (m-AMSA), and TAS stabilize TOP2cc by inhibiting the DNA relegation step of TOP2 catalytic cycle. On the other hand, quinolone CP-115,953, ellipticines and azatoxins enhance the formation of TOP2cc by stimulating DNA cleavage. A second class of compounds inhibits TOP2 catalytic activity but does not generate an increase in the levels of TOP2cc complexes. DNA intercalators can also act as TOP2 catalytic inhibitors when used at drug concentrations that modify DNA structure, thereby avoiding TOP2 binding to DNA or forming TOP2cc. Other molecules prevent TOP2 activity by inhibiting ATP hydrolysis, such as merbarone and bisdioxopiperazines (ICRF 159, 187 [dexrazoxane], and 193) which generate "closed clamps" without trapping TOP2cc (Pommier et al., 2010). Bisdioxopiperazines are considered the only TOP2 catalytic inhibitors that are specific, as compounds do not induce DNA damage response after short-term exposure. Nevertheless, they could trigger this signalling cascade following long-term exposure. Notably, catalytic inhibitors of TOP2 antagonize the toxicity of TOP2 poisons, indicating that the mechanism of action is different and the main toxicity of poisons derives from the formation of DSBs and not from a lack of TOP2 activity (J. L. Nitiss, 2009b).

3.2 Tyrosyl-phoshodiestesarse 2 (TDP2)

Tyrosyl DNA phosphodiesterase 1 (TDP1), was the first enzyme found that shows robust activity against 3′ phosphotyrosyl linkage generated by type IB topoisomerases (Pouliot, Yao, Robertson, & Nash, 1999). In yeast, Tdp1 can also remove the 5′phosphotyrosyl-linked peptides derive from the proteolytic degradation of Top2 (K. C. Nitiss, Malik, He, White, & Nitiss, 2006), but the capacity of its mammalian

counterpart was controversial (Interthal et al., 2005; Murai et al., 2012). It took additional 10 years after Tdp1 discovery to identify a second tyrosyl DNA phosphodiesterase, Tdp2, with activity against 5′phosphotyrosyl linkages (Cortés-Ledesma et al; 2009). In higher eukaryotes, TDP2 is the only known enzyme with the physiological capacity to process the 5′phosphotyrosyl bond between DNA and TOP2 peptide, converting the ends into 5′ phosphate/3′ hydroxyl ligatable termini (Gómez-Herreros et al., 2013; Cortés-Ledesma et al., 2009; Zeng, Cortés-Ledesma, El Khamisy, & Caldecott, 2011).

Structural and biochemical studies indicate that TDP2 is closely related to the AP endonuclease APE1 and the superfamily of Mg^{2+}/Mn^{2+}-dependent phosphodiesterases (R. Gao, Huang, Marchand, & Pommier, 2012; Rodrigues-Lima, Josephs, Katan, & Cassinat, 2001; Schellenberg et al., 2012). Human TDP2 shows a molecular mass of 41 kDa with two domains. The C-terminus harbours its catalytic domain, whereas N-terminal domain bears an ubiquitin-associated (UBA) domain, which probably has a regulatory function (Schellenberg et al., 2012). TDP2 is considered a multitask protein as it was previously known as a factor involved in other processes beyond DNA repair. Indeed, it was also termed TTRAP (TRAF and TNF receptor-associated protein), due to its possible function as regulatory factor that is involved in signal transduction by distinct members of the TNF receptor family (Pype et al., 2000), and EAPII (ETS1-associated protein 2), as it was identified as an interactor of the ETS1-transcription factor (C. Li et al., 2011; Pei et al., 2003). Additionally, TDP2 has been related with viral infection. First, it was shown that TDP2 interacts with HIV-1 integrase and facilitate lentiviral vector integration, however it remains to be determined if this role of TDP2 depends on its catalytic activity (J. qi Zhang et al., 2009). On the other hand, TDP2 has been involved in Hepatitis B infection. Specifically, TDP2 releases the viral polymerase (P protein) that is attached to relaxed circular DNA through a tyrosyl–DNA phosphodiester, which is an essential event for the formation of covalently closed circular (ccc) DNA, a viral persistence reservoir of Hepatitis B (Königer et al., 2014). However, although this TDP2 activity has been confirmed, TDP2 depletion has

insignificant effect on Hepatitis B infection *in vivo* (Xiuji Cui et al., 2015). Similarly, TDP2 is able to remove small viral protein (VPg) that act as a primer for viral RNA synthesis during picornavirus infection. VPg is linked to nascent viral RNAs via an O4-(5′-uridylyl)tyrosine bond, and its removal though VPg unlinkase activity is essential during viral replication (Virgen-Slane et al., 2012).

Besides these additional roles of TDP2, it seems that its principal function is the repair of DSBs generated by the aberrant action of TOP2. Its mechanism of action has been dissected in an extensive work (Gómez-Herreros et al., 2013, 2014, 2017; Ledesma et al., 2009; Schellenberg et al., 2017; Zeng et al., 2011). First, in standard activity assays employing dsDNA with 5′ phosphotyrosyl termini, extracts from TDP2 deleted DT40 or murine cells did not show any residual 5′TDP activity, suggesting that TDP2 is the major if not the only 5′-TDP activity in vertebrates, at physiologically relevant enzyme concentrations at least. Consistent with *in vitro* results, TDP2 deleted DT40 and murine cells and TDP2-depleted human cells show hypersentitivity to TOP2-induced damage, but not to other types of DNA damage such IR, methylmethane sulphonate or camptothecin. The observed hypersensitivity to TOP2 damage correlates with a defect in the repair of etoposide-induced DSBs in *Tdp2*$^{-/-}$ primary mouse embryonic fibroblasts (MEFs), which suggests that TDP2-mediated repair promotes tolerance to TOP2-induced DNA damage in mammalian cells. Based on the epistatic relation between KU70 loss and TDP2 deficiency in promoting survival upon etoposide exposure, it has been suggested that TDP2 participates in the NHEJ repair pathway. Further work identified a new TDP2-dependent DNA repair pathway that involves and requires TOP2 sumoylation and is independent of proteosome activity (Schellenberg et al., 2017). In this process, TDP2 interacts with ZATT, a SUMOE3/E4 ligase/elongase that promotes sumoylation of TOP2ccs and their remodelling to allow access of TDP2 phosphodiesterase activity. This TDP2-dependent pathway seems to be the major if not the only mechanism for removal of TOP2cc in the absence of proteasome activity. The relative contribution of proteolytic and non-proteolytic pathways in the processing of

TOPccs and how cells choose which mechanisms to deal with DNA damage due to abortive TOP2 activity remain to be figured out.

It seems that the tolerance to TOP2-induced damage is not specific to *ex vivo* cell cultures, but also at the whole-organism level, as etoposide administration to *Tdp2*$^{-/-}$ mice results in both increased mortality due to intestinal damage, and elevated toxicity in lymphoid tissue, which are established *in vivo* targets of etoposide. Despite the fact that TDP2 is a critical factor in the cellular and physiological response to TOP2 poisons, results from *Tdp2*$^{-/-}$ mice suggest that alternative TDP2-independent mechanisms of DSB repair are enough to cope with the endogenous level of TOP2 damage that arise during normal mouse development and life. In this regard, TDP2 deficient cells still repair a significant fraction of etoposide-induced DSBs, supporting again that there are TDP2-independent mechanisms to process TOP2-linked termini, although they most likely do so at expense of increasing genetic instability (Gómez-Herreros et al., 2013; Zeng et al., 2011). Consistent with this idea, further studies demonstrated that TDP2 suppresses chromosome rearrangements induced by TOP2 and reduces TOP2-induced chromosome translocations that arise during gene transcription (Gómez-Herreros et al., 2017).

In contrast to the normal development of TDP2 deficient mice, TDP2 homozygous mutations that cause an inactivation of the protein were found in patients that developed neurological disease characterized by cognitive defects, seizures and progressive ataxia (Gómez-Herreros et al., 2014). This suggests that TDP2 contributes to counteract the endogenous threat posed by TOP2 activity during neuronal development and maintenance in humans.

4. Regulation of end-processing during NHEJ

Although NHEJ is considered a single pathway, different factors are needed depending on the different DNA end configurations of the DSB and the regulation of the action of these factors in each situation is key to ensure an efficient and accurate repair outcome

(Pannunzio, Watanabe, & Lieber, 2018). As explained above, DSBs can harbour different structures at the ends and must be restored to conventional 5′ phosphate and 3′ hydroxyl ligatabe termini either by unblocking or end-processing, and the choice between both pathways is relevant. If complex ends are unblocked by a specific enzymatic activity, repair is more likely to be accurate, while ends that need processing may involve nucleotide loss or gain, entailing sequence modification. Thus, there must be a precise repair regulation to distinguish between clean and complex ends and, among the latter ones, ends that can be unblocked from ends that require nucleolytic processing. In this regard, how cells minimize and modulate processing to maintain genome integrity is a key question in the field.

4.1. NHEJ: an iterative vs a hierarchical process

It is worth noting that there is some controversy about how NHEJ accessory factors operate. On one hand, it has been proposed that they all operate in an iterative way without an established order. This model highlights the flexibility of NHEJ and explains the diversity of repair products generated for the same type of DSB induced. This is supported by different studies that claim that different routes of DNA end processing can give rise to a ligatable joint. This iterative nature of NHEJ implies that multiple components can act on the same DSB during numerous rounds of processing and the involvement of factors is not mutually exclusive to the usage of other ones, all of them remaining active as long as the DSB continues unrepaired (Gu et al., 2010; Gu & Lieber, 2008; Lieber, 2008).

On the other hand, it has been proposed that there is a hierarchy by which cells give precedence to resolution paths with the fewest number of enzymatic steps. This way, direct ligation (one step) is favoured over synthesis and ligation (two steps), which is favoured over more complex paths that include end-processing (Waters et al., 2014). Consequently, the first step would be the attempted formation of a close configuration by LIG4 and, if it is not achieved, the open-configuration of the complex LIG4-substrate would be recognised by end processing factors. This mechanism is permitted by the

tethering of LIG4 to the paired-end complex even in its open configuration (Waters et al., 2014). Consistent with this idea, LIG4 is the most flexible ligase known, and it is also able to ligate one strand when the other one harbours a complex configuration (Gu et al., 2007; Ma et al., 2004). In this regard, further work demonstrated that differences in how LIG4 catalytic domains interact with different end structures trigger dramatic changes in the dynamics of the entire NHEJ complex, determining the steps taken to complete repair (Conlin et al., 2017). This remodelling of end alignment carried out by LIG4 would be essential to the proficiency of NHEJ in repairing DSBs with complex ends. Therefore, apart from its catalytic role, LIG4 helps to modulate and decide the repair path that is more appropriate for the given end structure (Conlin et al., 2017). Accordingly, both X family polymerases (Tseng & Tomkinson, 2002) and ARTEMIS nuclease (Malu et al., 2012) directly interact with LIG4.

A hierarchical order in the action of NHEJ components is also supported by the formation of a synapsis with two different stages. First, DNA ends are initially tethered in a long-range complex, formed by Ku and DNA-PKcs, in which DNA ends are held sufficiently far apart. Then, the ends are closely aligned. This conversion of the long-range to the short-range synaptic complex is mediated by the activity of DNA-PK, XLF and LIG4-XRCC4 complex, however the catalytic function of LIG4 is not required (Graham, Walter, & Loparo, 2016). These findings suggest that this structural transition in end bridging can be coordinated with end-processing. Accordingly, DNA-PKcs phosphorylations at different clusters induce conformational changes that regulate end-processing (see below), which could be also involved in this conformational transition observed in the two-step synaptic complex (Graham et al., 2016). Consistent with this idea, XRCC4-LIG4 complex, and XLF are also reported to be involved in DNA-PKcs phosphorylation and some sorts of end trimming (Akopiants et al., 2009; Cottarel et al., 2013; J. W. Lee, Yannone, Chen, & Povirk, 2003).

Although both models could seem contradictory, they may not be mutually exclusive. While, NHEJ could behave as an iterative process in which various components can be loaded and act in various combinations without an established order, providing

flexibility and efficiency to the repair process, the decision whether and how to repair complex ends by unblocking or end-procesing should not be stochastically determined. In this context, different mechanisms that inhibit or delay the action of nucleases during NHEJ have been reported, that could give time to other factors to resolve complex ends without processing, promoting the maintenance of genome integrity. These mechanisms are fine-tuned regulated, as will be explained below, by the action of PI3 Kinase related protein kinases DNA-PKcs and ATM.

4.2. Nucleases in NHEJ

Under certain circumstances, such as the presence of complex lesions, DSBs require end-processing by the action of nucleases before ligation. Usually, these nucleases remove chemical modifications and blockages or cleave mismatched ends by trimming 5′ or 3′ termini through exo or endonucleolytic processing to expose short regions of microhomology between strands and promote end joining (Pannunzio et al., 2018).

When NHEJ requires end-processing, ARTEMIS is recruited (Goodarzi et al., 2006; Ma, Pannicke, Schwarz, & Lieber, 2002; Yannone et al., 2008), which is considered as the major nuclease implicated in end-processing during NHEJ. Its main activity is hairpin opening during V(D)J recombination, and this endonucleolytic function has been well dissected, which is promoted by the phosphorylation at the ABCDE cluster of DNA-PKcs. However, it also shows intrinsic 5′ exonuclease activity on ssDNA, which is DNA-Pkcs independent (S. Li et al., 2014; Pawelczak & Turchi, 2010). Its contribution in NHEJ is still being studied, recent analysis demonstrated that the ARTEMIS-DNA-PKcs complex also promotes the ligation of incompatible overhangs *in vitro*. Specifically, Artemis was reported to preferentially cut at the ss-dsDNA boundary in the case of 5′ overhangs, on the contrary, when processing 3′ overhangs and DNA hairpins, Artemis leaves a 4-nt 3′ overhang (Chang et al., 2016; Pannunzio et al., 2018). Besides this versatility to act at many different types of ends, there is a common feature in all substrates: a ss-dsDNA boundary, which is present in a wide variety of different DNA end configurations, such as 5′ and 3′ overhangs, hairpins, gaps, loops, blunt ends

in an open state and bubbles that may arise due to mismatches between the two DNA ends being joined (Chang & Lieber, 2016; Chang, Watanabe, & Lieber, 2015). On the other hand, as previously mentioned, C-terminal domain of Artemis interacts with the N-terminal region of LIG4 (De Ioannes, Malu, Cortes, & Aggarwal, 2012; Malu et al., 2012; Ochi, Gu, & Blundell, 2013). Interestingly, a 3′ endonuclease activity of ARTEMIS independent of DNA-PKcs has been recently described which is promoted by XRCC4-LIG4 complex (Gerodimos, Chang, Watanabe, & Lieber, 2017). The stimulation of this activity could be as a result of a conformational change due to the interaction with LIG4 (Pannunzio et al., 2018).

Another factor involved in the repair of complex ends requiring end-processing is the MRE11 protein from the MRN complex (consisting of MRE11, RAD50 and NBS1). The MRN complex acts as a sensor of DSB and promotes repair by NHEJ or HR. Specifically, MRE11 exhibits 3′-5′exonuclease and single-stranded and DNA hairpin endonuclease activities (Lisby, Barlow, Burgess, & Rothstein, 2004; Paull & Gellert, 1998; Stracker & Petrini, 2011; Trujillo et al., 2003; Williams et al., 2011). Inhibitors which primary inhibit exonuclease activity (MIRIN and PFM39) or endonuclease activity (PFM01 and PFM03) of the complex have been developed (Dupré et al., 2008; Shibata et al., 2014). Experiments performed with these compounds suggest that MRE11 endonuclease activity is required to initiate resection, followed by MRE11 exonuclease activity and EXO1/BLM bidirectional resection, which commits to HR (Shibata et al., 2014). Additionally, the endonucleolytic cleavage may be of particular importance for DNA ends covalently-bound to Spo11 (Neale, Pan, & Keeney, 2005), terminated by hairpins (Lobachev, Gordenin, & Resnick, 2002) or generated by TOP1 and 2 poisons (Hartsuiker, Neale, & Carr, 2009; Hoa et al., 2016a; Quennet, Beucher, Barton, Takeda, & Löbrich, 2011). In particular, a recent study showed the repair of etopside-induced TOP2cc is compromised by MRE11 deficient cells. These cells showed accumulations of high levels of TOP2cc even in the absence of exogenous damage. Furthermore, the genome instability and mortality observed in MRE11 deficient cells were significantly reversed upon TDP2 overexpression, suggesting that MRE11 could have a role in

removing lesions from TOP2 abortive activity (Hoa et al., 2016a). Furthermore, recent *in vitro* studies described that NBS1 is essential to promote MRE11 nuclease activities on DNA ends containing protein adducts, while it inhibits MRE11 3′ to 5′ exonuclease degradation of clean ends. Additionally, the function of the MRN complex is further stimulated by the phosphorylated form of CtIP (Deshpande, Lee, Arora, & Paull, 2016a). This endonuclease is consider a relevant factor in the regulation of end-processing, not only stimulating the MRN complex but also the long range resection by BLM and DNA2 during HR (James M. Daley et al., 2017).

4.3. End-protecting factors

On the other hand, factors that restrict or inhibit resection are involved in NHEJ to avoid an excessive degradation of DNA ends. In this regard, modifications at the chromatin flanking DSB, such as for example γH2AX (Helmink et al., 2011), and the subsequent recruitment of downstream factors, such as MDC1, 53BP1 and BRCA1 (Bekker-Jensen & Mailand, 2010) are reported to be crucial events for the choice of repair pathway, regulating to which extent ends are processed. Accordingly, H2AX deficient mice show an increase in genome instability and, in the absence of P53, are prone to tumour development (Craig H. Bassing et al., 2003; Celeste et al., 2003, 2002). Indeed, in Artemis deficient cells, H2AX was reported to limit CtIP-dependent end-processing upon induction of blocked DSBs during V(D)J recombination (Helmink et al., 2011); this function of H2AX being mediated by MDC1. Specifically, it was proposed that MCD1 binds to NBS1, inhibiting the CtIP-NBS1 interaction required for resection (J. Ross Chapman & Jackson, 2008; You & Bailis, 2010). In addition, 53BP1 is also reported to regulate end-processing during V(D)J and CSR recombination (Bothmer et al., 2010; Difilippantonio et al., 2008) and to inhibit CtIP-dependent resection in Brca1 deficient cells at post-replicative stages of cell cycle, suggesting that γH2AX may restrict resection by the recruitment of 53BP1 (Bunting et al., 2010). Since 53BP1 does not show enzymatic activity, its role in protection of DNA ends has been proposed to be mediated by downstream factors such as PTIP (Munoz, Jowsey, Toth, & Rouse, 2007) and RIF1 (J.

Ross Chapman et al., 2013; Escribano-Díaz et al., 2013; Zimmermann, Lottersberger, Buonomo, Sfeir, & De Lange, 2013). Both are also adaptor proteins involved in restricting end resection independently of each other (Callen et al., 2013). In this context, the recent discovered shieldin complex, which shows single-stranded DNA-binding activity, has been proposed to act as ultimate effector of the 53BP-RIF1 pathway for end protection (Dev et al., 2018; S. Gao et al., 2018; Noordermeer et al., 2018). On the other hand, ARTEMIS was identified as a PTIP-binding protein, and strikingly, as one of main downstream effectors of 53BP1-PTIP pathway (Jiadong Wang et al., 2014). This way, 53BP1 would be promoting limited end-trimming and the repair of DSBs through NHEJ, and therefore directly competing with the HR repair pathway that would entail long resection.

Besides the role of 53BP1 through downstream interacting factors, it interacts with a protein called DYNLL1 (Rapali et al., 2011), which partially mediates the oligomerization of 53BP1 (Becker et al., 2018), required for its recruitment to chromatin flanking DSBs (Fradet-Turcotte et al., 2013; Zgheib, Pataky, Brugger, & Halazonetis, 2008). DYNLL1 was also proposed to interact with the MRN complex and to inhibit its nuclease activity (He et al., 2018). Nevertheless, it is not known how these different 53BP1 pathways functionally interact, which would give clues into understanding the regulation of end-processing.

4.4. DNA-PKcs, a master regulator of end-access

DNA-PKcs, with a molecular weight of approximately 460 kDa, is one of the largest kinases within the PI3K-like kinase family (Dobbs, Tainer, & Lees-Miller, 2010). Despite not being conserved in lower eukaryotes, its enzymatic activity is a clear requisite for its function during NHEJ in mammalian cells (Kienker, 2000; Kurimasa et al., 2015). Because of this, significant efforts have been made to determine functionally crucial targets of DNA-PKcs. Despite the long list of factors that are excellent *in vitro* and *in vivo* substrates of DNA-Pkcs, mutational analysis conclude that their phosphorylations are not functionally relevant, at least for NHEJ (Pauline Douglas, Gupta, Morrice, Meek,

& Lees-Miller, 2005; Goodarzi et al., 2006; K. J. Lee, Jovanovic, Udayakumar, Bladen, & Dynan, 2004; Katheryn Meek, Dang, & Lees-Miller, 2008), being DNA-PKcs itself the only NHEJ factor that has shown to be a functionally relevant target of its own kinase activity (Chan et al., 2002; X. Cui et al., 2005; Ding et al., 2003; P. Douglas et al., 2007; K. Meek, Douglas, Cui, Ding, & Lees-Miller, 2007; Katheryn Meek et al., 2008; Soubeyrand, Pope, Pakuts, & Haché, 2003). As mentioned above, during the initiation of NHEJ, DNA-Pkcs is recruited to broken ends by KU bound DNA. In the absence of DNA-PKcs, KU interacts with the extreme termini of DNA ends. In the presence of DNA-PKcs, KU translocates inward and DNA-PKcs has direct contacts with ~10 bp at the terminus of DNA ends (Meek et al., 2008).

The most well-accepted consequence of DNA-PKcs autophosphorylation is its inactivation and dissociation from DNA end-bound KU, allowing the joining of DNA ends by LIG4 (Chan & Lees-Miller, 1996; Pauline Douglas, Moorhead, Ye, & Lees-Miller, 2001). Despite the fact that DNA end binding by DNA-PK is indifferent to distinct DNA end structures, some studies indicate that DNA ends with canonical 5′ phosphate and 3′hydroxyl termini are required for its autophosphorylation (Pawelczak, Andrews, & Turchi, 2005; Turchi, 2002). It has been suggested that kinase activation occurs in trans, linking autophosphorylation of DNA-Pkcs to synapsis. Although this point is still a matter of debate, this may provide an important mechanism by which DNA-PKcs protects DNA-ends to maintain genomic integrity.

However, extensive studies have shown that DNA-PKcs can be autophosphorylated, as well as phosphorylated by other kinases, in many residues upon DNA-damage, and each event has specific consequences (Davis, Chen, & Chen, 2014; Katheryn Meek et al., 2008). Clusters ABCDE, flanking Thr2609, and PQR, around the Ser2056 on human DNA-PKcs, are the two major phosphorylation clusters with a clear relevant function (Block et al., 2004; X. Cui et al., 2005; Ding et al., 2003; K. Meek et al., 2007; Reddy, Ding, Lees-Miller, Meek, & Ramsden, 2004). Although both clusters can be autophosphorylated by DNA-PKcs itself, but the ABCDE cluster is also phosphorylated by ATM or ATR under different cellular stresses (B. P. C. Chen et al., 2007; Davis, So, &

Chen, 2010; Katheryn Meek et al., 2008). Mutagenesis studies revealed that the specific defect imparted by blocking either ABCDE or PQR phosphorylation is dysregulated end processing. Both clusters show antagonistic functions, whereas phosphorylation of sites within the ABCDE cluster promotes end processing, phosphorylation of sites within the PQR cluster inhibits end-resection. Specifically, the ABCDE cluster is reported to promote end-processing by regulating the access of ARTEMIS to the ends (X. Cui et al., 2005; Goodarzi et al., 2006; Ma et al., 2002; Yannone et al., 2008).

Additionally, different mice have been generated as animal models of DNA-PKcs deficiency and their characterizations have also given important clues of its function in regulating end-processing and ligation (Figure 12). First of all, severe combined immunodeficiency (SCID) mice were found to homozygously express DNA-PKcs with a nonsense mutation at amino acid position 4046 (Araki et al., 1997; Blunt et al., 1996; Danska, Holland, Mariathasan, Williams, & Guidos, 1996). This mutation results in a truncated protein lacking the extreme C-terminal region which is expressed at very low levels with a dramatically impaired kinase activity (Beamish, 2000). Few years later, mice lacking DNA-PKcs (*DNA-Pkcs*$^{-/-}$) were generated. This mice are viable, with no overt phenotype other than SCID and radiosensitivity (Taccioli et al., 1998). In contrast, recent studies demonstrated that mice expressing catalytically inactive DNA-PKcs (*DNA-Pkcs*$^{KD/KD}$) show embryonic lethality (Jiang et al., 2015). Specifically, during V(D)J recombination, *DNA-Pkcs*$^{-/-}$ B cells cannot form CJs owing to hairpin-opening defects, but form SJs efficiently. This indicates that the presence of DNA-PKcs is only required for end-processing and not for end-ligation. In contrast, *DNA-Pkcs*$^{KD/KD}$ B cells accumulate SE-CE fragments, indicating severe ligation defects. This suggests that autophosphorylation of DNA-PKcs is required strictly for end-ligation, possibly by removing the physical blockage imposed by DNA-PKcs. However, *DNA-Pkcs*$^{KD/KD}$ B cells, although showing hairpin-opening, it is abolished upon the inhibition of ATM, suggesting that ATM could be phosphorylating DNA-PKcs in a redundant manner to allow the action of ARTEMIS.

On the other hand, alanine substitution at three of the five potential phosphorylation sites within the murine ABCDE cluster (referred to as the 3A allele) does not block hairpin opening, but this becomes hypersensitive to both ATM and DNA-PK kinase inhibitors. Notably, *DNA-Pkcs$^{3A/3A}$* mice developed bone marrow failure, are hypersensitive to DNA cross-linking agents and are defective in both homologous recombination and the Fanconi anemia DNA damage response pathways (S. Zhang et al., 2011), further supporting that the ABCDE cluster might regulate end-processing.

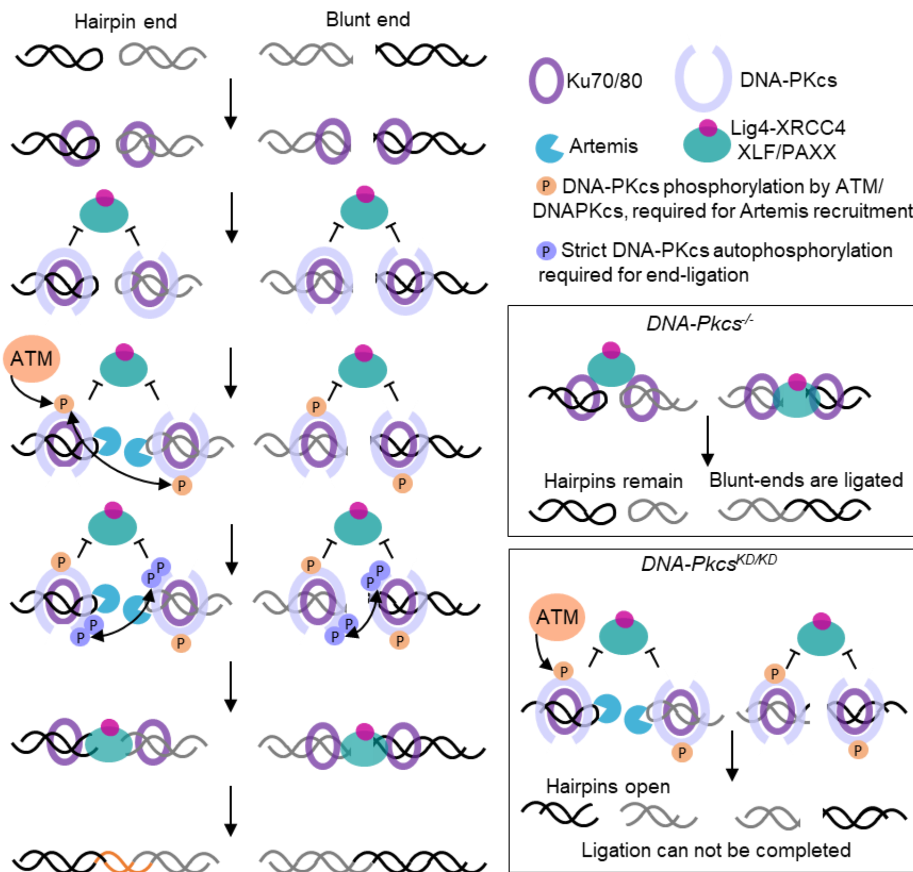

Figure 12: DNA-PKcs phosphorylations regulate end-ligation and end-processing. Normal vertebrate NHEJ mechanism during V(D)J recombination is illustrated (left) and in the context of *DNA-Pkcs$^{-/-}$* or *DNA-PKcs$^{KD/KD}$* (right). In this model is highlighted two kinds of DNA-PKcs phosphorylation: orange, ATM-mediated phosphorylation of DNA-PKcs; and blue, strict auto-phosphorylation of DNA-PKcs. First, the orange one is required for ARTEMIS endonuclease recruitment and subsequent end-processing. Second, the blue one indicates DNA-PKcs autophosphorylation required for end-ligation, (continue on the next page…)

(…continued) possibly by DNA-PKcs dissociation from DNA ends or by structure modification that allows joining by LIG4-XRCC4-XLF complex.

Together all these data suggest that distinct DNA-PKcs phosphorylations are required for end-processing and end-ligation. Phosphorylation events required in end-processing may be carried out either by ATM or DNA-PKcs itself at ABCDE cluster, while end-ligation requires a strict DNA-Pkcs autophosphorylation, possibly in PQR cluster, which is promoted by ligatable ends and synapsis. This way, possible unsuccessful ligation attempts are avoided. Thus, DNA-Pkcs can be considered a molecular shift that coordinates end-processing and ligation through its phosphorylation to maximize the efficiency of NHEJ pathway.

4.5. ATM, a key factor to orchestrate an efficient and accurate repair of DSBs with complex ends

As mentioned above, ATM is best known for its function as an apical activator of the DDR in response to DSBs (McKinnon, 2004). Loss-of-function mutations in ATM result in Ataxia telangiectasia (A-T), which is an autosomal-recessive neurodegenerative disease that manifests in early childhood. The prominent neurological sign of A-T is an inexorable loss of cerebellar function, leading to progressive dysarthria (speech defects) and choreoathetosis (abnormal body movements). In addition to this hallmark neurodegeneration, there are a number of other features that typify this debilitating disease. These include telangiectasia (dilated blood vessels), immune dysfunction, sterility, radiosensitivity and cancer predisposition (evidenced by an increased susceptibility to lymphoma and lymphocytic leukemia). Additionally, there are other diseases that share some symptoms with A-T, and which are caused by loss-of-function in other proteins involved in the DDR, such as Nijmegen breakage syndrome, ataxia telangiectasia-like disorder, etc. (McKinnon & Caldecott, 2007). Based on all of this, it is generally accepted that A-T symptoms result from an accumulation of unrepaired/misrepaired DSBs. However, ATM loss does not result in obvious DSB-

repair defects, with only a minor fraction of the breaks (10-20%) being affected in their repair after X- or γ-irradiation, respectively (Kühne et al., 2004; Riballo et al., 2004). This subset of DSBs whose repair is ATM-dependent were found to require ARTEMIS, functioning in a common pathway of DSB rejoining with H2AX, 53BP1, NBS1, MRE11, and DNA-PK. Identifying the nature of DSBs that specifically require ATM for their repair remained one of the main questions in the field, as it could provide important clues into disease pathogenesis. Two explanations have been put forward. On the one hand, ATM has been proposed to contribute specially to the repair of DSBs induced in heterochromatin by phosphorylating KAP1 (KRAB-associated protein1). This event induces the decompaction of heterochromatin in close proximity to the DSB and allows its repair (Goodarzi et al., 2008). On the other hand, ATM was proposed to specifically facilitate the repair of DSBs with damaged termini requiring end-processing (Riballo et al., 2004).

In our laboratory we could demonstrate that, at least, the structure of ends is a crucial factor which determines the requirement of ATM for the repair of a DSB. Specifically, ATM was shown to exclusively facilitate the repair of DSBs induced by TOP2 in a TDP2 deficient background, when the ends are irreversibly blocked. Consistent with this, we also demonstrated that ATM-mediated repair promotes cell survival and the maintenance of genome integrity, avoiding micronuclei and chromosomal aberration formation after the induction of DSBs harbouring termini that require end-processing (Alejandro Álvarez-Quilón et al., 2014).

Although the reasons underlying the molecular mechanisms by which ATM deals with blocked DNA ends are still unclear, it has been proposed two complementary manners (Figure 13) (Alejandro Álvarez-Quilón et al., 2014), that will be explained below.

On the one hand, ATM can promote limited resection to eliminate the complex structures at the ends through the action of nucleases. In this regard, ATM phosphorylates ARTEMIS and DNA-PKcs at the ABCDE cluster, the exclusive event required for ARTEMIS-mediated endonuclease activity (B. P. C. Chen et al., 2007; Davis,

So, & Chen, 2010; Katheryn Meek et al., 2008). In addittion, the interplay between ATM and the MRN complex is widely reported. Indeed, the three components of the complex are all phosphorylated by ATM, which has been proposed as a modulator of its processing activity (Kijas et al., 2015). Furthermore, ATM full activation upon DSB-induction seems to require its interaction with the MRN complex through NBS1 (Difilippantonio et al., 2007; Lavin, Kozlov, Gatei, & Kijas, 2015). Then, the MRN complex interacts with CtIP, which is also positively regulated by ATM to promote end-resection (You & Bailis, 2010) (H. Wang et al., 2013). Finally, ATM regulates other nucleases that could be involved in resolving incompatible ends. This includes APLF (Aprataxin and PNKP-like factor) (Fenton, Shirodkar, MacRae, Meng, & Anne Koch, 2013; Macrae, McCulloch, Ylanko, Durocher, & Koch, 2008); DNA replication helicase/nuclease 2 (DNA2) (Paudyal, Li, Yan, Hunter, & You, 2017) or EXO1 (Bolderson et al., 2010; Tomimatsu et al., 2017). In addition to nucleases, the Werner syndrome ATP-dependent helicase/nuclease (WRN) and the Bloom syndrome RecQ-like helicase (BLM), which are also related with or phosphorylated by ATM, could have a role in processing incompatible ends by generating a substrate for the nucleases previously mentioned (Ababou et al., 2000; Cheng et al., 2008).

On the other hand, ATM could restrict excessive nucleolytic degradation of DNA ends (Rahal et al., 2008). This can actually operate by a direct inhibitory action on aforementioned nucleases such as MRE11 (Rahal et al., 2010) or EXO1 (Bolderson et al., 2010), and/or by promoting modifications at the chromatin flanking the DSB and the recruitment of protecting factors. In this regard, the protective function of H2AX depends on its phosphorylation at Ser139 to form γ-H2AX in chromatin flanking DNA DSBs (Helmink et al., 2011), which is preferentially carried out by ATM (Takahashi et al., 2010). The γ-H2AX downstream factor MDC1 is also phosphorylated by ATM, promoting its oligomerization and spreding on chromatin (Maréchal & Zou, 2013). In addition, ATM phosphorylates 53BP1 on over 25 residues in the N-terminal half of the protein (Anderson, Henderson, & Adachi, 2002; Jowsey et al., 2007). Interestingly, these phosphorylations are required for 53BP1 interaction with PTIP (Munoz et al., 2007) and

RIF1 (J. Ross Chapman et al., 2013), as well as the recruitment to DSB sites in the case of RIF1. Nonetheless, ATM is not essential for PTIP localization after DNA damage (Gong, Cho, Kim, Ge, & Chen, 2009).

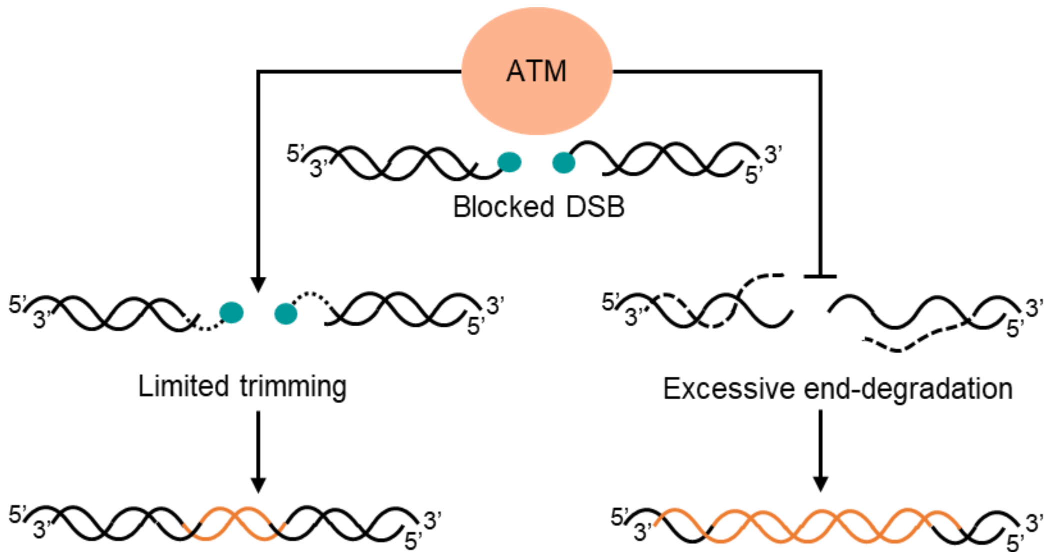

Figure 13: Model for the involvement of ATM in blocked DSB repair. ATM is proposed to promote limited end-trimming and to inhibit excessive end degradation of blocked ends when requiring end-processing. This could avoid deletions and gross chromosomal rearrangements that are associated with genome instability.

Thus, ATM could modulate the function of antagonistic pathways that both positively and negatively regulate DNA end processing so it is restricted to the minimal resection necessary to allow joining. In addition to this dual end-processing/protective roles, ATM could operate at later a stage. Both ATM and DNA-PKcs have been recently reported to phosphorylate Pol λ at threonine 204 *in vitro*, and this event to form part of the regulation of NHEJ. *In vivo*, after ionizing radiation treatment, this event is mainly performed by ATM, and it could promote gap-filling DNA synthesis during NHEJ. This phosphorylation could induce conformational changes in Pol λ that can facilitate its interaction with KU (Sastre-Moreno et al., 2017), providing another mechanism by which ATM is coordinating the processing of ends to be efficiently and accurately ligated.

4.6. Interplay of ATM and DNA-PKcs in the repair of DSBs

Up to date, extensive work has been performed to elucidate both specific and/or overlapping functions of DNA-PKcs and ATM (i.e. Callén et al., 2009; Caron et al., 2015; Riballo et al., 2004). Remarkably, although several redundant functions of these PI3K-like kinases during the DDR have been described, their regulation and the impact of their loss largely differ. In this regard, while, as mentioned above, the role of ATM is specifically required for the repair of specific types of DSBs, DNA-PKcs activity has been reported essential for the c-NHEJ pathway.

Some studies have indicated that a crosstalk takes place between these kinases, and they could cooperate during DSB signalling and the regulation of pathway choice. As previously mentioned, the phosphorylation of the DNA-PKcs ABCDE cluster can be performed by either by DNA-PKcs itself or ATM, which is essential for end-processing mediated by ARTEMIS endonuclease activity (Goodarzi et al., 2006). Furthermore, DNA-PKcs has been recently reported to phosphorylate ATM, negatively regulating its function in signalling and repair upon DNA damage (Y. Zhou et al., 2017). Since ATM promotes the HR pathway, these phosphorylations have been proposed to function as a relevant mechanism to regulate the choice of repair pathway. Despite these data, how DNA-PKcs and ATM work independently and cooperate to promote repair upon the induction of DSBs under different circumstances remains unknown.

II. OBJETIVES

II. OBJECTIVES

The understanding of the molecular mechanisms that govern DSB repair is extremely relevant. Notably, the structures that are present at DSB ends define their complexity and are considered putative determinants for repair pathway choice and outcome. This question, however, has not been sufficiently elucidated due to the difficulty to induce homogeneous populations of DSBs with defined end structures. Recently, we developed a genetic strategy to specifically induce clean (5′-phosphate and 3′-hydroxyl termini) or blocked DSBs by promoting the aberrant action of TOP2 in TDP2 proficient and deficient background, respectively. This provides an excellent tool to address the relevance of DNA end structure on DSB repair and whether there is a preference for "unblocking" the ends over "end-processing" as well as determining which are the consequences of each choice. Based on this, the objectives of this thesis are as follows:

1. Identify factors involved in the cellular response to DSBs induced by TOP2 in human cells and determine which factors are specifically required in TDP2-deficient background.
2. Characterize the involvement of the identified factors in the repair of TOP2 induced-DSBs, their impact in genome instability and their genetic relationships.
3. Characterize the potential involvement of PIK Kinases in the regulation of the identified factors.
4. Study whether the contribution of the identified factors to the repair TOP2-induced DSBs is conserved in rodent cells.
5. Study of the impact of TDP2 loss in malignant transformation and cancer development.

III. RESULTS

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1. CRISPR-Cas9 screen for etoposide sensitivity.

The induction of TOP2-mediated DSBs in TDP2 proficient and deficient background can be used to the development of a new genetic strategy to induce homogeneous populations of clean or blocked DSBs (Álvarez-Quilón et al., 2014). We decided to take the advantage of this genetic tool to identify unknown factors involved in the cellular response to DSBs induced by the aberrant action of TOP2, and to study how the structure of DNA ends affects such response. For this, CRISPR-Cas9 screens were carried out, which allowed us to perform unbiased analyses and identify genes that differentially affected etoposide sensitivity in TDP2 proficient and deficient backgrounds.

Currently, genome wide CRISPR-Cas9 screens are extensively used to identify potential determinants of drug sensitivity. Generally, cells expressing Cas9 nuclease are infected with a library of gRNAs targeting protein-coding genes of interest. After selection for infected cells and population sampling at time zero (t0), the starting cells populations are divided into two groups. One of them is the untreated control and the other one is chronically treated with concentrations of the drug that kills 20% of uninfected cells (LD_{20}). Then the evolving populations are sampled every 3 days from day 6 to day 21. Genomic DNA is isolated and gRNA abundance in each sample is measured by deep sequencing of the integrated gRNA cassettes in order to monitor the change in abundance of each gRNA between the initial cell population and t18. Finally, scores in samples arising from drug-treated vs. untreated cells are compared (Figure 14A).

Specifically, to identify unknown factors involved in the cellular response to DSBs induced by the aberrant action of TOP2, this approach was carried out in TDP2 proficient and deficient hTERT immortalized RPE1 retinal epithelial cells stably

expressing the Cas9 nuclease. These cell lines were generated in P53-deficient background to avoid confounding effects from P53-dependent responses. To generate

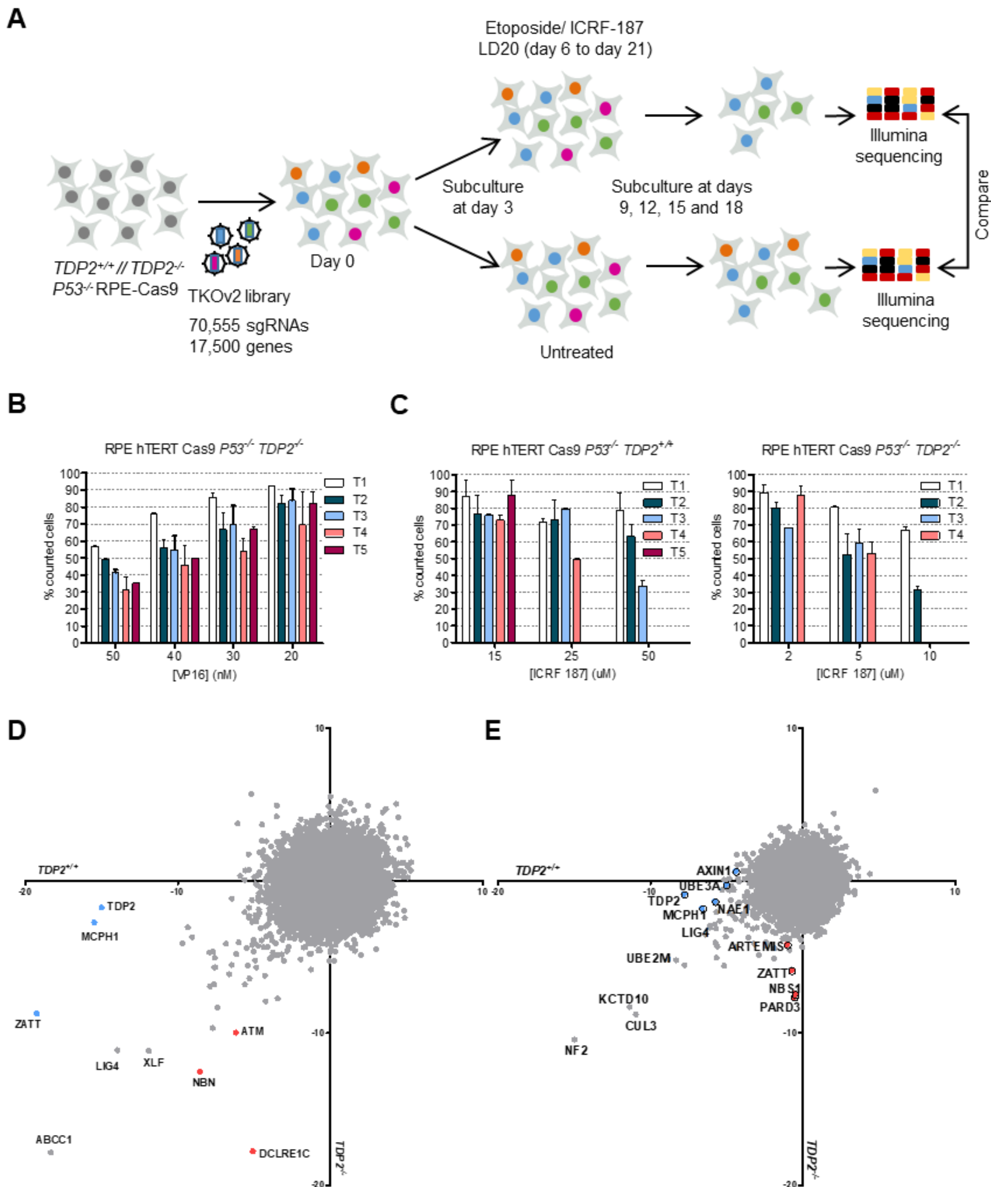


Figure 14: CRISPR screens identify determinants of etoposide and ICRF187 sensitivity in TDP2 proficient and deficient background. A, Schematic of the screen pipeline. **B**, Etoposide concentration test to identify LD_{20} in *TDP2*$^{-/-}$ *P53*$^{-/-}$ RPE1-hTERT cells. **C**, ICRF-187 concentration test to find LD_{20} in TDP2 proficient and deficient *P53*$^{-/-}$ RPE1-hTERT cells. Cells were maintained for at least (continue on next page…)

(…continued) 12 days and subcultured every 3 days. "T1" stands for the first passage and the percentage of survival comparing to untreated conditions is represented. The experiments were repeated in duplicate. **D**, Representation of etoposide screens. Results from TDP2 proficient cells are plotted in X axis and DrugZ scores from TDP2 deficient cells are plotted in Y axis. Genes encoded by gRNAs that show a specific underrepresentation in TDP2 proficient cells are circled in blue in contrast to the ones underrepresented in *TDP2*$^{-/-}$ cells which are circled in red. **E**, As above, with ICRF-187 treatment.

these cell lines, *P53*$^{-/-}$ RPE1-hTERT Cas9 cells (a gift from Dr. Daniel Durochers's laboratory) were transfected either with a negative control or *TDP2* targeting gRNAs. Cells transfected with the gRNA targeting *TDP2* showed ~90% of gene editing efficiency, as assessed by TIDE software (Brinkman, Chen, Amendola, & van Steensel, 2014). Then, cells were sorted to isolate individual clones which were selected on the basis of successful gene editing determined by individual TIDE analysis, showing a homozygous single nucleotide insertion. Clones from cells transfected with the negative gRNA control were also isolated and were used in the CRISPR-screen as *P53*$^{-/-}$ *TDP2*$^{+/+}$ cells. In order to generate TOP2-induced DSBs a treatment with the paradigmatic TOP2 poison etoposide was used. Something that must be taken into consideration is that, apart from the induction of DSBs, etoposide treatment implies TOP2 inhibition, as TOP2 functions are abrogated as a consequence of TOP2 cleavage-complexes trapping. In order to focus on DSB-repair candidates and rule out factors related with the catalytic inhibition of TOP2, CRISPR-Cas9 screens were also carried out with dexrazoxane (ICRF-187) so we could compare results from both treatments. ICRF-187 generates "closed clamps" without covalently trapping TOP2cc (Pommier et al., 2010) and it is considered a specific TOP2 catalytic inhibitor, as it does not induce DNA damage response after short-term exposure, although, it could trigger this signalling cascade following long-term exposure (J. L. Nitiss, 2009b). To test which drug-dose was required to kill specifically 20% of cells in each genotype, TDP2 proficient and deficient *P53*$^{-/-}$ RPE1-hTERT cells were maintained with different drug concentrations for at least 12 days, mimicking screen conditions. The cells from each treatment were split, counted and $3x10^6$ cells were seeded again every 3 days, monitoring which percentage comparing to untreated sample had died at each concentration. A 100nM etoposide treatment was determined as LD_{20} for *TDP2*$^{+/+}$ cells, this experiment was carried out by Dr. Daniel Durocher's laboratory and therefore it is

not included in this thesis. In contrast, *TDP2*$^{-/-}$ cells required a 20nM concentration (Figure 14B), consistent with the reported hypersensitivity to TOP2-poisons caused by TDP2 deletion in avian and murine cells (Gómez-Herreros et al., 2013). On the other hand, a 25μM ICRF-187 concentration was required to kill 20% of TDP2 proficient cells (Figure 14C, left) vs 2μM required for cells from TDP2 deficient background, suggesting that TDP2 is also involved in cellular response to TOP2-inhibition by ICRF-187 treatment (Figure 14C, right).

In order to perform our screens, a second generation of Toronto KnockOut lentiviral library (TKOv2) was employed. This library contains 70,555 gRNA targeting 17,942 protein-coding genes, as well as 142 sequences targeting LacZ, luciferase, and eGFP (Noordermeer et al., 2018). During screens, the complexity of the gRNA pool in the population was maintained at >400-fold coverage, and the screens were repeated in duplicate. The gene-based score were obtained using a newly developed algorithm DrugZ (Colic et al., 2019) which allowed us to compare scores in samples arising from etoposide/dexrazoxane-treated cells with those from untreated cells (Figure 14D,E).

First of all, with this CRISPR screen strategy, factors that are generally involved in the cellular response to etoposide were identified (from gRNAs showing differential abundance between untreated and etoposide-treated samples in TDP2 proficient cells). This screen in *TDP2*$^{+/+}$ cells was carried out by Dr. Daniel Durocher's laboratory. As expected, the gRNAs that showed a higher difference in abundance in etoposide-treated *TDP2*$^{+/+}$ cells (Figure 14D), compared with those from untreated cells, were those corresponding to genes encoding for TDP2 and ZATT. Additionally, gRNAs targeting genes coding factors of the NHEJ pathway, such as XLF and LIG4 were also less represented after etoposide treatment. Interestingly, apart from these expected results, one of the most underrepresented gRNAs were those targeting microcephalin 1 (*MCPH1*). MCPH1 is mutated in human primary microcephaly and it is reported to be involved in chromatin remodelling upon DNA damage (Peng et al., 2009) and in the decatenation checkpoint (Arroyo et al., 2019). Furthermore, some of the highest underrepresented gRNAs upon etoposide treatment were the ones that target *ABCC1*,

which encodes a member of the superfamily of ATP-binding cassette (ABC) transporters and it is associated with multidrug resistance (Robey et al., 2018).

In contrast to the results obtained in TDP2 proficient background, gRNAs showing specific changes in TDP2-deficient cells allowed us to identify factors specifically required for the repair of blocked lesions. As expected, in etoposide-treated *TDP2*$^{-/-}$ cell populations some the highest underrepresented gRNAs were the ones that target *ATM*, consistently with its already reported role in blocked DSB repair (Álvarez-Quilón et al., 2014). Interestingly, gRNAs targeting genes encoding for ARTEMIS and NBS1 (from the MRN complex) showed even a higher underrepresentation than ATM, suggesting a key role of these factors in the response to the TOP2-DSBs in the absence of TDP2. Furthermore, TDP2 deficient and wild-type cells shared factors from the NHEJ pathway and ZATT (Figure 14D), but the latter showed less underrepresentation than in TDP2 proficient cells, in agreement with previous results that suggest, both TDP2-dependent and, in a lesser degree, independent mechanisms for ZATT functions (Schellenberg et al., 2017). Strikingly, TDP2 deficient cells did not show the underrepresentation of gRNAs targeting MCPH1 upon etoposide treatment observed in *TDP2*$^{+/+}$ cells. This result suggests that MCPH1 functions in the cellular response to etoposide-induced TOP2-breaks could be related to TDP2. Finally, the fact that gRNAs targeting *TDP2* were not found underrepresented in TDP2 deficient background supports and validates the results obtained from this screen.

Furthermore, ICRF and etoposide screens shared some hits. First of all, gRNAs targeting *TDP2* also showed a high underrepresentation upon ICRF treatment (Figure 14E). This observation is consistent with the hypersensitivity to this TOP2 inhibitor previously observed in *TDP2*$^{-/-}$ cells (Figure 14C) and validated the results obtained in this screen. Moreover, as happened with the etoposide screen, again gRNAs targeting the gene encoding for MCPH1 were specifically underrepresented in TDP2 proficient background upon ICRF treatment. This suggests that MCPH1 function could be related with TOP2 activity instead with DNA damage response to etoposide-induced TOP2-breaks. The specificity of this hit in TDP2 proficient background further supports the

idea that MCPH1 could be involved in a TDP2-mediated response to this treatment. Additionally, gRNAs targeting genes encoding for LIG4 were also underrepresented upon ICRF treatment, although to a lesser degree than in the etoposide screen. This suggests that TOP2 inhibition by ICRF-187 could also lead to some level of DSB induction.

On the other hand, different results were also obtained from the ICRF compared to etoposide screen. Interestingly, both *TDP2*$^{+/+}$ and *TDP2*$^{-/-}$ cell populations showed an underrepresentation of gRNAs that targeted the gene *NF2*, which encodes for MERLIN, a factor involved in limiting proliferation and promoting apoptosis (Petrilli & Fernández-Valle, 2016). Interestingly, gRNAs targeting genes encoding for UBE2M, KCTD10 and CUL3, which are involved in ubiquitination and neddylation, were also underrepresented after ICRF treatment in both TDP2 proficient and deficient backgrounds (Kovačević et al., 2018; Petroski & Deshaies, 2005; W. Zhou et al., 2018). However, other gRNAs targeted genes encoding UBE3A and NAE1, which are also related with ubiquitination (Sun et al., 2018) and neddylation (T. C. Delgado et al., 2018), were specifically underrepresented in TDP2 proficient cell populations. The highest specific hit in *TDP2*$^{-/-}$ cell populations was PARD3, involved in asymmetrical cell division and cell polarization processes (Hapak, Rothlin, & Ghosh, 2018). Strikingly, in contrast to results obtained from the etoposide-screen, upon ICRF treatment, ZATT was only a hit in TDP2 deficient background suggesting that the ZATT function that is independent from TDP2 may be related with the inhibition of TOP2 activity. Moreover, in this background, NBS1 and ARTEMIS-targeting gRNAs were also specifically underrepresented in TDP2 deficient background upon ICRF treatment, although with a much lower score than in the etoposide-screen. Additionally, ATM was not underrepresented in any background. Altogether, these data strongly support that NBS1 and ARTEMIS, together with ATM, are factors particullary related to the cellular response to TOP2-induced DSBs in TDP2 deficient background.

While it will be of interest to analyse many factors identified in our screens, we first chose to focus on the role of these nucleases in the repair of TOP2-induced DSBs.

2. ARTEMIS is specifically required to repair TOP2-breaks in the absence of TDP2.

Since the CRISPR-Cas9 screens provided us candidate genes possibly involved in the cellular response to etoposide, we first wondered whether this feature was due to a direct role in the repair of these lesions. Because of this reason, we decided to measure the disappearance of γH2AX foci after the induction of TOP2-DSBs in cells that were deficient for individual candidates. An etoposide dose that induced a small amount of DSBs was used to recapitulate more physiological conditions, and to not overwhelm cellular repair capacity. In order to enhance the relevance of the structure of the ends, we decided to assess repair rate during G0/G1, as repair of DSBs is limited to NHEJ in this cell cycle phase and the influence of DNA end structure is expected to be maximal (Rothkamm, Kruger, Thompson, & Lobrich, 2003).

To assess the contribution of our candidates to the repair of TOP2-DSBs, TDP2 proficient and deficient RPE1-hTERT cells stably expressing active Cas9 nuclease were generated in P53 proficient background. For this, *P53*$^{+/+}$ RPE1-hTERT Cas9 cells (a gift from Dr. Daniel Durochers's laboratory) were transfected with a gRNA targeting *TDP2* or a negative control and clones were isolated in a similar way as *P53*$^{-/-}$ cells used in the screens. Once generated, *TDP2*$^{+/+}$ and *TDP2*$^{-/-}$ RPE-Cas9 cells were transfected with gRNAs targeting genes encoding for the different candidates. Gene editing efficiency was analysed by TIDE, obtaining an efficiency ~60-90%, depending on the sgRNA. This good gene editing efficiency provided advantages for experiments in which individual cells are analysed, i.e. immunofluorescence, as clone selection was not required because cells that have been effectively knocked-out can be distinguished from wildtype cells. This allowed us to analyse the role of essential factors and avoids possible artefacts due to clone variability and adaptive responses.

After seeding transfected cells, they were arrested in G0 cell cycle phase by FBS starvation. To address the importance of these factors in the repair of DSBs generated by TOP2, we measured DSBs repair at different time-points after 30 min of etoposide treatment. For this, immunofluorescence of γH2AX was performed. Furthermore,

during immunofluorescence, an antibody that recognizes our tested candidate was also added that could let us distinguish knock-out cells (Figure 15). An additional advantage of this approach is the fact that, as clonal selection is not necessary, it significantly reduces time.

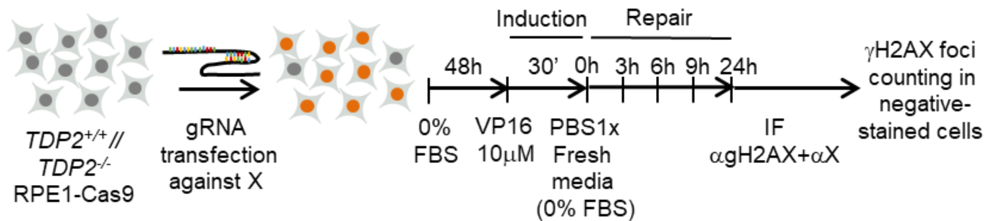


Figure 15: Experiment pipeline to address repair contribution of different factors. Schematic of CRIPR-Cas9 based-tool to assess contribution of the candidates of the screen (X) in the repair of DSBs induced by TOP2.

We first decided to apply the experimental setup described above to analyse the impact of ARTEMIS loss on the repair of either clean or blocked TOP2-DSBs. As can be seen in Figure 16A, at this dose of etoposide, wild-type cells repaired all DSBs induced before 3h. In contrast, *TDP2*$^{-/-}$ human cells showed a significant repair defect even at this low etoposide dose, as cells need more than 6 hours to repair all DSBs induced. These data are consistent with previous observations in which DSB repair was diminished in *Tdp2*$^{-/-}$ murine and avian cells and in TDP2-depleted human cells upon etoposide treatment (Gómez-Herreros et al., 2013). Furthermore, the absence of ARTEMIS did not affect the rate of etoposide-induced DSB repair in a TDP2-proficient background. Therefore, ARTEMIS does not influence repair of clean DSBs, which is consistent with previous observations of a limited DSB-repair defect for ends not requiring trimming before ligation (Kurosawa et al., 2007). In contrast, a striking drop in the repair kinetics was observed in double *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ cells, with most of the breaks remaining unrepaired even after 24h, suggesting that ARTEMIS is specifically required to repair TOP2-DSBs when TDP2 is not present. Thus, these data define a role of ARTEMIS in DSB repair besides its generic role in VDJ recombination and further support the idea

that there is a strong preference for repair TOP2-DSBs though TDP2 pathway and, only when TDP2 is not available, ARTEMIS nuclease is required.

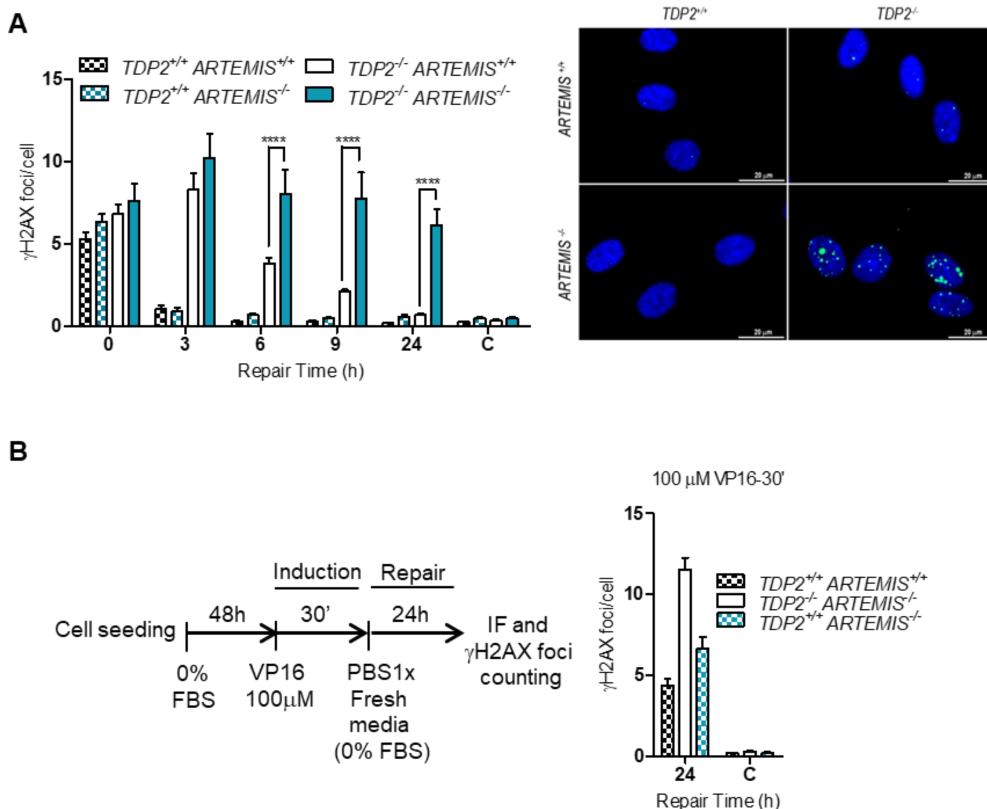


Figure 16: ARTEMIS is specifically required to repair TOP2-breaks in the absence of TDP2. A, γH2AX foci induction after 30 min 10 μM etoposide treatment and repair at different times following drug removal in the indicated serum starved arrested RPE1-hTERT Cas9 cells. Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments and the minimal statistical significance between cells deficient in both *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ and *TDP2*$^{-/-}$ single mutants by two-way ANOVA test with Bonferroni post-test are shown (*P≤0.05; **P≤0.01; ***P≤0.001) (left). Representative images of γH2AX foci (green) and DAPI counterstain (blue) for the 9 h repair time point are shown. Scale bar, 20 μm. (right). **B,** Schematic of the experiment pipeline (right), γH2AX foci remaining after 30 min 100 μM etoposide treatment and repair at 24 hours following drug removal in the indicated serum starved arrested RPE1-hTERT cells. Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments is shown (left).

In order to further confirm that the negligible impact of ARTEMIS loss in TDP2 proficient cells is not due to the dose of etoposide being too low, wild-type, *TDP2*$^{-/-}$ and

ARTEMIS$^{-/-}$ cells were treated with 100 µM etoposide for 30 min. At this dose, a high amount of TOP2-DSBs were induced, precluding γH2AX for scoring. Due to this reason, cells were allowed to repair for 24 h and only this time-point was analysed. As can be seen in Figure 16B, wild-type cells were not able to repair all damage under these circumstances, suggesting that the activity of TDP2 is limited upon high dose of etoposide treatment. On the other hand, TDP2 deficient cells showed a much higher repair defect compared to wild-type cells, further supporting its crucial role in the repair of TOP2-DSBs. Remarkably, even with this high dose of etoposide, *ARTEMIS*$^{-/-}$ single mutant showed no repair defect, when compared to wild-type cells. This further supports that ARTEMIS exclusively facilitates repair of TOP2-DSBs in the absence of TDP2 and, even with a high dose of etoposide treatment that could saturate the activity of TDP2, ARTEMIS deficiency does not have a significant impact, at least when cells are allowed to repair for a long period.

3. The nuclease activity of the MRN complex is specifically required to repair TOP2-breaks in the absence of TDP2.

Despite the fact that ARTEMIS loss caused a strong defect in the repair of TOP2-DSBs when TDP2 is absent, some breaks were still repaired. This led us to wonder whether there are other nucleases involved in this process. In this regard, MRE11, which harbours the nuclease activities of the MRN complex, has been described to play major roles in the cellular response to etoposide (Hoa et al., 2016). In fact, the NBS1 subunit from the MRN complex was also a top hit in the etoposide screen in TDP2 deficient cells (Figure 14D), although underrepresentation of sgRNAs targeting *NBS1* was not as high as those targeting ARTEMIS. Taking the advantage that our experimental approach allows the analysis of essential factors (Figure 15), we decided to assess the contribution of MRE11 to the repair of etoposide-induced DSBs. Surprisingly, MRE11 deficiency did not involve a repair defect at all in TDP2 proficient background after etoposide treatment (Figure 17A). This is highly striking as previous studies claimed that MRE11 has a major role in the cellular response to etoposide in wild-type cells (Hoa et al., 2016).

Our results indicate that this is not the case, at least in our experimental conditions. On the contrary, removing MRE11 implied a striking drop in the repair kinetics of *TDP2$^{-/-}$* cells, with most of the breaks remaining unrepaired even after 24h, in a similar way as occurred in *TDP2$^{-/-}$ ARTEMIS$^{-/-}$*. Thus, this indicates that MRE11 is uniquely necessary to repair TOP2-breaks in TDP2 deficient cells, when the ends are blocked. These data again support the idea that there is a strong preference for the repair of TOP2-DSBs through the TDP2 pathway and, only when TDP2 activity is compromised, nuclease functions are relevant.

Furthermore, this specific repair defect in TDP2 deficient cells was also observed upon inhibition of either the exo or endonucleolytic activities of MRE11 by MIRIN or PFM01 incubation, respectively (Figure 17B,C). Nevertheless, it must be taken into consideration the exonucleolytic degradation from a nick towards a blocked end was demonstrated to be sensitive to the allegedly endonuclease-specific inhibitor PFM01, even when the nick is already generated in the substrate (Deshpande, Lee, Arora, & Paull, 2016b). We therefore cannot draw definitive conclusions regarding the endonuclease activity of MRE11 but our results indicate that at least its exonuclease activity is required to repair TOP2-DSBs specifically in TDP2 deficient cells.

In order to further confirm that the negligible impact of the inhibition of nuclease activities of MRE11 in TDP2 proficient cells is not due to the dose of etoposide being too low, wild-type cells incubated with PFM01 inhibitor were treated with 100 μM etoposide for 30 min and were allowed to repair for 24h. As can be seen in Figure 17D, inhibition of MRE11 in wild-type cells showed a negligible impact, showing similar levels of repair as controls without inhibitor incubation. This further supports that MRE11 nuclease activity exclusively facilitates repair of TOP2-DSBs in the absence of TDP2 and not in wild-type cells.

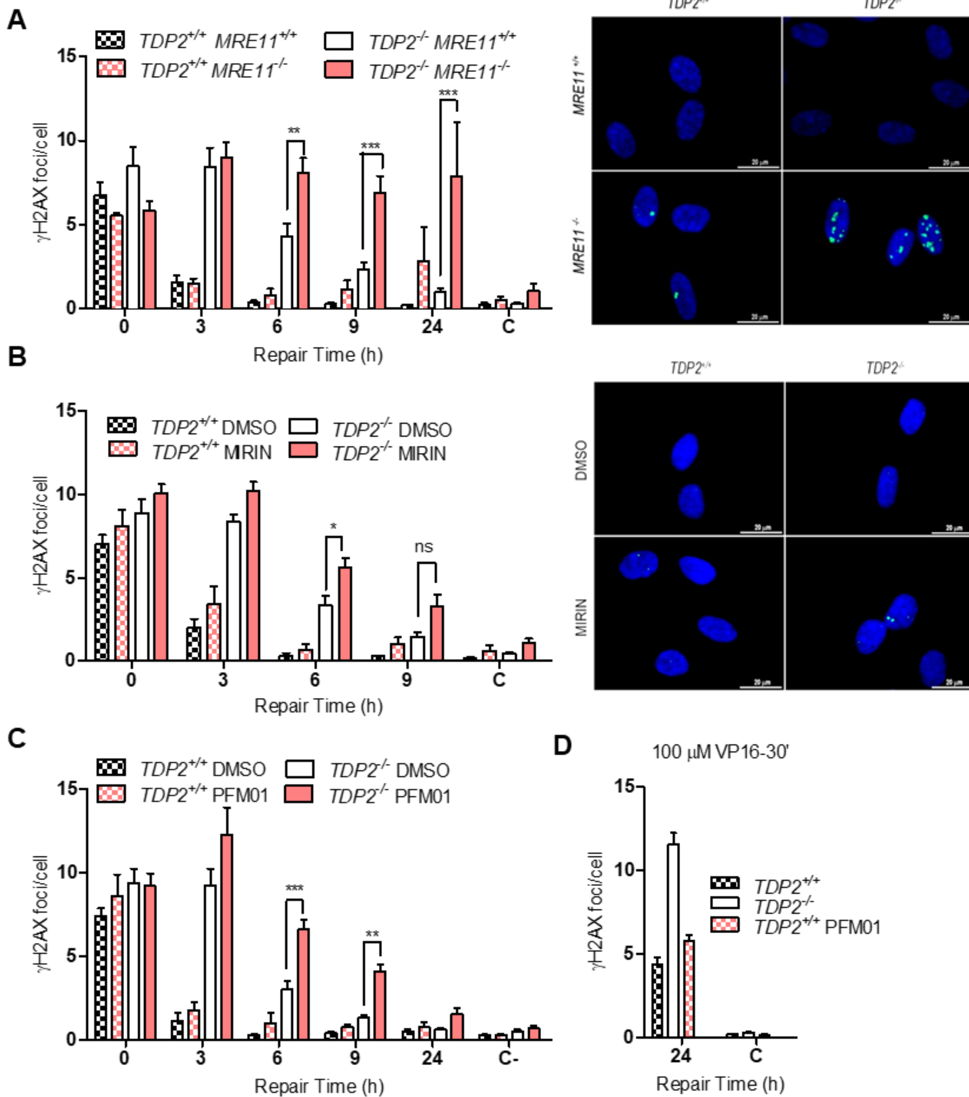


Figure 17: Nuclease activity of the MRN complex is specifically required to repair TOP2-breaks in the absence of TDP2. A, γH2AX foci induction after 30 min 10 μM etoposide treatment and repair at different times following drug removal in the indicated serum starved arrested RPE1-hTERT Cas9 cells (right). **B**, As above, in *TDP2*$^{+/+}$ and *TDP2*$^{-/-}$ serum starved-arrested RPE1-hTERT cells with or without 50 μM exonuclease activity inhibitor of MRN complex (MIRIN) (right). Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments and the minimal statistical significance by two-way ANOVA test with Bonferroni post-test are shown (*P≤0.05; **P≤0.01; ***P≤0.001). In **A**, **B**, representative images of γH2AX foci (green) and DAPI counterstain (blue) for the 9 h repair time point are shown. Scale bar, 20 μm (left). **C**, As above, with or without 4 μM endonuclease activity inhibitor of MRN complex (PFM01). **D**, γH2AX foci remaining after 30 min 100 μM etoposide treatment and repair at 24 hours following drug removal in the indicated serum starved arrested RPE1-hTERT cells with and without 4 μM (continue on next page…)

(…continued) endonuclease activity inhibitor of the MRN complex (PFM01). Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments is shown.

4. ARTEMIS and MRE11 nucleases function together to promote the repair of TOP2-DSBs in the absence of TDP2.

Given the specific roles of both ARTEMIS and MRE11 nucleases in the repair of TOP2 breaks when TDP2 is not present, we wondered whether they contributed to the same pathway or were acting independently. In order to avoid the simultaneous generation of double knock-out cells by transfection with two gRNAs, *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ RPE1-hTERT-Cas9 clones were generated and isolated by transfecting TDP2 deficient cells with a gRNA targeting *ARTEMIS*. To avoid possible artefacts due to clonal variations, 5 clones were selected and mixed. These *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ generated mutants and *TDP2*$^{-/-}$ RPE1-hTERT-Cas9 single mutants were transfected with a gRNA targeting *MRE11*. As previously observed, ARTEMIS or MRE11 loss involved a strong repair defect in TDP2 deficient cells. Interestingly, we found that MRE11 deficiency did not increase the repair defect observed in *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ double knockout cells (Figure 18A). This epistatic effect demonstrates that both nucleases are involved in the

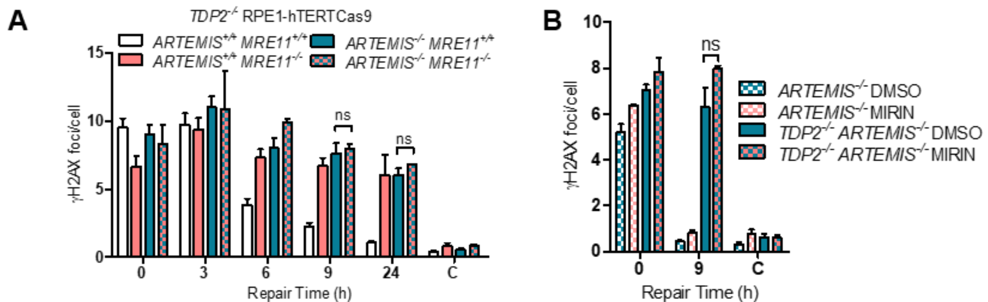


Figure 18: ARTEMIS and MRE11 function together in the same pathway to repair TOP2-DSBs in the absence of TDP2. A, γH2AX foci induction after 30 min 10 µM etoposide treatment and repair at different times following drug removal in the indicated serum starved arrested RPE1-hTERT cells. Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments and the minimal statistical significance between *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ *MRE11*$^{+/+}$ cells and *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ *MRE11*$^{-/-}$ triple mutant by two-way ANOVA test with Bonferroni post-test are shown (*P≤0.05). **B**, As indicated above, with or without 50 µM MIRIN inhibitor. Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments and the minimal statistical significance between *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ cells with or without inhibitor by two-way ANOVA test with Bonferroni post-test are shown (*P≤0.05).

same pathway to repair blocked TOP2-DSBs. A similar result was obtained upon inhibition of the exonuclease activity of MRE11 by MIRIN treatment instead of removing it in *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ double mutants (Figure 18B), further confirming that ARTEMIS and the exonuclease activity of MRN work together to repair etoposide-induced DSBs in TDP2 deficient cells.

5. The alternative ARTEMIS-MRN nucleolytic pathway compromises genome integrity.

At least in normal circumstances, our results indicate that cells show a strong preference for the repair of TOP2-DSBs by TDP2 unblocking activity over processing through an ARTEMIS-MRE11 pathway. Thus, we wondered which were the consequences for genome integrity of disrupting this established hierarchy. To address this question, we monitored the formation of micronuclei, which arise from the mis-segregation of chromosomes or acentric chromosomal fragments, and is a well-established indicator of genome instability (Fenech, 2000). To restrict our analysis to micronuclei arising due to TOP2-induced damage in G1, serum-arrested cells were treated with etoposide and allowed to repair for 24h. After repair time, serum was added for 48 hours so as to enable cells to entry into cell cycle again (Figure 19). As previously observed in TDP2-deficient primary MEFs (Gómez-Herreros et al., 2013), TDP2 deficiency increases micronuclei formation after a low-dose etoposide treatment in human RPE-hTERT

cells. This supports that the preferent TDP2 pathway is required to ensure the maintenance of genome integrity after induction of DSBs by TOP2. However, neither ARTEMIS loss nor the inhibition of nuclease activities of MRE11 showed a relevant increase in micronuclei formation in these conditions.

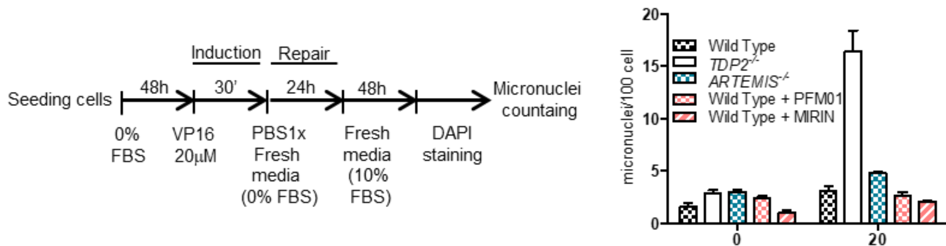


Figure 19: Upon a low dose of etoposide treatment, micronuclei formation depends on TDP2 deficiency and is independent of the lack of nucleases or DNA-PKcs. Schematic of the experiment pipeline (right); micronuclei accumulation monitored in the indicated RPE1-hTERT Cas9 cells after serum starvation arrest following 30 min 20 μM etoposide treatment, 24 h of repair and finally serum addition for 48 h. Histogram bars represent the average ± s.e.m. of n>500 cells from three independent experiments.

In order to analyse the consequences of the disruption of this hierarchy, micronuclei formation was analysed after a high dose of etoposide treatment (100 μM for 30'). We hypothesized that this high induction of TOP2-DSBs would overwhelm TDP2 unblocking capacity which could allow the action of nucleases to process the ends, unbalancing the preference for TDP2 and favouring end-processing instead. Indeed, wild-type cells showed a high induction of micronuclei formation after this high dose of etoposide treatment (Figure 20). Interestingly, this induction was greatly reduced in ARTEMIS deficient cells or upon inhibition of MRE11 nuclease activities by MIRIN or PFM01. This reduction in micronuclei formation is not likely due to a checkpoint arrest, as loss of ARTEMIS or incubation of wild-type cells with PFM01 showed no repair defect under these circumstances (Figures 16B, 17D). This suggests that the formation of micronuclei observed when TDP2 capacity is overwhelmed is due to the action of ARTEMIS and the nuclease activities of the MRN complex, supporting the requirement for TDP2 to ensure genome stability after TOP2-damage and it is consistent with TDP2 unblocking activity acting preferentially over the potentially deleterious nucleaolytic pathway.

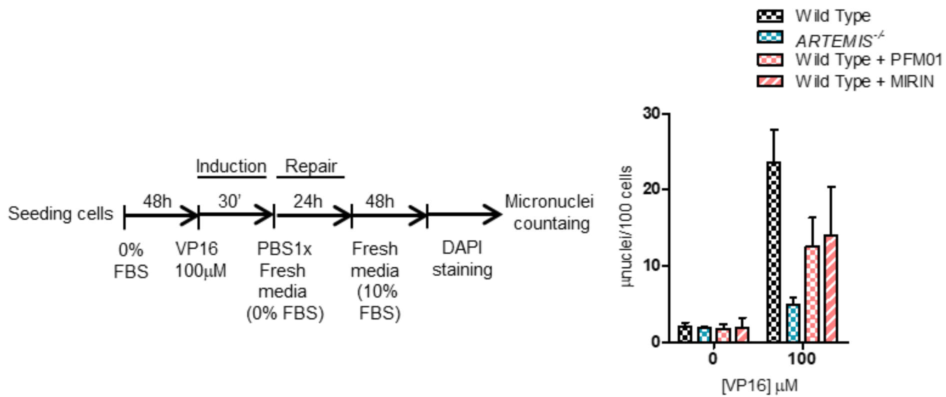


Figure 20: Micronuclei formation depends on nuclease pathway upon high doses of etoposide treatment. Schematic of the experiment pipeline (right), micronuclei accumulation monitored in the indicated RPE1-hTERT Cas9 cells after serum starvation arrest following 30 min 100 μM etoposide treatment, 24 h of repair and finally serum addition for 48 h. Histogram bars represent the average ± s.e.m. of n>500 cells from three independent experiments (left).

6. The presence of DNA-PKcs specifically facilitates repair of TOP2-DSBs in the absence of TDP2.

Given the preference to channel the repair of TOP2-DSBs through TDP2 unblocking activity instead of ARTEMIS-MRE11 pathway, we wondered how this hierarchy is regulated. In this regard, since the presence and phosphorylation of DNA-PKcs is necessary for ARTEMIS to acquire endonucleolytic activity (Goodarzi et al., 2006; Ma et al., 2002), we decided it would be of interest to examine the contribution of DNA-PKcs to the repair of TOP2-DSBs. To address this question, we transfected wild-type and *TDP2-/-* RPE1-hTERT-Cas9 cells with a gRNA targeting *DNA-PKcs*. After transfection, repair kinetics was determined as explained in Figure 15. Strikingly, as can be seen in Figure 21A, loss of DNA-PKcs did not affect repair of etoposide-induced DSBs in the presence of TDP2, at least with this low dose of etoposide treatment, regardless of its reported roles as a core NHEJ factor. Nevertheless, the absence of DNA-PKcs significantly increased the repair defect observed in *TDP2-/-* cells. These data suggest that DNA-PKcs is only necessary to repair TOP2-DSBs when the ends are blocked, similarly to what occurs upon ARTEMIS and MRE11 deficiency.

As previously performed with ARTEMIS- and MRE11-deficient cells, *DNA-PKcs$^{-/-}$* cells were then treated with a high dose of etoposide (Figure 21B) and were allowed to repair for 24h. Strikingly, in these conditions the *DNA-PKcs$^{-/-}$* single mutant showed a dramatic repair defect that was even higher than in TDP2-deficient cells. This is in stark contrast with the complete lack of repair defect observed at low doses of etoposide, suggesting that in DNA-PKcs-deficient cells, although efficient, repair occurs through a fundamentally different mechanism that is strongly affected by the amount of damage induced. We therefore decided to explore the function of DNA-PKcs in the repair of TOP2-induced DSBs further.

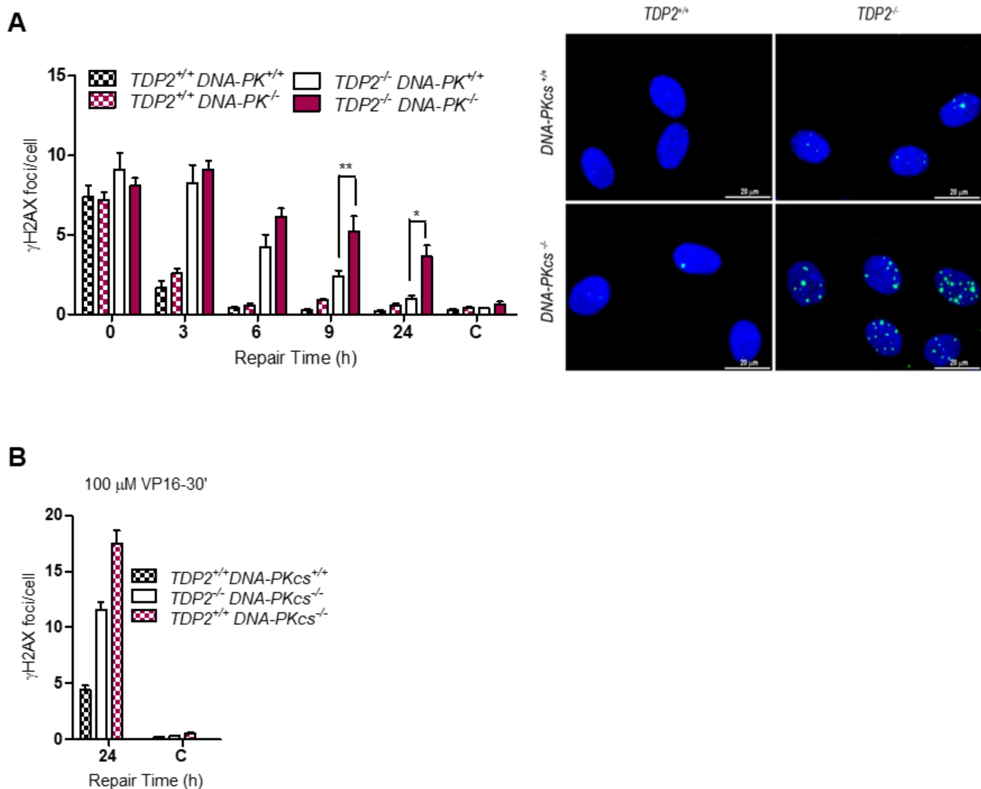


Figure 21: At a low doses of etoposide treatment, DNA-PKcs loss specifically impairs repair of TOP2-DSBs in the absence of TDP2. A, γH2AX foci induction after 30 min 10 μM etoposide treatment and repair at different times following drug removal in *TDP2$^{+/+}$ DNA-PKcs$^{+/+}$*, *TDP2$^{+/+}$ DNA-PKcs$^{-/-}$*, *TDP2$^{-/-}$ DNA-PKcs$^{+/+}$* and *TDP2$^{-/-}$ DNA-PKcs$^{-/-}$* serum starved arrested RPE1-hTERT cells. Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments and the minimal statistical significance between cells deficient in both *TDP2$^{-/-}$ DNA-PKcs$^{-/-}$* and *TDP2$^{-/-}$* single mutant by (continue on next page…)

(…continued) two-way ANOVA test with Bonferroni post-test are shown (*P≤0.05; **P≤0.01) (left). Representative images of γH2AX foci (green) and DAPI counterstain (blue) for the 9 h repair time point are shown. Scale bar, 20 µm (right). **B,** γH2AX foci remaining after 30 min 100 µM etoposide treatment and repair at 24 hours following drug removal in the indicated serum starved arrested RPE1-hTERT cells. Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments is shown.

In order to determine the contribution of DNA-PKcs activity, the disappearance of γH2AX foci was monitored after etoposide treatment upon DNA-PKcs inhibition in TDP2 proficient and deficient RPE1-hTERT-Cas9 cells. As can be seen, DNA-PKcs inhibition completely abolished repair already in TDP2 proficient cells and was epistatic over removing TDP2 (Figure 22). These data suggest that DNA-Pkcs always binds to DSBs, regardless of the type of end configurations, and that removing physical blockade imposed by the DNA-PKcs protein itself through its autophosphorylation is a requisite for repair.

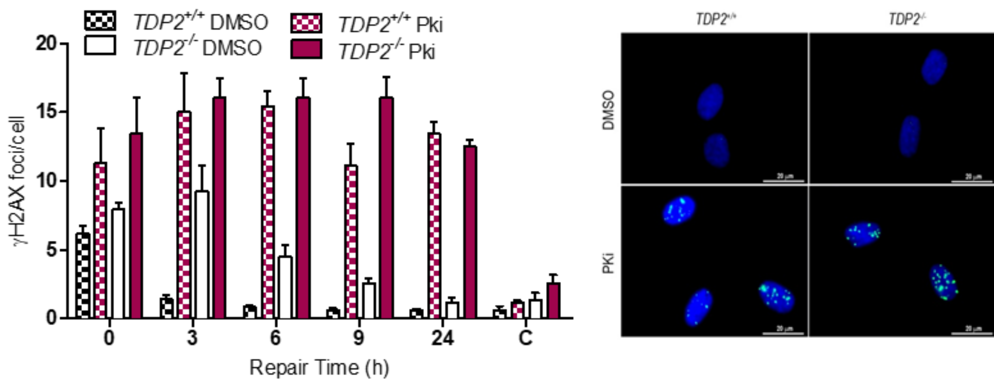


Figure 22: DNA-PKcs inhibition completely abolishes repair of TOP2-DSBs regardless of TDP2 background. γH2AX foci induction after 30 min 10 µM etoposide treatment and repair at different times following drug removal, in *TDP2*$^{+/+}$ and *TDP2*$^{-/-}$ serum starved-arrested RPE1-hTERT cells with or without 10 µM DNA-PKcs inhibitor (NU7441 (KU-57788)). Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments is shown (left). Representative images of γH2AX foci (green) and DAPI counterstain (blue) for the 9 h repair time point are shown. Scale bar, 20 µm (right).

7. DNA-PKcs and ARTEMIS operate together to promote the repair of TOP2-DSBs in the absence of TDP2.

Once we determined the role of DNA-PKcs in the repair of TOP2-DSBs, we aimed to address whether DNA-PKcs operates in the ARTEMIS-MRE11 pathway to remove TOP2 adducts in the absence of TDP2. For this, we first studied the genetic relationship between DNA-PKcs and ARTEMIS. For this, the previously generated *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ mix of 5 clones and *TDP2*$^{-/-}$ RPE-Cas9 cells were transfected with a gRNA targeting *DNA-PKcs*, and a repair time course was performed as described above (Figure 15). As we had observed previously, ARTEMIS loss involved a strong repair defect in TDP2 deficient cells. Similarly, although to a lesser extent than upon ARTEMIS deletion, DNA-Pkcs deficiency induced a relevant repair defect in *TDP2*$^{-/-}$ cells (Figure 23). Strikingly, DNA-PKcs loss was epistatic over removing ARTEMIS in TDP2 deficient cells, showing the same repair defect as *TDP2*$^{-/-}$ *DNA-PKcs*$^{-/-}$ double knock-out cells. This result is consistent with the requirement of DNA-PKcs for ARTEMIS function, and further suggests that DNA-PKcs somehow avoids repair through pathways that are independent of ARTEMIS. Thus, DNA-PKcs would be committing the repair of TOP2-induced DSBs through an ARTEMIS dependent pathway when TDP2 is not present, avoiding the access of other alternative pathways.

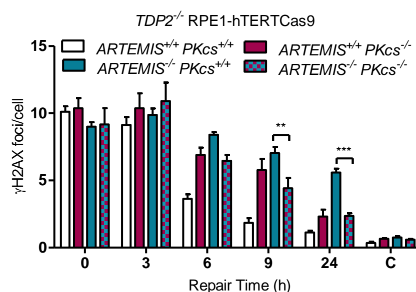


Figure 23: The loss of DNA-Pkcs is epistatic over removing ARTEMIS in TDP2 deficient background. γH2AX foci induction after 30 min 10 μM etoposide treatment and repair at different times following drug removal in *TDP2*$^{-/-}$ *ARTEMIS*$^{+/+}$ *DNA-PKcs*$^{+/+}$, *TDP2*$^{-/-}$ *ARTEMIS*$^{+/+}$ *DNA-PKcs*$^{-/-}$, *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ *DNA-PKcs*$^{+/+}$ and *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ *DNA-PKcs*$^{-/-}$ serum starved arrested RPE1-hTERT cells. Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments and the minimal statistical significance between *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ *DNA-PKcs*$^{+/+}$ cells and *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ *DNA-PKcs*$^{-/-}$ triple mutant by two-way ANOVA test with Bonferroni post-test are shown (*P≤0.05; **P≤0.01; ***P≤0.001).

8. In the absence of DNA-PKcs, MRE11 is required to repair TOP2-DSBs.

We then aimed to assess the relationship between MRE11 and DNA-PKcs in the repair of DSBs induced by TOP2. For this, *TDP2$^{+/+}$ DNA-PKcs$^{-/-}$* and *TDP2$^{-/-}$ DNA-PKcs$^{-/-}$* RPE1-hTERT-Cas9 clones were generated and isolated by transfecting TDP2 proficient and deficient cells with a gRNA targeting *DNA-PKcs*. In order to avoid artefacts due to clonal variations, 3 *TDP2$^{+/+}$ DNA-PKcs$^{-/-}$* and 3 *TDP2$^{-/-}$ DNA-PKcs$^{-/-}$* RPE1-hTERT-Cas9 clones were selected and mixed. These mutants were transfected with a gRNA targeting *MRE11*, and a repair time course was performed as previously described (Figure 15). Strikingly, despite *MRE11$^{-/-}$* or *DNA-PKcs$^{-/-}$* single mutants not showing a repair defect as we had previously determined, the double mutant *DNA-PKcs$^{-/-}$ MRE11$^{-/-}$* displayed a dramatic repair defect (Figure 24A). This suggests that, in the absence of DNA-PKcs, MRE11 plays a relevant role in the repair of TOP2-DSBs even when TDP2 is present and, therefore, regardless of end configuration. This was really surprising, as it was the first time that the loss of any factor showed an impact in TDP2 proficient background.

Furthermore, the absence of TDP2 further increased this repair defect observed, leading to a complete lack of γH2AX focus disappearance similar to that observed upon DNA-PKcs inhibition, indicating that TDP2 and MRE11 are acting independently to repair TOP2-breaks also in DNA-PKcs deficient background. This suggests that DNA-PKcs could be regulating the established hierarchy by which TDP2 is preferred to repair TOP2-induced DSBs over the nucleolytic pathways, avoiding somehow the action of MRE11.

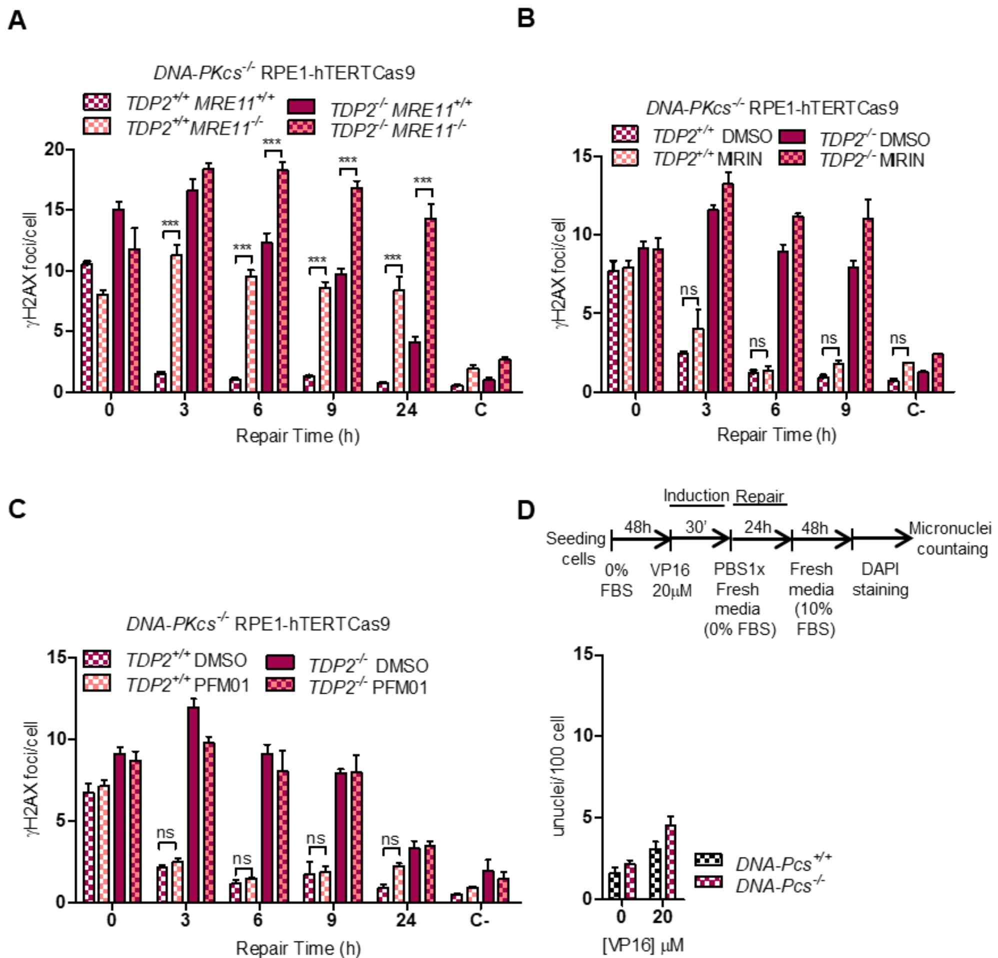


Figure 24: In DNA-PKcs absence, MRE11 facilitates repair of TOP2-DSBs regardless TDP2 background. A, γH2AX foci induction after 30 min 10 µM etoposide treatment and repair at different times following drug removal in the indicated serum starved arrested RPE1-hTERT cells. Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments and the minimal statistical significance by two-way ANOVA test with Bonferroni post-test are shown (*P≤0.05; **P≤0.01; ***P≤0.001). **B**, As indicated above, with or without 50 µM MIRIN inhibitor. **C,** As above, with or without 4 µM endonuclease activity inhibitor of MRN complex (PFM01). **D,** Micronuclei accumulation monitored in the indicated RPE1-hTERT Cas9 cells after serum starvation arrest following 30 min 20 µM etoposide treatment, 24 h of repair and finally serum addition for 48 h. Histogram bars represent the average ± s.e.m. of n>500 cells from three independent experiments.

Surprisingly, *DNA-PKcs-/-* cells did not show a significant repair defect upon the inhibition of either the exo or endonucleolytic activities of MRE11 by MIRIN or PFM01 incubation, respectively (Figure 24B,C), suggesting that the role of MRE11 in the repair

of TOP2-breaks when DNA-PKcs is absent is not related to its nuclease activities but to additional structural or regulatory functions. Since ARTEMIS activity absolutely requires DNA-PKcs, it is very likely that this MRE11 function is different from the one observed in ARTEMIS-dependent pathway. This suggests that MRE11 can function in two independent pathways to repair TOP2-DSBs. The first ARTEMIS-MRE11 pathway that we described above and is DNA-PKcs dependent, and a second function of MRE11 that would be avoided by the presence of DNA-PKcs and is not related with its nuclease activities (we will refer to it as “MRE11 only” pathway). Summing up, these data indicate that TOP2-DSBs are preferentially repaired by TDP2 unblocking activity. In the absence of TDP2, DNA-PKcs would commit repair through an ARTEMIS-MRE11 nucleolytic pathway, avoiding an MRE11-only pathway that is only relevant when DNA-PKcs is not present.

Because of this observed function of MRE11 in DNA-PKcs deficient cells, we wondered whether the loss of DNA-PKcs had an impact in maintaining genome integrity. To address this question, as described above, we monitored formation of micronuclei after induction of TOP2-breaks by a low dose of etoposide treatment (Figure 24D). Unexpectedly, DNA-PKcs deficiency showed a negligible impact on the induction of micronuclei formation compared to wild-type cells, suggesting that this MRE11-only alternative pathway does not compromise genome integrity in the form of gross chromosomes rearrangements. This, of course, does not rule out the accumulation of other deleterious events, such as small deletions, that are undetectable with the micronuclei assay, and that are expected to arise from unscheduled and excessive processing of DSBs.

9. ATM is involved in the ARTEMIS-MRE11 nucleolytic pathway to repair TOP2-blocked DSBs.

Previous work from the lab has shown that, in primary MEFs, ATM facilitates the repair of TOP2-DSBs specifically in TDP2 absence, when the ends are blocked (A. Álvarez-Quilón et al., 2014). In order to deeply assess the contribution of human ATM to this

process and analyse its relationship with nucleases, *ATM*$^{-/-}$ single mutants and *TDP2*$^{-/-}$ *ATM*$^{-/-}$ double knock-out RPE-hTERT Cas9 cells were generated. Three clones were selected and characterized in each genotype. As can be seen, Western blot analysis using an antibody that was raised against an epitope of ATM demonstrated that none of the selected clones expressed the ATM protein (Figure 25A). Thus, we first decided to confirm the role of ATM in the repair of blocked-DSBs in our experimental conditions (Figure 15). First of all, we observed that the absence of ATM caused a marked reduction in the intensity of etoposide-induced γH2AX foci. This is not surprising given the key role of ATM in H2AX phosphorylation in response to DSBs (Kim et al., 1999). However, in contrast to the intensity, the number of γH2AX foci induced was not affected, validating the use of this parameter as a tool to score DSBs in our conditions. Consistent with previous observations in primary MEFs, ATM loss did not display a repair defect in TDP2 proficient cells after etoposide treatment (Figure 25C). Nevertheless, it significantly affected repair in TDP2 knockout cells. This, therefore, confirms the specific requirement of ATM in the repair of blocked-DSBs in human cells. Moreover, cell survival was monitored in response to etoposide treatment (Figure 25B). As can be observed, removing ATM had a negligible effect on etoposide sensitivity in TDP2 proficient cells. In contrast, sensitivity of *TDP2*$^{-/-}$ cells, which was mild at this low etoposide dose, was significantly increased by the loss of ATM, correlating with the observed DSB-repair defect and the previous results in primary MEFs (Alejandro Álvarez-Quilón et al., 2014). This further confirms a similar effect of ATM deficiency in human and rodent cells regarding the repair of TOP2-blocked DSBs.

Secondly, we wondered whether ATM involvement in the repair of blocked-DSBs is related to the ARTEMIS-MRE11 pathway. Therefore, one of previously characterized *TDP2*$^{-/-}$ *ATM*$^{-/-}$ RPE-Cas9 clones (#13) and *TDP2*$^{-/-}$ single mutant were transfected with gRNAs targeting either *ARTEMIS* or *MRE11*, and γH2AX foci disappearance was monitored after etoposide treatment, as previously described (Figure 15). The impact of removing ATM in the repair of TOP2-DSBs in *TDP2*$^{-/-}$ cells was not as strong as the one observed upon the loss of ARTEMIS or MRE11. Remarkably, removing ATM in *TDP2*$^{-}$

-/- *ARTEMIS*-/- double mutants has only a minor effect compared to the one observed in *TDP2*-/- *ARTEMIS*+/+ background (Figure 26A).

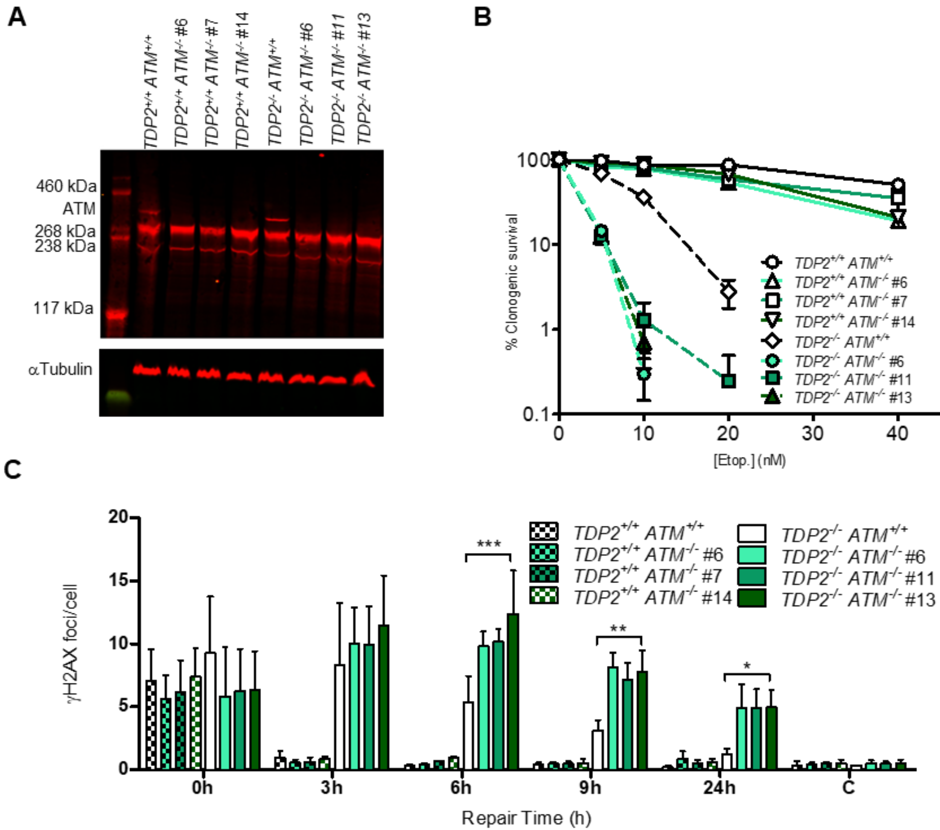


Figure 25: ATM facilitates repair of TOP2-induced DSBs and promote cell survival but exclusively when TDP2 is not present in human cells. A, Lack of ATM expression in TDP2 proficient and deficient RPE1-hTERT selected clones assessed by immunoblot. α-Tubulin was used as a loading control. **B**, Clonogenic survival of *TDP2*+/+ *ATM*+/+, *TDP2*+/+ *ATM*-/-, *TDP2*-/- *ATM*+/+ and *TDP2*-/- *ATM*-/- RPE1-hTERT Cas9 clones following chronic treatment with the indicated concentrations of etoposide. Average ± s.e.m. of at least three independent experiments is shown. **C**, γH2AX foci induction after 30 min 10 µM etoposide treatment and repair at different times following drug removal in the indicated serum starved arrested RPE1-hTERT cells. Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments and the minimal statistical significance between cells deficient in both *TDP2*-/- *ATM*-/- and *TDP2*-/- single mutant by two-way ANOVA test with Bonferroni post-test are shown (*P≤0.05; **P≤0.01; ***P≤0.001).

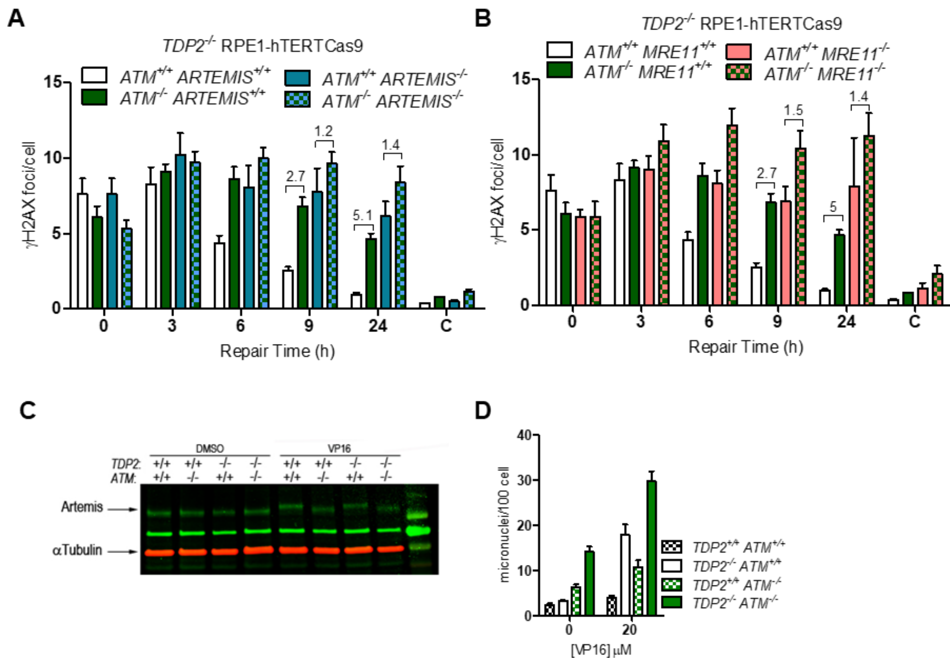


Figure 26: ATM is involved in nuclease pathway to repair TOP2-blocked DSBs. A, γH2AX foci induction after 30 min 10 µM etoposide treatment and repair at different times following drug removal in *$TDP2^{-/-}$ $ATM^{+/+}$ $ARTEMIS^{+/+}$*, *$TDP2^{-/-}$ $ATM^{-/-}$ $ARTEMIS^{+/+}$*, *$TDP2^{-/-}$ $ATM^{+/+}$ $ARTEMIS^{-/-}$* and *$TDP2^{-/-}$ $ATM^{-/-}$ $ARTEMIS^{-/-}$* serum starved arrested RPE1-hTERT Cas9 cells. Fold increase of removing ATM in ARTEMIS proficient and deficient background is indicated. Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments is shown. **B**, γH2AX foci induction after 30 min 10 µM etoposide treatment and repair at different times following drug removal in *$TDP2^{-/-}$ $ATM^{+/+}$ $MRE11^{+/+}$*, *$TDP2^{-/-}$ $ATM^{-/-}$ $MRE11^{+/+}$*, *$TDP2^{-/-}$ $ATM^{+/+}$ $MRE11^{-/-}$* and *$TDP2^{-/-}$ $ATM^{-/-}$ $MRE11^{-/-}$* serum starved arrested RPE1-hTERT Cas9 cells. Fold increase of removing ATM in MRE11 proficient and deficient background is indicated. Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments is shown. **C**, Shift in the mobility of ARTEMIS monitored by immunoblot, after 1h incubation with 100 mM etoposide of *$TDP2^{+/+}$ $ATM^{+/+}$*, *$TDP2^{+/+}$ $ATM^{-/-}$*#7, *$TDP2^{-/-}$ $ATM^{+/+}$* and *$TDP2^{-/-}$ $ATM^{-/-}$*#13 RPE1-hTERT cells. α-Tubulin was used as a loading control. **D,** Micronuclei accumulation monitored in the indicated RPE1-hTERT Cas9 cells after serum starvation arrest following 30 min 20 µM etoposide treatment, 24h of repair and finally serum addition for 48h. Histogram bars represent the average ± s.e.m. of n>500 cells from three independent experiments.

In more detail, removing ATM causes a 5,1-fold increase in the number of unrepaired γH2AX foci remaining following 24h of repair in *$TDP2^{-/-}$* cells but only 1,4-fold in the *$TDP2^{-/-}$ $ARTEMIS^{-/-}$* mutant. This suggests that the main contribution of ATM is related to ARTEMIS function, although it also has additional roles. In similar way, the loss of ATM in *$TDP2^{-/-}$ $MRE11^{-/-}$* did not show a substantial effect compared to the impact observed in *$TDP2^{-/-}$* single mutant (Figure 26B). At 24h after repair, again fold change in DSBs remaining unrepaired between *$TDP2^{-/-}$* single mutants and *$TDP2^{-/-}$ $ATM^{-/-}$*

double mutants was 5, while fold change between *TDP2$^{-/-}$ MRE11$^{-/-}$* double mutant and *TDP2$^{-/-}$ MRE11$^{-/-}$ ATM$^{-/-}$* triple mutant was 1,4. This indicates that, as in the case of ARTEMIS, the main contribution of ATM is also related to MRE11. This is consistent with the observations that ARTEMIS and MRE11 are working at the same pathway to remove the blockage in TOP2 breaks in the presence of DNA-PKcs. Nevertheless, as mentioned above, ATM seems aso to be involved in ARTEMIS-MRE11-independent functions. Furthermore, the fact that the impact of removing ATM in TDP2 deficient cells is lower than ARTEMIS or MRE11 suggests that there could be possible redundancy between ATM and other factors.

Since ATM was reported to be required for radiation-induced hyperphosphorylation of ARTEMIS (Riballo et al., 2004), it is conceivable that ATM could be regulating ARTEMIS in TOP2-blocked DSB repair through phosphorylation. Consistent with this idea, upon a high dose of etoposide exposure, ARTEMIS showed a shift in its mobility, that likely reflects hyperphosphorylation events and which was ATM dependent (Figure 26C). Nevertheless, although ARTEMIS is only necessary to repair etoposide-induced breaks in the absence of TDP2, the mobility shift was similar in wild-type and *TDP2$^{-/-}$* cells. Thus, ATM-mediated ARTEMIS phosphorylation is not dependent on the type of the structures generated at DNA ends. However, we cannot rule out the possibility that this event is specifically required for the repair of blocked-DSBs although occurring constitutively after DSB induction.

In addition, as described above, we studied the impact of removing ATM in genome integrity by analysing the induction of micronuclei formation after a low dose of etoposide treatment. As can be seen in Figure 26D, *ATM$^{-/-}$* single mutant showed slightly higher levels of micronuclei than wild-type cells after etoposide incubation, but these likely represented increased basal levels of genome instability and not specific etoposide-induced events, since micronuclei in untreated samples were similarly increased.This is consistent with the fact that ATM does not have a role in the repair of TOP2-breaks in TDP2 proficient background, but it specifically facilitates repair in the absence of TDP2. However, removing ATM in TDP2 deficient cells further increased

the induction of micronuclei formation observed in *TDP2*$^{-/-}$ single mutant, as previously observed in primary MEFs (Álvarez-Quilón et al., 2014). This indicates a role of ATM in maintaining genome integrity when TDP2 is not present, as previously suggested (Álvarez-Quilón et al., 2014), and is thus, consistent with an additional role of ATM besides its contribution on end-processing by ARTEMIS and MRE11 nucleases.

10. ATR activity is specifically required to repair TOP2-induced DSBs in TDP2 deficient background and is more relevant in the absence of ATM.

As mentioned above, ATM does not seem to be absolutely required for the ARTEMIS-MRE11pathway, suggesting the possible existence of redundant activities. ATR is another PIK Kinase that shares some substrates with ATM and is activated upon ssDNA generation, so we reasoned that it could also be involved in the repair of TOP2-blocked DSBs. As can be observed (Figure 27), ATR inhibition by AZ-20 (Foote et al., 2013) did not affect the repair rate of etoposide-induced DSBs in TDP2 proficient cells, regardless of the presence of ATM. Nevertheless, *TDP2*$^{-/-}$ cells showed an increased repair defect upon ATR inhibition. This supports that ATR could be activated after the generation of ssDNA due to the action of nucleases, which remove TOP2 blockages exclusively in the absence of TDP2. In addition, the loss of ATM increased this repair defect, suggesting that ATR activity could be somewhat redundant with ATM in the regulation of this nucleolytic pathway, and also indicating that ssDNA generation may be exacerbated in the absence of ATM, which would entail an additional function of ATM regarding end protection.

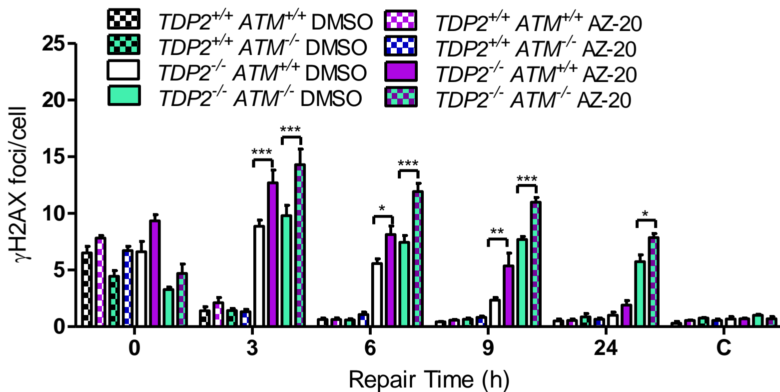


Figure 27: ATR activity is specifically required for repair TOP2-induced DSBs in TDP2 deficient background. A, γH2AX foci induction after 30 min 10 µM etoposide treatment and repair at different times following drug removal in *TDP2*$^{+/+}$ *ATM*$^{+/+}$, *TDP2*$^{+/+}$ *ATM*$^{-/-}$#7, *TDP2*$^{-/-}$ *ATM*$^{+/+}$ and *TDP2*$^{-/-}$ *ATM*$^{-/-}$#13 serum starved-arrested RPE1-hTERT Cas9 clones with or without 10 µM ATR inhibitor (AZ-20). Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments and the minimal statistical significance by two-way ANOVA test with Bonferroni post-test are shown (*P≤0.05; **P≤0.01; ***P≤0.001).

11. RNF168 facilitates repair of TOP2-DSBs exclusively in TDP2 deficient background and this function is partially mediated by ATM.

Beyond its contribution to end-processing, we therefore, turned our attention to end protection as an additional function of ATM in blocked-DSB repair. This can be operated by promoting modifications at the DSB, such as for example γH2AX (Helmink et al., 2011), and 53BP1 recruitment (Anderson et al., 2002), which are actually ATM substrates. To address the contribution of the 53BP1-mediated end protection to TOP2-blocked break repair, *RNF168* (coding for an upstream factor in 53BP1 recruitment pathway), *53BP1* and *RIF1* (downstream factor of 53BP1) were disrupted by transfecting *TDP2*$^{-/-}$ RPE1-hTERT-Cas9 cells with gRNAs targeting these genes and γH2AX disappearance was monitored after etoposide treatment as previously described (Figure 15). As can be seen, the absence of RNF168 did not affect the rate of repair in *TDP2*$^{+/+}$ background. In contrast, a significant decrease in the repair rate was observed in the combined mutant *TDP2*$^{-/-}$ *RNF168*$^{-/-}$ (Figure 28A), supporting that RNF168 has a role in TOP2-blocked DSB repair. Unfortunately, although the loss of

53BP1 or RIF1 showed a similar impact, it did not reach statistical significance (Figure 28B,C). These data support the idea that RNF168 is involved in TOP2-DSB repair when the ends are blocked, although more experiments should be performed to conclude if 53BP1-RIF1 are downstream factors for this pathway, or the role of RNF168 is related to an additional function.

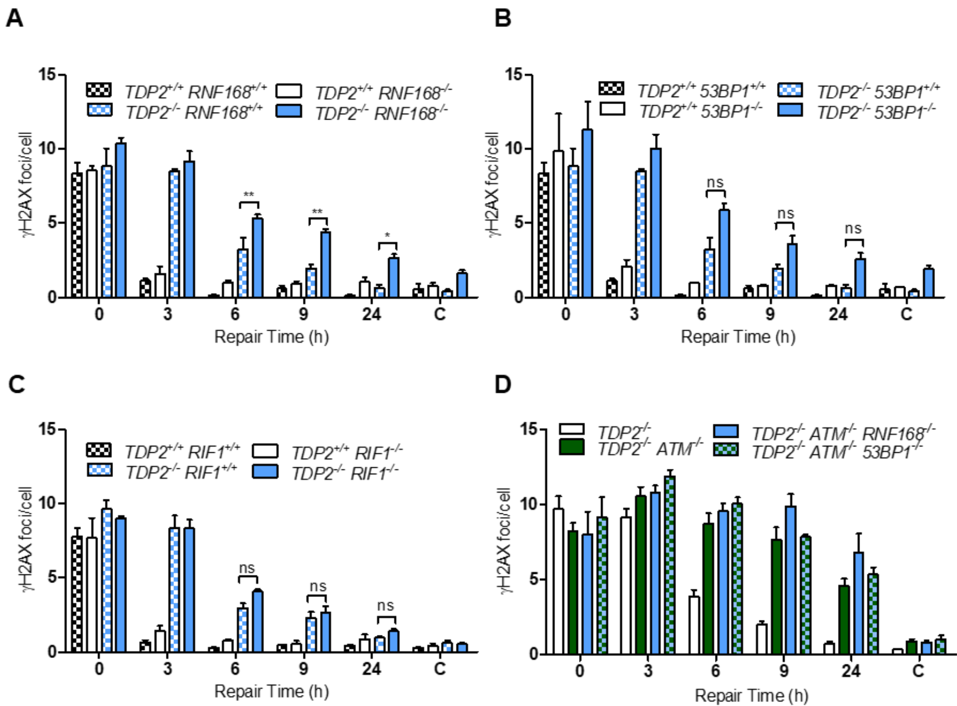


Figure 28: RNF168 contributes to the repair of TOP2-induced DSBs but exclusively when TDP2 is absent. A, B, C, D, γH2AX foci induction after 30 min 10 μM etoposide treatment and repair at different times following drug removal in the indicated serum starved arrested RPE1-hTERT cells. Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments and the minimal statistical significance by two-way ANOVA test with Bonferroni post-test are shown (*P≤0.05; **P≤0.01; ***P≤0.001).

Additionally, to address the contribution of this pathway in the repair of blocked DSBs at ATM deficient background, *TDP2-/- ATM-/-* RPE1-hTERT-Cas9 cells were transfected with gRNAs targeting *RNF168* or *53BP1* (RIF1 was not analysed as its recruitment is totally dependent on ATM (J. Ross Chapman et al., 2013)) and γH2AX disappearance

was monitored after etoposide treatment as previously explained (Figure 15). As can be seen, in contrast to the strong deficiencies caused in *TDP2$^{-/-}$* cells, loss of RNF168 only slightly increased the repair defect observed in *TDP2$^{-/-}$ ATM$^{-/-}$*, whereas 53BP1 deficiency showed almost negligible effect (Figure 28D). This suggests that the function of RNF168 is partially mediated by ATM, suggesting that, in addition to facilitating the ARTEMIS-MRE11 pathway to repair TOP2-blocked DSBs, ATM could prevent excessive end processing through the action of RNF168 and possibly 53BP1-RIF1.

12. The preference to repair TOP2-DSBs by TDP2 over nucleases is conserved in murine cells.

Up to date, mice have been widely used as model organisms to study human biology due to the relatively genetic and physiological similarities between these species (Perlman, 2016). Thus, they could be a useful tool to study the physiological consequences that pathway choice has in the repair of TOP2-DSBs. Because of these reasons, first we decided to characterize the involvement of aforementioned factors in the repair of TOP2-blocked DSBs in primary mouse embryonic fibroblast (MEFs) and analyse whether the established hierarchy previously observed is conserved.

In order to determine the impact of the loss of DNA-PKcs in the repair of TOP2-DSBs, MEFs from SCID mice, harbouring a DNA-PKcs defective protein (Blunt et al., 1996), and *Tdp2$^{-/-}$ DNA-Pkcs$^{scid/scid}$* double mutants were obtained and were confluency-arrested before performing the experiments. As can be seen, *DNA-Pkcs$^{scid/scid}$* did not show a repair defect upon etoposide treatment in TDP2 proficient background. In contrast, cells from *Tdp2$^{-/-}$ DNA-Pkcs$^{scid/scid}$* double mutants showed a significant increase in repair defect observed in *Tdp2$^{-/-}$* single mutants (Figure 29A). Thus, this suggests that the presence of DNA-PKcs is only required in the repair of TOP2-breaks when the ends are blocked, sharing a similar phenotype with DNA-PKcs absence in RPE cells, and indicating a conservation in rodent cells.

On the other hand, although TDP2 proficient cells showed an important repair defect upon DNA-PKcs inhibition, the repair defect is higher in TDP2 deficient background (Figure 29B). This suggests a difference between response to etoposide from human and mouse cells regarding DNA-Pkcs activity, that could be due to a strong difference in expression levels of the protein in both species (Finnie, Gottlieb, Blunt, Jeggo, & Jackson, 2006). In this context, whereas DNA-PK is relatively abundant in all mammalian cells, primate cells express ~50 times more DNA-PK activity than rodent cells (Finnie et al., 2006).

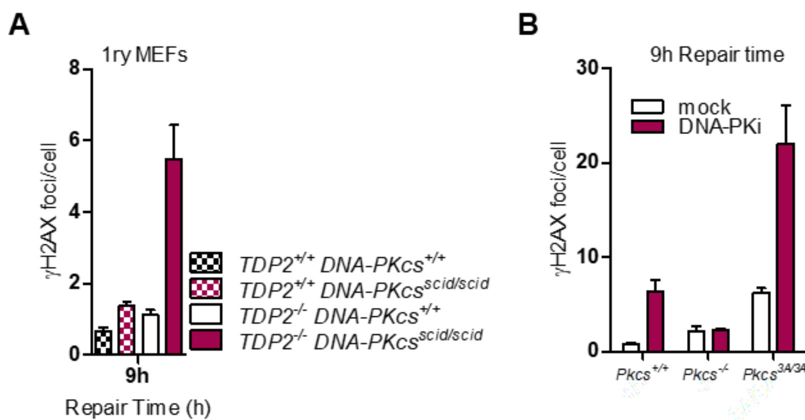
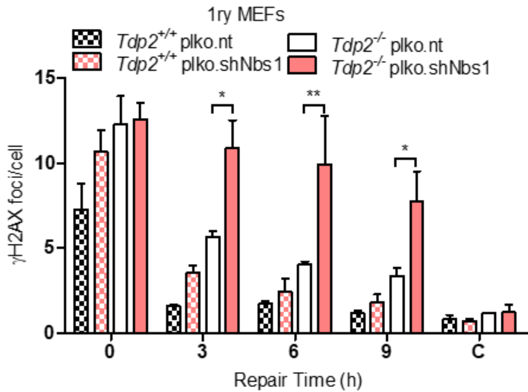


Figure 29: Analysis of DNA-PKcs contribution in the repair of TOP2-DSBs in 1ry MEFS. A, γH2AX foci remaining at 9h repair time after 30 min 10 μM etoposide treatment following removal in *Tdp2*$^{+/+}$ *PKcs*$^{+/+}$, *Tdp2*$^{+/+}$ *PKcs*$^{scid/scid}$, *Tdp2*$^{-/-}$ *PKcs*$^{+/+}$ and *Tdp2*$^{-/-}$ *PKcs*$^{scid/scid}$ confluency-arrested primary MEFs. Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments is shown. **B**, γH2AX foci remained at 9h repair time after 30 min 10 μM etoposide treatment following removal in *PKcs*$^{+/+}$, *PKcs*$^{-/-}$ and *PKcs*$^{3A/3A}$ confluency-arrested primary MEFs with or without 10 μM DNA-PKcs inhibitor (NU7441 (KU-57788)). Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments is shown.

Furthermore, as it was reported that DNA-PKcs phosphorylation at ABCDE cluster mediates conformational change in the DNA-PK complex that it is critical for end processing (Ding et al., 2003; Reddy et al., 2004), we wondered which was the contribution of the DNA-PKcs phosphorylation at the cluster ABCDE in etoposide-induced DSB repair. As can be seen in Figure 29B, mutant primary MEFs with three

alanine substitutions in the ABCDE cluster (*DNA-PKcs$^{3A/3A}$* mutant) (S. Zhang et al., 2011) displayed an increase in repair defect upon etoposide treatment, suggesting that phosphorylation at these residues is relevant for the repair of TOP2-DSBs. Nevertheless, other autophosphorylation events must be required, as *DNA-PKcs$^{3A/3A}$* mutant presented a dramatic repair defect upon DNA-PKcs inhibition, reaching levels of almost non repair at all. Moreover, *DNA-Pkcs$^{-/-}$* primary MEFs did not show repair defects after etoposide treatment neither with nor without DNA-PK inhibitor, further confirming that DNA-Pkcs presence is only required to repair TOP2-DSBs in the absence of TDP2, and ruling out possible artefacts due to unspecificity of the inhibitor.

Next, we aimed to analyse if the function of the MRN complex in TOP2-break repair was conserved. A marked increase in DSB-repair defect was observed when Tdp2 deficient cells were depleted for NBS1, and not in TDP2 proficient background (Figure 30A). This suggests that in primary MEFs, the MRN complex is specifically required to repair TOP2-DSBs in the absence of TDP2 in a similar way as in RPE human cells. This specific repair defect for TDP2 deficient cells was also observed upon inhibition of the exonuclease activity with MIRIN, although to a lesser extent than in RPE human cells (Figure 30B). Nonetheless, this inhibition highly increased the repair defect observed in *Tdp2$^{-/-}$ Atm$^{-/-}$* double knock-out primary MEFs, suggesting that in mouse cells the exonuclease activity of the MRN complex is only required upon TDP2 deficiency and is more relevant in ATM deficient background. Thus, although the individual function of ATM and MRN in the repair of TOP2-blovked DSBs is similar, their relationship is not fully conserved between human and murine cells.



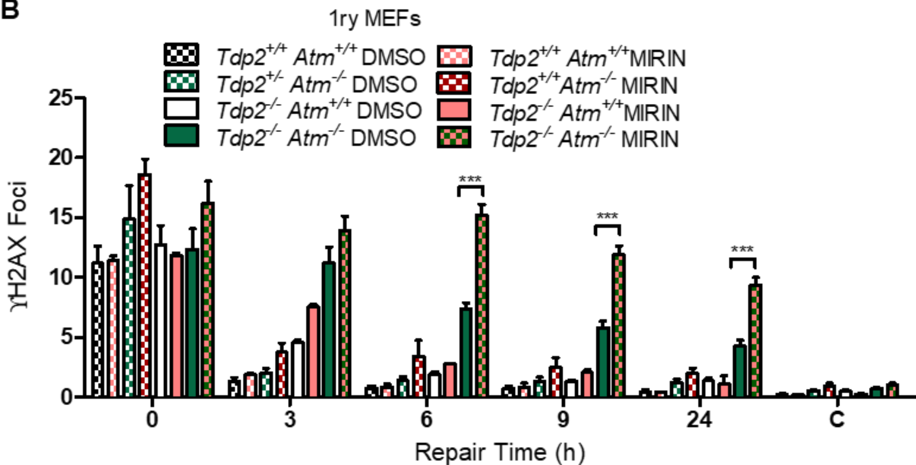


Figure 30: Analysis of the contribution of the MRN complex in the repair of TOP2-DSBs in 1ry MEFS. **A,** γH2AX foci induction after 30 min 10 µM etoposide treatment and repair at different times following drug removal in confluency-arrested TDP2 proficient or deficient primary MEFs depleted (shNbs1) or not (nt) for NBS1. Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments and the minimal statistical significance by two-way ANOVA test with Bonferroni post-test are shown (*P≤0.05; **P≤0.01; ***P≤0.001). **B**, As above, in the indicated confluency-arrested with or without 100 µM exonuclease activity inhibitor of MRN complex (MIRIN).

In the same line of the results obtained upon MIRIN treatment, incubation with the ATR inhibitor AZ-20 slightly diminished the repair rate of TOP2-induced DSBs in primary MEFs, but had a specifically relevant effect in *Tdp2-/-* background (Figure 31). In addition, this increase in the repair defect in TDP2 deficient cells was exacerbated by ATM deficient cells, further confirming their functional redundancy and the ssDNA

generation is more relevant for the repair of blocked-TOP2 DSBs when ATM is absent in this organism.

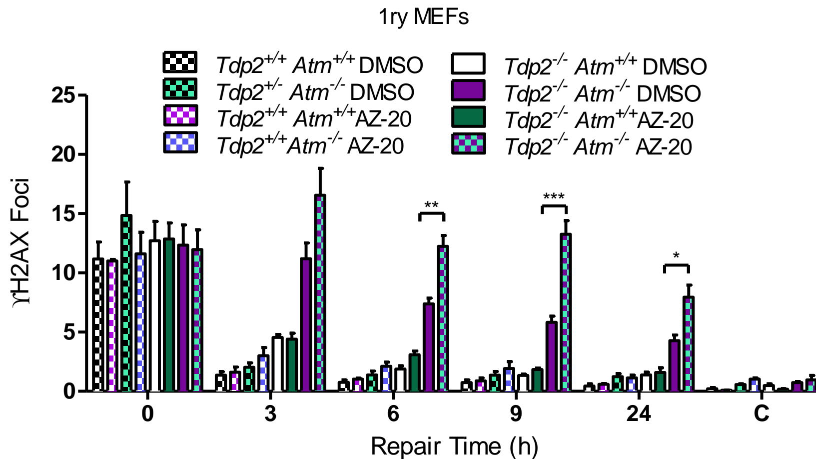


Figure 31: Inhibition of ATR activity in 1ry MEFS facilitates the repair of TOP2-DSBs exclusively in the absent of both TDP2 and ATM. γH2AX foci induction after 30 min 10 μM etoposide treatment and repair at different times following drug removal in the indicated confluency-arrested primary MEFs with or without 10 μM ATR inhibitor (AZ-20). Average ± s.e.m. of the total number of foci per cell remaining from at least three independent experiments and the minimal statistical significance by two-way ANOVA test with Bonferroni post-test are shown (*P≤0.05; **P≤0.01; ***P≤0.001).

Overall, we can conclude that, despite some differences, the strong preference for TDP2 unblocking activity over end-processing by nucleases is also observed in mouse cells, and, therefore, this established hierarchy is conserved in mouse.

13. TDP2 avoids malignant transformation

Given the conservation of this hierarchy in rodent cells, primary MEFs were used to analyse how the increase in genome instability, that is caused by TDP2 deficiency, affects malignant transformation. To address this, *Tdp2*$^{+/+}$ and *Tdp2*$^{-/-}$ primary MEFs were used to perform a 3T3 protocol that relies on spontaneous mutation to achieve immortalization (Xu, 2005). Results showed that cells from both genotypes proliferated with comparable rates at first passages, as measured by cells counting (Figure 32).

However, *Tdp2*$^{-/-}$ primary MEFs escaped senescence earlier than TDP2 proficient cells, as a difference in cell growth can be observed from 10th passage and had an increased colony-forming capacity. This early immortalization supports that the lack of TDP2 increases the probability of malignant transformation, possibly through the observed compromise of genome integrity.

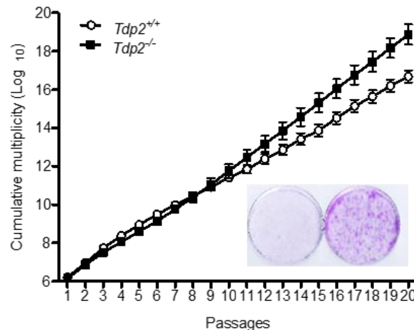


Figure 32: TDP2 deficient involves an earlier immortalization in primary MEFs. 3T3 analysis of *Tdp2*$^{+/+}$ and *Tdp2*$^{-/-}$ primary MEFs. Graph indicates the passage numbers at which wild-type or TDP2 deficient MEFs became immortalized.

Considering that TDP2 deficiency is related with an increase in genome instability and malignant transformation, we decided to assess the physiological consequences of TDP2 absence *in vivo*. Previously, it was demonstrated that *Tdp2*-deleted mice are sensitive to TOP2-damage induced by a single injection with a high dose of etoposide, displaying marked lymphoid toxicity, severe intestinal damage, and increased genome instability in the bone marrow, as compared to wild type animals (Gómez-Herreros et al., 2013). To address the implications of TDP2 in cancer, we analysed the incidence of tumours in 24 months-lifespan experiments in the TDP2 mouse model. A group of mice (8 weeks) were additionally treated with intraperitoneal injections of etoposide in order to mimic chemotherapeutical doses. As observed in Figure 33A, TDP2 deficient mice displayed a lower lifespan not only after etoposide treatment (showing a median survival of 475,5 days, in contrast to 571 days in *Tdp2*$^{+/+}$ mice), but also in non-treated controls (TDP2 deficient animals exhibited a median survival of 581,5 days in contrast

to wild-type mice that showed 668,5 days). This reduced lifespan was due to an increased tumour incidence (Figure 33C,D) either after etoposide treatment or in non-treated controls. We found that they underwent tumours of a very wide origin and, strikingly, almost 30% of TDP2 deficient mice developed more than one tumour of a different origin (Figure 33B), which is unusual in wild-type animals. Therefore, these data support an important tumour suppressor role of TDP2, suggesting that a hyerarchical response to TOP2-induced DSBs is important to prevent tumorigenesis. Noteworthy, mice experiments were performed together with Dr. Rocío Romero-Granados and technicians from Dr. Felipe Cortés-Ledesma's laboratory.

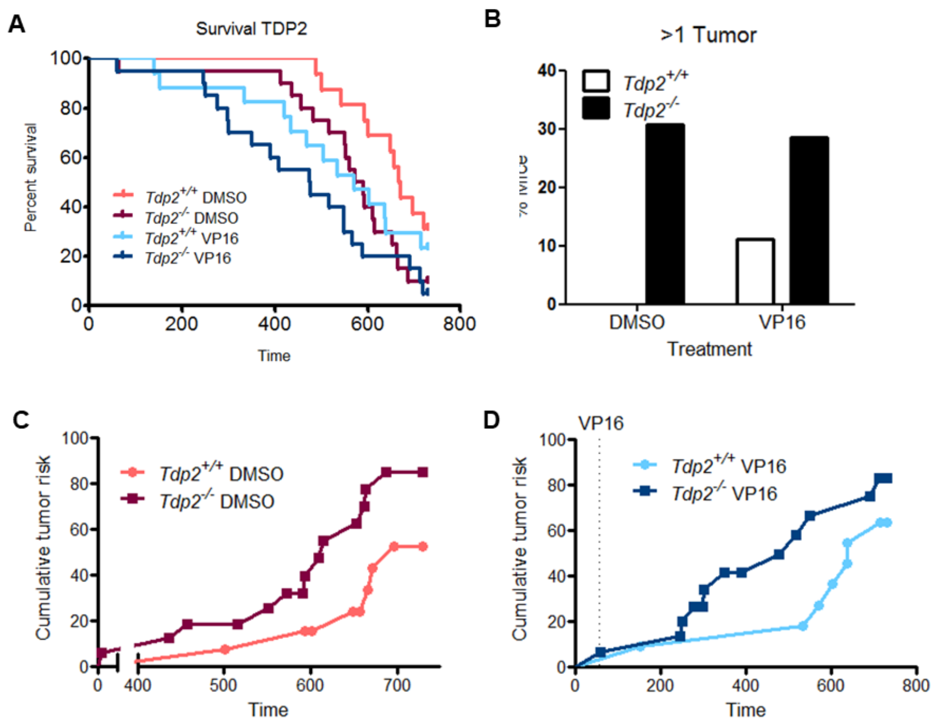


Figure 33: TDP2 deficient mice display a lower lifespan due to an increase in tumor incidence. A, Kaplan-Meier survival curves of *Tdp2*$^{+/+}$ and *Tdp2*$^{-/-}$ mice following treatment with 4mg/kg etoposide or DMSO for 5 days. *Tdp2*$^{-/-}$ mice showed a shortened life span compared with TDP2 proficient mice in non-treated control (median survival = 668,5 days in *Tdp2*$^{+/+}$ vs. 581,5 days in *Tdp2*$^{-/-}$) as well as in etoposide-treated mice (median survival = 571 days in *Tdp2*$^{+/+}$ vs. 475,5 days in *Tdp2*$^{-/-}$). At least (continue on next page…) (…continued) 16 animals per group were included in the analysis. **B**, Percentage of TDP2 proficient and deficient mice showing more than one tumor from different origins after etoposide treatment described above or non-treated controls. **C**, Representation of percentage of mice that underwent tumor in *Tdp2*$^{+/+}$ and *Tdp2*$^{-/-}$ DMSO-treated controls. **D**, As above, cumulative tumor risk after etoposide treatment previously described.

To determine whether changes in the response to TOP2-induced DSBs could indeed be linked to cancer in human patients, Oncomine database (https://www.oncomine.org/resource/main.html) was used and an analysis of published gene expression data was performed. Interestingly, the mRNA expression of *TDP2* in colorectal cancers was found to be markedly lower than in matched normal tissue (Figure 34). Moreover, the same type of cancers exhibited an upregulation of MRE11, which would suggest an imbalance in the repair of these breaks towards the nucleolytic pathway (Figure 34).

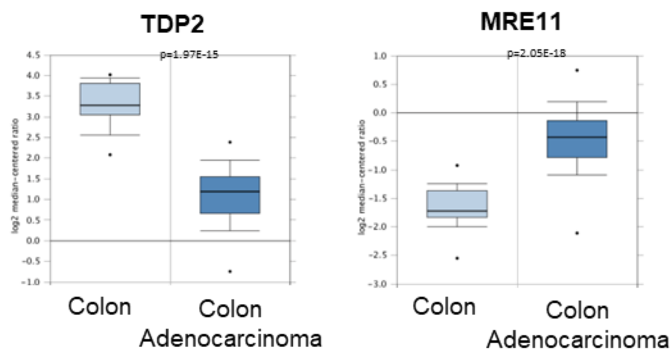


Figure 34: Colorectal cancers show a markedly lower mRNA expression of TDP2 and an upregulation of MRE11. TDP2 and MRE11 gene expression in colorectal cancer using the Oncomine gene expression tool (https://www.oncomine.com).

This further support the crucial relevance of the established hierarchy in which TDP2 preferentially operates to unblock TOP2-DSBs which would avoid end-processing through nucleases and the subsequent increase in genome instability. Furthermore, these data suggest that endogenously-occurring TOP2 breaks significantly contribute to tumour development.

IV. DISCUSSION

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Historically, the study of how the different configurations of DNA ends affect the repair of DSBs and its outcome has been impeded by the difficulty to induce homogeneous populations of lesions with the desired structures. Indeed, the majority of DNA damaging agents used for this purpose induce a wide variety of chemical structures at DNA ends, IR being an example of this. Besides DSBs, IR generates a plethora of nucleotide and base modifications, as well as SSBs (Hagen, 1994; J. F. Ward, 1988), and a small fraction of DSBs induced by IR would harbour complex ends. This could be the reason why IR-induced DSBs are repaired with biphasic kinetics involving a fast and a slow process (DiBiase et al., 2000; Riballo et al., 2004). The slow component would represent the repair of DSBs harbouring complex ends or the ones induced in heterochromatin. Additionally, high-energy radiation increases the frequency of the induction of clustered DSB with aberrant structures (Brenner & Ward, 1992). Therefore, following exposure to α particles, the majority of DSBs are repaired with slow kinetics (Barton et al., 2014; Shibata et al., 2014). Thus, α-IR has been used as a tool to analyse the repair of DSBs harbouring complex or blocked structures at the ends. Nevertheless, the heterogeneity of lesions generated impedes a clear interpretation of the results. In addition, there are more molecularly controlled systems that have shed some light on the process of the repair of blocked DSBs. For instance, V(D)J recombination, which relies on RAG recombinase in specific loci during lymphocyte development, generates at the same time two types of DSBs, a hairpin blocked DSB in the coding end and a clean blunt-ended DSB in the signal end, allowing the comparison of the contribution of different NHEJ factors. Several *in vitro* and *in vivo* systems are based on this process, with a main focus on immunology research (Bredemeyer et al., 2008; Ramsden, Paull, & Gellert, 1997). These tools however, are limited by the peculiarities of the V(D)J recombination process. Additionally, several *in vitro* studies have been performed to evaluate the contribution of each NHEJ component to the efficiency of the repair in different NHEJ substrates harbouring various types of structures at DNA ends (Chang

et al., 2016; Deshpande et al., 2016b). However, *in vivo* analyses that provide a more representative situation of physiological conditions are lacking.

It is tempting to think that specific unblocking activities that restore complex ends to conventional 5′ phosphate and 3′ hydroxyl ligatable termini must be preferred over end-processing by general nucleases in order to avoid sequence modification. In this regard, there is some controversy about the existence of a hierarchical order in which NHEJ accessory factors operate. On one hand, it has been proposed that they all behave in an iterative way without an established order (Lieber, 2010). Alternatively, it has been also proposed that there is a hierarchy in which cells give preference to resolution paths with the fewest number of enzymatic steps (Waters et al., 2014).

In any case, under certain circumstances, complex DSBs can require end-processing. Therefore, cells must have evolved mechanisms to avoid sequence changes or loss by restricting the action of nucleases to the minimum possible when the ends require trimming. Consistent with this putative threat of end-processing to genome stability, DSBs generated during metabolism, such as the ones generated by TOP2 activity, are usually characterized by compatible ends with complementary overhangs that ensure easy joining, except in those cases in which sequence variability is required, as it happens in V(D)J recombination, where incompatible ends that need end-processing for repair are generated. In this regard, how cells minimize and modulate processing to maintain genome integrity is still a key unanswered question in the field.

Taking the advantage of the recently developed genetic strategy to induce populations of DSBs that are homogeneous in end-structure, in our study we have dissected pathways required to repair TOP2-DSBs harbouring specifically clean or blocked-ends in G0/G1. We have characterized candidates from unbiased analyses, which allowed us to identify unsuspected factors, and we have performed a candidate based approach to address the contribution of known factors that are related with our previous candidates. Furthermore, we have been able to distinguish the relevance of each factor according to the type of DNA ends generated, and the relationship between them.

Finally, we have found that there is an established hierarchical sequence of different pathways to repair TOP2-DSBs which contributes to ensure genome stability. In this regard, we have also assessed the consequences of disrupting this established hierarchy.

1. Molecular characterization of repair of TOP2-DSBs

1.1. Preference of the unblocking activity of TDP2 over end-processing

The nucleolytic activity of ARTEMIS in opening hairpin-sealed coding ends during V(D)J recombination has been well dissected (Ma et al., 2002). Besides its role in V(D)J recombination, in the context of NHEJ, ARTEMIS-deficient cells are radiosensitive, but the majority of the DSBs in these cells are repaired efficiently (Moshous et al., 2001; Nicolas et al., 2002). Specifically, only ~20% of X ray IR-induced DSBs require ARTEMIS for repair, corresponding to the fraction of DSBs repaired by the slow repair process (Riballo et al., 2004). Additionally, the requirement of ARTEMIS is more relevant after α particle-IR in G1 (Shibata et al., 2017), suggesting that ARTEMIS is required for the repair of DSBs with complex end-configurations. However, the interpretation of these observations could be confounded by the heterogeneity of lesions generated. Moreover, *in vitro* analysis determine that ARTEMIS is necessary for NHEJ of DNA substrates with incompatible 5′ and 3′ overhangs and some blunt ends that form transient single to double strand boundaries that have structural similarities to hairpins (Chang et al., 2016, 2015). Through CRISPR-Cas9 genetic screening and following studies, we have established that inactivation of gene encoding ARTEMIS results in a strong repair defect of TOP2-DSBs exclusively when TDP2 is not present. This leads us to conclude that ARTEMIS is only required to repair blocked DSBs and there is a strong preference to repair TOP2-DSBs through the TDP2 pathway over this nucleolytic end-processing pathway. Our results show for the first time that the requirement for ARTEMIS depends on structure of the ends *in vivo*, further confirming that it has a wider function in the NHEJ pathway besides V(D)J recombination, specifically when the ends need the end processing because of their complexity.

In addition to ARTEMIS-dependent processing, which is restricted to a few nucleotides away from the ends, the MRN complex can act hundreds of nucleotides upstream of the DSB termini (Garcia, Phelps, Gray, & Neale, 2011). Remarkably, MRE11 loss shows a negligible impact on repair rate of etoposide-induced DSBs when TDP2 is present. As happened with ARTEMIS deficient cells, MRE11 only facilitates the repair of TOP2-DSBs in TDP2 deficient background. These data are consistent with the fact that the NBS1 subunit of the MRN complex is a top hit from the etoposide screen exclusively in *TDP2*$^{-/-}$ cells. Similarly, NBS1 depletion in primary MEFs only has a relevant effect in TDP2 deficient background, which leads us to think that this specific role of MRN complex in the repair of blocked-DSBs is conserved in mice. In addition, this specific repair defect caused by the deficiency of MRN complex in TDP2 deficient cells is also observed upon exo or endonuclease activity inhibition. Nevertheless, the repair defect levels are lower than in MRE11 deficient cells. This could be due to these drugs not completely inhibiting nuclease activities. However, other possibilities cannot be ruled out such as MRE11 having additional structural function that is distinct from its enzymatic activity or both nuclease activities independently collaborating in processing the ends in TDP2 deficient cells.

Previously, the role of MRE11 nuclease activity was described during repair of other sorts of blocked-DSBs that require end-processing, such as DNA ends covalently-bound to SPO11, a relative of type 2 topoisomerases (Neale et al., 2005), or terminated by hairpins (Lobachev et al., 2002). Interestingly, these kinds of end-configurations lack an unblocking activity to become canonical 5′ phosphate and 3′ hydroxyl termini, which supports the role of MRN complex specifically when the ends are irreversibly blocked and require end-processing. In addition, recent *in vitro* studies described that NBS1 is essential to promote MRE11 nuclease activities on DNA ends containing protein adducts, while it inhibits MRE11 3′ to 5′ exonuclease degradation of clean ends (Deshpande et al., 2016b), further supporting NBS1 being a top hit from the etoposide screen specifically in TDP2 deficient background.

In contrast, the nucleolytic activity of MRE11 was reported to function in the cellular response to etoposide also in a TDP2 proficient background in previous studies (Hoa et al., 2016b; Quennet et al., 2011). Our results indicate that this is not the case, and, at least in these circumstances cells show a strong preference for repairing TOP2-DSBs by the unblocking activity of TDP2 in G0/G1 cell cycle phase. Exclusively when TDP2 is not present, MRE11 has a crucial function (Figure 35). In the case of Hoa et al., 2016, this contradictory observations could be due to the fact that, although they analyse repair rate in G1 cells, they do not arrest the cells. Therefore, cells in the G1 cell cycle phase at repair time point analysed could have been in S/G2 phases when receiving the damage, as cells can progress through mitosis with a relatively low number of DSBs. Moreover, in their study they do not show total number of γH2AX foci remaining unrepaired but the percentage of γH2AX foci-positive cells, which could lead to misinterpretations as we consider that repair rate can be hardly assessed by this observation and relevant information could be missed. Additionally, the type of cells could also affect. In Hoa et al., 2016 TK6 human and DT40 chicken cells are used, and could not represent normal physiological conditions. For instance, the regulation of the preference for TDP2 could be disrupted. In this regard, the overexpression of TDP2 in these cell lines significantly reverses genome instability and mortality caused by deficiency of MRE11, supporting a putative imbalance in the pathway choice for repair towards end-processing over unblocking, which could be a consequence of the specific circumstances of these transformed and tumour cells. Secondly, in Quennet et al., 2011 it is also shown that confluency-arrested cells from ATLD2 (Ataxia-telangiectasia-like disorder) patients, in which MRE11 is mutated, display a repair defect upon induction of DSBs by etoposide. However, due to the lack of negative controls without treatment, we cannot rule out that these cells have accumulated spontaneous lesions before being arrested. In addition, another study reported the role of MRE11 nuclease activity in removing TOP2 adducts from 5′ ends in fission yeast (Hartsuiker et al., 2009). Nevertheless, since the yeast genome does not encode for TDP2 protein, MRE11 could be operating the function of TDP2 in this organism.

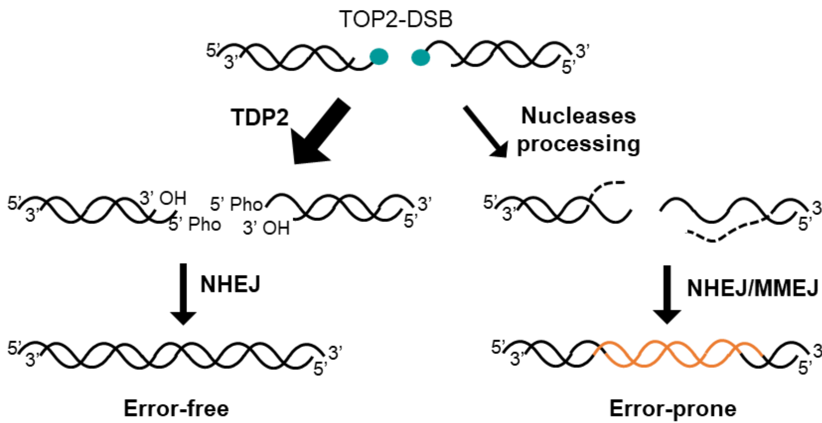

Figure 35: Established hierarchy for the repair of TOP2-DSBs. TDP2 unblocking activity, which directly convert ends into 5'phospahte and 3' hydroxyl and the nucleotide sequence remaining intact (left), is preferred over end-processing, which can remove TOP2 adducts from DNA ends by nucleotide trimming (right). This pathway can lead to error-prone repair when NHEJ or MMEJ are used.

Interestingly, we see an epistatic relationship between ARTEMIS and MRE11, indicating that they both work in the same pathway for repairing TOP2-DSBs in the absence of TDP2. Under these circumstances, one could think that ARTEMIS is recruited to induce an initial endonucleolytic cleavage event followed by exonucleolytic degradation by MRN complex towards the blocked end (Figure 36). This would be contradictory with the fact that the endonuclease inhibition of MRE11 also shows a repair defect in TDP2 deficient cells when ARTEMIS is present. However, it has been demonstrated that the 3′-to-5′ exonucleolytic degradation from a nick towards a blocked end is sensitive to the endonuclease inhibitor PFM01, even when the nick is already generated in the substrate (Deshpande et al., 2016b). Therefore, we cannot conclude whether the endonucleolytic activity of MRE11 is relevant for the repair of TOP2-DSBs, but we do demonstrate that its exonucleolytic activity is required. Nevertheless, the fact that ARTERMIS preferentially cuts directly at the ss/dsDNA boundary at 5′ overhangs (Chang et al., 2015) could contradict this model as an exonuclease activity would not be necessary. Alternatively, the exonucleolytic activity of the MRN complex could act before, promoting the formation of a hairpin which would be a substrate for ARTEMIS (Figure 36). Unfortunately, we cannot establish the

exact molecular mechanism by which these nucleases are operating with our current data and additional experiments would be required to shed light on to these questions.

Our results strongly support the notion that cells have a robust preference for the repair of TOP2-DSBs by tyrosyl DNA phosphodiesterase activity of TDP2. Exclusively when TDP2 is not available, end-processing nucleases function to repair DSBs harbouring blocked ends. Although in a less efficient manner, end-processing pathways are able to repair most of TOP2-DSBs induced when TDP2 is not present. However, we demonstrate that they do so at the expense of increasing genetic instability. Specifically, upon a high dose of etoposide treatment, wild-type cells show a high induction of micronuclei. Surprisingly, these levels of micronuclei formation are significantly lower in the absence of ARTEMIS or upon MRE11 nucleolytic activities inhibition. We reasoned that, upon such a high dose of etoposide treatment, TDP2 activity could be overwhelmed, and, therefore, nuclease pathways could operate even in *TDP2*$^{+/+}$ cells. Thus unbalancing the established hierarchy to repair TOP2-DSBs preferentially by TDP2, and compromising the maintenance of genome integrity. It worth noting that the levels of DSBs remaining at 24 hours after repair, time point in which we added serum to allow cells to proliferate and form micronuclei, are very similar between all conditions. This supports that the action of nucleases contributes to an increase in genome instability and suggests that the difference in micronuclei induction observed is not due to a check-point activation that would avoid cell proliferation and would impede micronuclei formation. The same repair defect between wild-type, *ARTEMIS*$^{-/-}$ or cells treated with inhibitors of MRE11 makes sense, as although TDP2 capacity is initially overwhelmed upon this high dose of etoposide, repair for 24 hours allows TDP2 taking over if the other pathway has not engaged. Therefore, under these conditions deficiency in nucleases does not have deleterious consequences for repair and, paradoxically, protects genome integrity. Consistent with this, deletion of *Tdp2* also causes an increase in etoposide-induced micronuclei in transformed MEFs (A. Álvarez-Quilón et al., 2014) and TDP2 is reported to supress chromosome rearrangements induced by TOP2 and reduce TOP2-induced chromosome

translocations that arise during transcription (Gómez-Herreros et al., 2017), further supporting our observations.

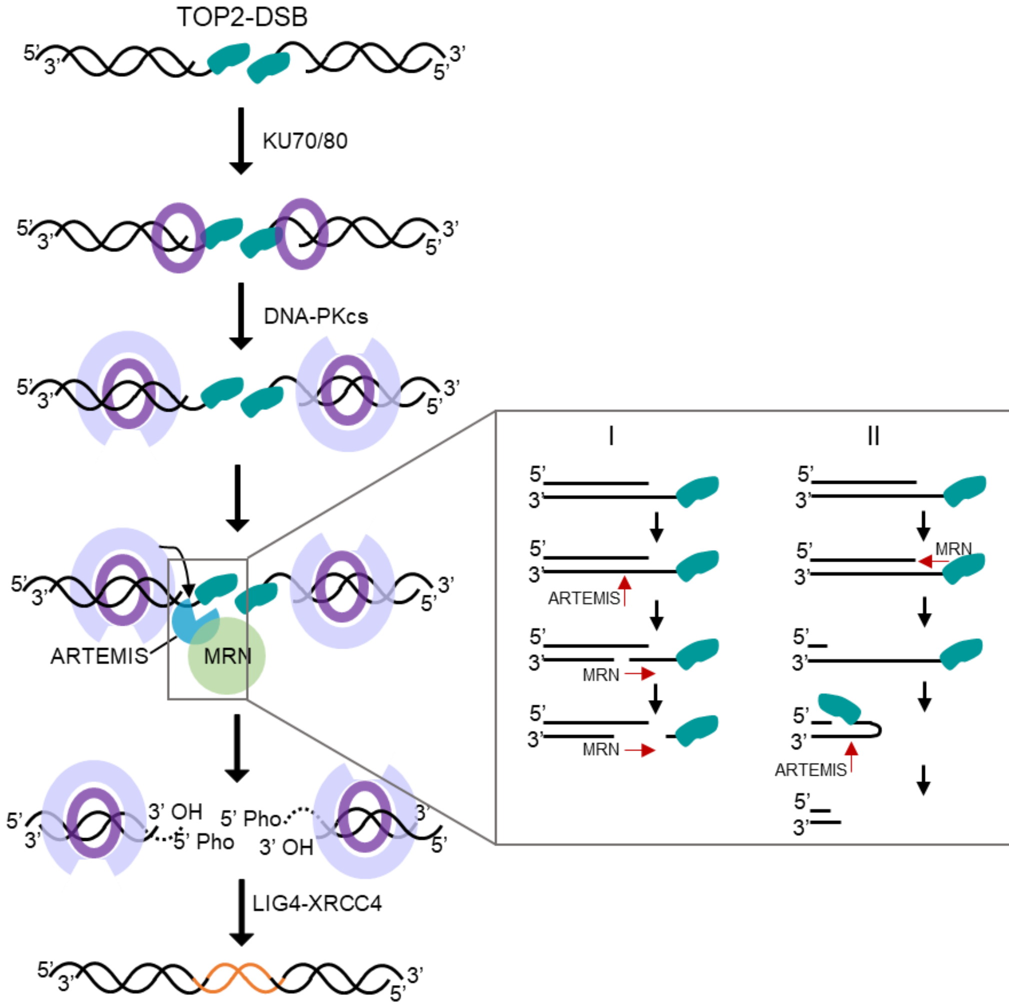

Figure 36: Model for the repair of TOP2-DSBs in TDP2 deficient background. TOP2-DSBs are recognized by the ring-shaped KU70/80 heterodimer. Then, DNA-PK catalytic subunit (DNA-PKcs) is recruited, translocating KU70/80 ring inward the break. DNA-PKcs commits the repair through ARTEMIS, which works together with MRE11. ARTEMIS can induce an initial endonucleolytic cleavage followed by exonucleolytic degradation by the MRN complex towards the blocked end (scenario I), or on the other hand, the MRN complex can act before, somehow generating a substrate for ARTEMIS (scenario II). As a consequence of end-trimming, this repair pathway is prone to sequence modification.

1.2. Regulation of the repair of TOP2-DSBs by PIK Kinases.

The clear preference for the repair of TOP2-DSBs by TDP2 over ARTEMIS-MRE11 pathway, lead us to wonder how this established hierarchy could be regulated. First, this made us turn our attention to DNA-PKcs, which is required to recruit and promote the endonuclolytic and hairping opening activities of ARTEMIS (Goodarzi et al., 2006; Gu et al., 2010; Ma et al., 2002; Niewolik et al., 2006). Unfortunately, the library of sgRNAs (TKOv2) in the CRISPR-Cas9 screens performed does not contain sgRNAs targeting the *DNA-PK* gene. Thus, we decided to address the contribution of DNA-PKcs directly. Interestingly, DNA-PKcs deficiency only entails a relevant defect in the repair of TOP2-induced DSBs in TDP2 deficient background, showing an effect in the same line as those cause by the loss of ARTEMIS or MRE11, although the level of the repair defect was lower in this case. This supports that, despite the fact that DNA-PKcs has been reported to be a core NHEJ factor involved in synapsis and the stimulation of XRCC4-LIG4 liagase activity (Lu et al., 2008; Mahaney et al., 2009; Katheryn Meek et al., 2008), its presence is only necessary for the repair of blocked DSBs that require end-processing. Accordingly, DNA-PKcs scid mutation in mice does not imply a repair defect in TDP2 proficient primary MEFs but it does in the absence of TDP2. This is consistent with previous studies indicating that, in V(D)J recombination, *DNA-PKcs*$^{-/-}$ or scid cells are much more defective for coding joint than signal joints formation (Shin, Rijkers, Pastink, & Meek, 2000). ARTEMIS null mice behave similarly in this respect (Rooney et al., 2002), indicating, that DNA-PKcs and ARTEMIS are exclusively essential for opening hairping during this process. Therefore, our data demonstrate that these factors function specifically when DSBs repaired by NHEJ harbour complex ends requiring processing before ligation, and not specifically on hairpins.

In contrast to the loss of DNA-PKcs, inactivation of DNA-PKcs by inhibition of its kinase activity completely abolishes repair already in TDP2 proficient cells. This suggests that DNA-PKcs always binds to the ends of TOP2-DSBs regardless of its structure, in agreement with previous data (Smider, Rathmell, Brown, Lewis, & Chu,

1998). Since up to date, DNA-PKcs itself is the most functionally relevant target of its own enzymatic activity, it is tempting to think that its autophosphorylation is required to allow ligation. Accordingly, B cells expressing catalytically inactive DNA-PKcs (*DNA-Pkcs*$^{KD/KD}$) accumulate signal end and coding ends fragments during V(D)J recombination, indicating severe ligation defects (Jiang et al., 2015). This further supports that autophosphorylation of DNA-PKcs is required for end-ligation by removing the physical blockage imposed by itself. Despite the fact that DNA end binding by DNA-PK is indifferent to distinct DNA end structures, some studies indicated that DNA ends with canonical 5′ phosphate and 3′hydroxyl termini are required to activate its kinase activity (Pawelczak et al., 2005; Turchi, 2002). Moreover, it has been suggested that kinase activation occurs in trans, linking synapsis to kinase autophosphorylation (Jovanovic & Dynan, 2006; Pawelczak et al., 2005; Reddy et al., 2004). This would provide a mechanism by which DNA-PKcs coordinates end-processing with end-ligation, ensuring that ends are clean and in close proximity before attempting ligation. However, TDP2 deficient primary MEFs show a higher repair defect upon DNA-PKcs inhibition than wild-type cells. The fact that in wild-type murine cells DNA-PKcs inhibition does not completely abolish repair could be due to differences in DNA-PKcs abundance. Since human cells express ~50 times more DNA-PK activity than rodent cells (Finnie et al., 2006), it is tempting to think that DNA-PKcs in human cells always binds to DNA ends while in primary MEFs, DNA-PKcs expression could be limiting. Therefore, in TDP2 proficient background, primary MEFs occasionally repair DSBs induced by etoposide before DNA-PKcs can bind. In contrast, as TDP2 deficient cells show delayed repair, DNA-PKcs, could have enough time to bind a larger proportion of DNA ends. This could explain the difference regarding the impact of DNA-PKcs inhibition between human and rodent cells.

Strikingly, the loss of DNA-PKcs was epistatic over removing ARTEMIS in TDP2 deficient cells, showing the same repair defect as *TDP2*$^{-/-}$ *DNA-PKcs*$^{-/-}$ double knockout cells. This observation is key to understand the role of DNA-PKcs during this process, as it indicates that when ARTEMIS is absent, DNA-PKcs deficiency results in a milder

repair defect, suggesting that DNA-PKcs avoids the function of alternative pathways and commits the repair of blocked DSB through the ARTEMIS-dependent pathway. Furthermore, this epistatic interaction is consistent with aforementioned studies claiming that both proteins work together in hairping opening. Remarkably, during hairpin opening there is no specific unblocking activity, in contrast to TOP2-breaks, where TDP2 is able to remove TOP2 adducts without end-processing, which indicates the difference between the distinct requirements for these factors in each process.

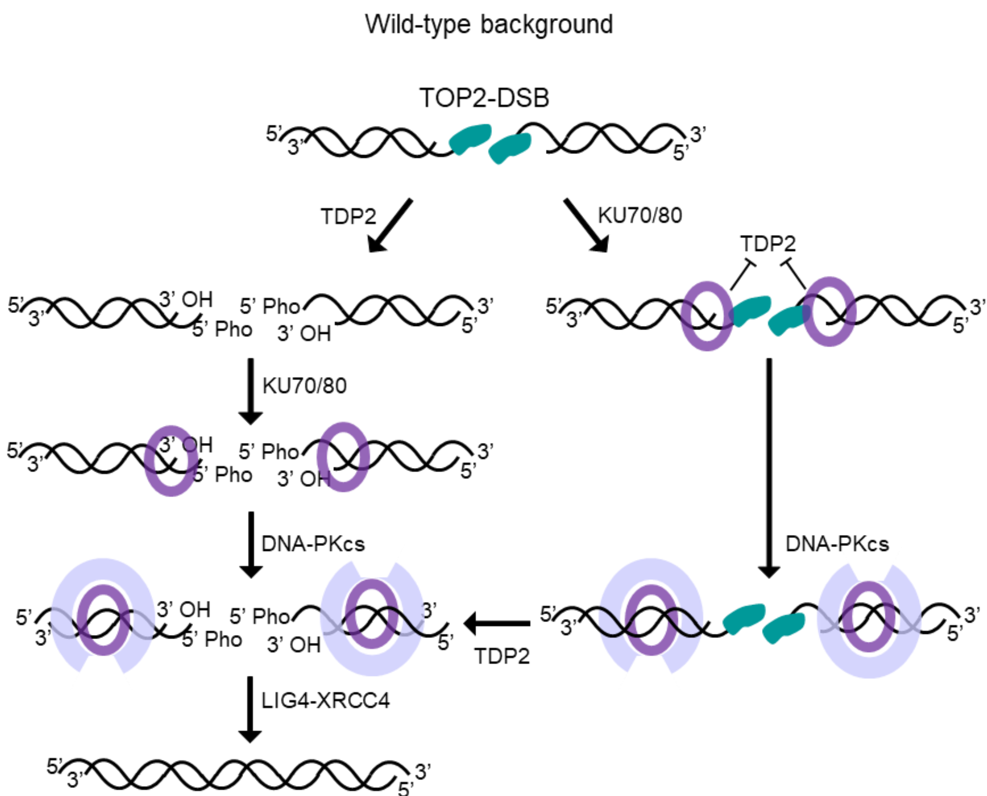

Figure 37: Model for the repair of TOP2-DSBs in wild-type background. TOP2-DSBs are recognized by KU70/80 heterodimer. Then, DNA-PK catalytic subunit (DNA-PKcs) is recruited, translocating KU70/80 ring inward the break, which allows the access of TDP2 to unblock the ends (right). In some cases, TDP2 can remove TOP2 blockage very quickly even before KU binds to the ends (left), then KU70/80 and DNA-PKcs can promote repair by NHEJ pathway.

Surprisingly, in the absence of DNA-PKcs, MRE11 is required to repair TOP2-induced DSBs even when TDP2 is present. This result is really striking considering that ARTEMIS shows an epistatic relationship with both MRE11 and DNA-PKcs. This leads us to think that MRE11 could be involved in two different pathways to repair TOP2-DSBs: in first place, an ARTEMIS-MRE11 pathway that is DNA-PKcs-dependent and, secondly, a pathway that is only active in the absence of DNA-PKcs ("MRE11-only pathway"). This suggests that the presence of DNA-PKcs avoids the MRE11-only pathway, while committing repair of TOP2-DSBs to ARTEMIS function (Figure 39). Furthermore, the fact that this efect of MRE11 deficiency in *DNA-PKcs*$^{-/-}$ cells is also observed in TDP2 proficient background, entails that the preference for the repair of

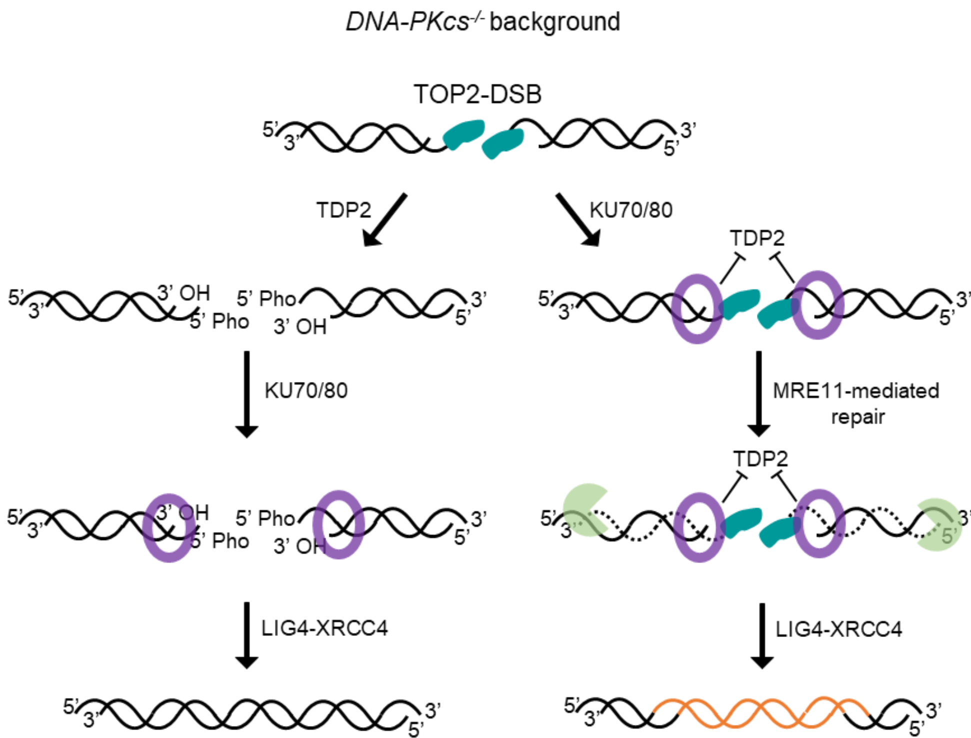

Figure 38: Model for the repair of TOP2-DSBs in DNA-PKcs deficient background. In the absence of DNA.PKcs KU70/80 is not translocated inward the break, avoiding the access of TDP2. This leads to the function of the MRE11-only pathway, which is independent of DNA-PKcs and can involve a large loss of sequence. On the other hand, due to the lack of DNA.PKcs, TDP2 has a narrow window to remove the TOP2-adducts before the binding of KU70/80 (left), avoiding sequence modification.

TOP2-DSBs though TDP2 is disrupted in the absence of DNA-PKcs, indicating a putative role of DNA-PKcs in the regulation of this hierarchy. In addition, although in DNA-PKcs deficient cells both TDP2 or MRE11 loss show a repair defect, the repair kinetics are completely different: *DNA-PKcs$^{-/-}$ MRE11$^{-/-}$* cells repair a fraction of induced DSBs very quickly (before 3h of repair time point), then the fraction of DSBs that has not been repaired at that moment remains unrepaired following 24 hours. On the other hand, *TDP2$^{-/-}$ DNA-PKcs$^{-/-}$* cells show a slow repair rate (at 3h repair time point all DSB remained unrepaired) but finally most of them are repaired after 24 hours. This suggests that when DNA-PKcs is not present, while the contribution of TDP2 to the repair of TOP2-DSBs occurs at very early stages of the process but is precluded from action at later times, MRE11 operates slower but at constant rate independent of time. These interesting results lead us to propose a model in which, in DNA-PKcs deficient background, when a TOP2-DSB is induced, TDP2 could remove TOP2 blockage very quickly even before KU binds to the ends. After KU binding, since DNA-PKcs is not present, the complex does not translocate inward (Katheryn Meek et al., 2008). This fact could avoid the access of TDP2, which implies that in the absence of DNA-PKcs, TDP2 has a narrow window to operate before KU binding. Therefore, DSBs that have not been unblocked by TDP2 before KU binds can be only repaired by MRE11 (Figure 38). On the contrary, when DNA-PKcs is present, KU translocates inward and DNA-PKcs has direct contacts with ~10 bp at the terminus of the DNA ends (Figure 37), this event allowing access to DSB ends even after KU binding. In TDP2 proficient background, TOP2 blockages are going to be preferentially removed by its tyrosyl phosphodiesterase activity. In TDP2 deficient background, DNA-PKcs allows a restrictive end trimming through ARTEMIS and MRE11 pathway while avoiding the action of MRE11 far away from the DNA termini (Figure 39). It would be particularly interesting to analyse ssDNA generation in DNA-PKcs deficient background. Based on our results, it is tempting to think that when DNA-PKcs is not present, a long resection of the ends occurs. Strikingly, DNA-PKcs deficient cells do not show a relevant induction of micronuclei formation after etoposide treatment. Nevertheless, we cannot rule out the

possibility that the genome instability is increased, although not leading to the induction of micronuclei. This occurs in the case of deletions, which are highly likely to appear due to the activity of MRE11. Therefore, additional experiments should be carried out to address the impact of DNA-PKcs deficiency in genome integrity. On the other hand, although our proposed model seems contradictory with previous observations of an epistatic relationship of KU70 over TDP2 deletion in DT40 cells (Gómez-Herreros et al., 2013), this is not the case as KU is likely to be required for downstream events of the NHEJ process such as ligation.

Strikingly, MRE11 function in DNA-PKcs deficient background does not depend on its nucleolytic activities. This suggests that an additional structural or regulatory function of MRE11 could be involved in removing TOP2 adducts under these conditions and another nuclease would be required in this process. In this regard, during the initiation of resection in HR, the MRN complex interacts with and is stimulated by the CtIP endonuclease (Anand, Ranjha, Cannavo, & Cejka, 2016; Sartori et al., 2007). Furthermore, CtIP also stimulates MRN endonucleolytic activity on DNA termini harbouring protein adducts *in vitro* (Deshpande et al., 2016b). Moreover, CtIP-deficient cells are reported to be sensitive to etoposide (Huertas & Jackson, 2009; Nakamura et al., 2010). Consistently, CtIP is needed for the NHEJ-mediated repair of etoposide-induced DSBs in G1 (Quennet et al., 2011) and promotes the removal of topoisomerase II adducts in *Xenopus* extracts collaboratively with MRN (Aparicio, Baer, Gottesman, & Gautier, 2016). Remarkably, its nuclease activity has been reported to be specifically required for the removal of DNA adducts at sites of DSBs and not for resection of unmodified DNA breaks (Makharashvili et al., 2014). This differentiates catalytic and non-catalytic roles of this factor during end-resection, which requirement would be end-structure dependent. Therefore, one could speculate that CtIP could trigger end-resection when DNA-PKcs is absent to remove TOP2-blockages and this activity could be promoted by the MRN complex. Unfortunately, due to the lack of time we could not address the contribution of CtIP in our experimental conditions. Thus, it would be

particularly interesting to address the impact of CtIP loss in DNA-PK deficient cells to address if it is related with MRE11 function in this genetic background.

On the other hand, our observations indicate that the main function of ATM in the repair of TOP2-DSBs in TDP2 deficient cells is related to the DNA-PKcs-ARTEMIS-MRE11 pathway. In this regard, the recruitment of ARTEMIS requires DNA-PKcs phosphorylation at ABCDE cluster, which can be autophosphorylated by DNA-PKcs but can also be phosphorylated by ATM and ATR under different cellular stresses (B. P. C. Chen et al., 2007; Katheryn Meek et al., 2008; Yajima, Lee, & Chen, 2006). Thus, the role of ATM to facilitate repair of blocked TOP2-DSBs could be the phosphorylation of DNA-PKcs on this cluster. Additionally, ATM hyperphosphorylates ARTEMIS upon DNA damage (L. Chen et al., 2005; Ma, Schwarz, & Lieber, 2005; Poinsignon et al., 2004; Riballo et al., 2004; Junhua Wang et al., 2005; X. Zhang et al., 2004). However, the shift in ARTEMIS mobility upon etoposide treatment is similarly observed in TDP2 proficient and deficient cells, which indicates that this event is not specific to blocked-DSBs. Nevertheless, although ocurring constitutively, it could be required exclusively when ends need processing. Yet, analysis of multisite phosphorylation mutants of ARTEMIS demonstrated that none of the nine canonical phosphorylation sites at the C-terminal are required for its endonuclease activity, implying that the phosphorylation of DNA-PKcs at ABCDE cluster is the target necessary for end-processing by ARTEMIS (Goodarzi et al., 2006). In this regard, MEFs with three alanine substitutions in the ABCDE cluster (*DNA-Pkcs*$^{3A/3A}$ mutant) display a relevant repair defect upon etoposide treatment. Nonetheless, it would be interesting to address whether this impact is higher in TDP2 deficient background.

The fact that the loss of ATM in TDP2 deficient background shows a lower repair defect than loss of ARTEMIS or MRE11 suggests that there could be redundancy between ATM and other factors. Presumably, this factor could be DNA-PKcs itself. Thus both ATM and DNA-PKcs could phosphorylate redundantly the ABCDE cluster of DNA-PKcs to promote the access of ARTEMIS. It would be important in the future to carry out experiments to study the relationship between ATM and DNA-PKcs that could

shed light on their possible partial redundant function during repair of blocked TOP2-DSBs. Due to the fact that both ATM and DNA-PKcs are the main kinases involved in H2AX phosphorylation at S139 (Firsanov et al., 2011), molecular assays to analyse DSBs would be crucial to address this question.

On the other hand, consistent with the fact that ATM is mainly working in the ARTEMIS-MRE11 nuclease pathway, the interplay between the MRN complex and ATM in regulating DSB repair has been widely reported. Indeed, every single component of the MRN complex can be phosphorylated by ATM, and ATM full activation upon DSB-induction seems to require its interaction with the MRN complex through NBS1 (Difilippantonio et al., 2007; Lavin et al., 2015). On the top of this, ATM has been proposed as a modulator of the processing activity of MRN (Kijas et al., 2015). Thus, it would be interesting to perform further work to test how ARTEMIS and MRE11 activities are regulated by ATM and address the crosstalk mechanism between both nucleases.

In addition to DNA-PKcs and ATM, ATR also belongs to PIK kinase family and has important roles in coordinating the DDR. Specifically, ATR it is activated by replication protein-A bound ssDNA (Maréchal & Zou, 2013; Nam & Cortez, 2011; B. Shiotani & Zou, 2009). Interestingly, ATR inactivation shows a significant repair defect specifically in TDP2 deficient cells, which is consistent with the fact that end-processing is only required when cells lack TDP2 and, due to the action of nucleases, ssDNA that activates ATR could be generated (FIG 39). This role of ATR is more relevant in ATM deficient cells, suggesting that in the absence of ATM, end-resection is less limited. This result supports a previous model (A. Álvarez-Quilón et al., 2014) which proposes ATM as a protector of DNA ends from an excessive degradation. Additionally, since we have demonstrated that in DNA-PKcs deficient cells the presence of MRE11 is critical for repairing TOP2-DSBs even in TDP2 proficient background, it would be really interesting to address what is the role of ATR in this context. Together, all these data would demonstrate functionally distinct, but cooperative, roles for each kinase in

promoting the repair of TOP2-DSBs in the absence of TDP2, when the ends are irreversibly blocked.

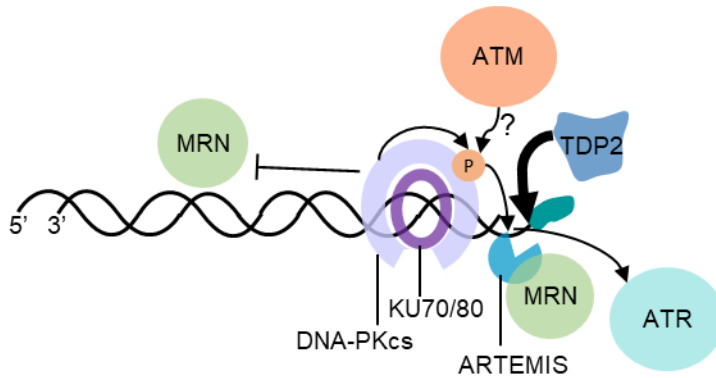

Figure 39: Integrated model for the repair of TOP2-DSBs. TOP2-DSBs are recognized by the ring-shaped KU70/80 heterodimer. Then, DNA-PK catalytic subunit (DNA-PKcs) is recruited, translocating KU70/80 ring inward the break. There is a strong preference for the unblocking activity of TDP2 to remove TOP2-adducts, which can act even before the binding of KU70/80. When the activity of TDP2 is compromised, DNA-PKcs commits the repair through ARTEMIS-MRE11 pathway, avoiding alternative pathways such as MRE11-only pathway. ATM is also involved in ARTEMIS-MRE11 pathway, possibly by promoting DNA-PKcs phosphorylation at the ABCDE cluster, which can also be autophosphorylated by DNA-PKcs itself. As a consequence of nucleolytic activities, ssDNA is generated that can activate ATR, which promotes somehow the repair of the DSBs. this repair pathway is prone to sequence modification.

On the other hand, as previously mentioned, in quiescent cells (G0/G1), processing activities must be closely regulated to avoid long-range resection of DSB ends, since sister chromatids are not present to be used as a template for HR during these cell cycle stages and most DSBs are repaired by NHEJ or MMEJ pathways. Thus, it is tempting to think that protecting factors could be specifically important when the ends are irreversibly blocked and require limited trimming. Since ATM deficiency slightly aggravates the repair defect observed in *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ or *TDP2*$^{-/-}$ *MRE11*$^{-/-}$ cells, one could speculate that it has additional roles regarding end-protection besides its main function in the ARTEMIS-MRE11 pathway. Due to this reason, we aimed to address the contribution of some ATM substrates which are related with restriction of end-resection. In this regard, ATM-dependent formation of γH2AX has been demonstrated to be essential for the recruitment of downstream factors, such as 53BP1,

another ATM substrate which has been widely related to avoid long range resection and regulate end-processing during V(D)J recombination and immunoglobulin class switch recombination (Bothmer et al., 2010; Bunting et al., 2010; Difilippantonio et al., 2008). Consistent with this, the 53BP1 upstream factor RNF168 contributes to the repair of TOP2-induced DSBs but exclusively when TDP2 is absent. Remarkably, the effect of removing RNF168 is less prominent in *TDP2$^{-/-}$ ATM$^{-/-}$* double knockout cells, suggesting that the main function of RNF168 in the repair of TOP2-DSBs is related to ATM. On the other hand, although the loss of 53BP1 or RIF1 display a similar impact of RNF168 deficiency in the absence of TDP2, statistical significance was not reached. Therefore, more experiments would be required to address whether 53BP1 and RIF1 operate to repair TOP2 breaks in the absence of TDP2 or the role of RNF168 is related to an additional function. In the former case, it would be also interesting to address the contribution of PTIP, another 53BP1 downstream factor, which interacts with ARTEMIS to promote limited end-trimming and the repair of DSBs through NHEJ (J. Wang et al., 2014), and could do operate as a bridge between processing and end-protecting factors.

On the other hand, the deficiency of 53BP1 could have a negligible effect in the efficiency of repair of TOP2-DSBs but could affect genome integrity. In this regard, it is tempting to think that, 53BP1 could be specially required to maintain genome stability in DNA-PKcs deficient cells, as we speculate that DSBs undergo longer resection mediated by MRE11-only pathway, instead of the DNA-PKcs-dependent limited trimming by ARTEMIS-MRE11. Thus, it would be interesting to analyse the impact of removing 53BP1 in DNA-PKcs deficient cells regarding not only repair but also genome instability.

2. The established hierarchy that prioritise TDP2 activity to repair TOP2-DSBs avoids malignant transformation.

Consistent with the increased genome instability observed upon TDP2 deficiency, the loss of TDP2 promotes an earlier immortalization of primary MEFs by 3T3 protocol. This further supports that TDP2 is a factor that must be taken into account in cancer

development, progression and treatment. To address the implication of TDP2 in cancer, we addressed the incidence of tumours in lifespan experiments in the *Tdp2*$^{-/-}$ mouse model. Remarkably, we have found that TDP2 deficient mice display a lower lifespan than wild-type animals, both upon untreated conditions or after an etoposide treatment that mimics chemotherapeutical doses. This reduced lifespan is due to an increase in tumour incidence. In this regard, 30% of TDP2 deficient mice develop more than one tumour from different origins even in untreated controls, which is an event extremely rare in wild-type animals. The fact that *Tdp2*$^{-/-}$ untreated controls already show this tumour incidence leads us to think that TOP2 activity could be a potential source of neoplastic changes and TDP2 would play a key role in avoiding them. This indicates a tumour suppressor role of TDP2 and supports that when TOP2-DSBs are repaired by nuclease pathways instead of TDP2, genome integrity is compromised. Together, these results are contradictory with arguments exposed by Hoa et al., 2016 that suggest that MRE11 activity, rather than TDP2, prevents spontaneously arising TOP2cc from becoming a serious threat to genome instability or cell viability. This is not the case, at least in mice, and it is consistent with our observations of genome instability in human cells. Thus, it would be really interesting to analyse whether this incidence of tumour is also observed in SCAR23 patients, which are TDP2-deficent and show severe neurological abnormalities (Zagnoli-Vieira et al., 2018). The low number of patients, however, preclude any conclusion regarding cancer epidemiology. In any case, we performed an analysis in the Oncomine database of differential TDP2 expression in cancer. Interestingly, mRNA expression of TDP2 is downregulated in colorectal cancers, which further supports the role of TDP2 in preserving genomic integrity. Remarkably, colorectal cancers also show markedly higher mRNA expression of MRE11. Again, this could support that a disruption in the hierarchy in which TDP2 activity is preferred to repair TOP2-DSBs over nucleases leads to genome instability, and potentially to cancer development. Intestinal tissue is an established *in vivo* target of etoposide, consistent with high proliferation rates and the associated requirement for TOP2 activity. This could be the reason why this tissue is more sensitive for the

unbalanced expression of TDP2. Thus, TDP2 downregulation could contribute to cancer development by compromising genome integrity in a tissue with a high load of accidental TOP2-DSBs.

Finally, given the established preference for TDP2 to repair TOP2-DSBs and the etoposide resistance observed upon its high expression (Ledesma et al., 2009), TDP2 can be considered a determinant factor in cancer treatment with TOP2-poisons (Pommier et al., 2010). Indeed, recent studies have put great efforts to develop TDP2 inhibitors (Kankanala et al., 2016, 2019; Kossmann et al., 2016; Laev, Salakhutdinov, & Lavrik, 2016; Marchand et al., 2016; Raoof et al., 2013; Ribeiro et al., 2018, 2019; Yu et al., 2018). Unfortunately, potent TDP2 inhibitors with enough cellular potency and/or permeability are not yet reported. We can anticipate that TDP2 inactivation will sensitize tumour cells to TOP2-poison chemotherapy. However, it is important to consider that based on our findings, inactivation of TDP2 would channel DSB-repair to the error-prone nuclease pathways, which could potentiate the development of treatment associated secondary leukemias (Pendleton, Lindsey, Felix, Grimwade, & Osheroff, 2014).

V. CONCLUSIONS

V. CONCLUSIONS

1. ARTEMIS nuclease, NBS1 from the MRN complex and ATM are the main genetic hits specifically promoting etoposide resistance of TDP2 deficient cells, as determined on a genome wide pooled CRISPR-Cas9 screen.

2. ARTEMIS and MRE11 work together to facilitate the repair of TOP2-induced DSBs exclusively when TDP2 is absent, and therefore the ends are blocked. At least the exonuclease activity of MRE11 is involved in this process.

3. TDP2 contributes to avoid the formation of micronuclei upon the induction of TOP2-induced DSBs. Saturation of TDP2 function by a high dose of damage induces micronuclei that are dependent on ARTEMIS and, at least, the exonuclease activity of MRE11.

5. DNA-PKcs facilitates the repair of TOP2-induced DSBs exclusively when TDP2 is absent, and therefore the ends are blocked. Nevertheless, the inhibition of its activity completely abolishes the repair regardless of background.

6. DNA-PKcs is epistatic over ARTEMIS in the repair of TOP2-induced DSBs in the absence of TDP2, partially suppressing the repair defects of *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ mutant.

7. In the absence of DNA-PKcs, independent TDP2 and MRE11 pathways are responsible for the repair of TOP2-induced DSBs.

8. ATM facilitates the repair of TOP2-induced DSBs and promote cell survival exclusively when TDP2 is not present. This contribution is mainly related with the ARTEMIS-MRE11 pathway, although it also displays independent functions.

9. ATR facilitates the repair of TOP2-induced DSBs exclusively in TDP2 deficient background, and its function is more relevant in the absence of ATM.

10. RNF168 facilitates the repair of TOP2-induced DSBs exclusively in TDP2 deficient background, this function is partially ATM-dependent.

11. Despite some differences in the genetic relationship between factors, the established preference for TDP2 to repair TOP2-induced DSBs is conserved in murine cells.

12. Tdp2 prevents malignant transformation during 3T3 protocol in primary MEFs and cancer incidence in mouse.

13. Expression of TDP2 is decreased in colorectal cancer when compared to healthy tissue, while expression of MRE11 is increased.

VI. MATERIALS AND METHODS

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1. Cell culture procedures

1.1. Cell lines and primary cell culture

Cells were maintained in HEPA class 100 incubators (Thermo) at 37ºC and 5% CO_2.

Primary MEFs were isolated from embryos at day 13 p.c. and cultures at 3% O_2 I Dubelcco's Modified Eagles's Medium (DMEM) supplemented with penicillin, streptomycin, 2 mM L-Glutamine, 15% FCS and non-essential aminoacids. All experiments were carried out between p1 and P5.

RPE-1 hTERT cells expressing Cas9 were maintained at 37ºC, 5% CO_2 in DMEM: F-12 Medium supplemented with penicillin, streptomycin, 2 mM L-Glutamine, 10% FCS and 2 μg/ml blasticidin.

HEK293T and U2OS were maintained at 37ºC, 5% CO_2 in DMEM supplemented with penicillin, streptomycin, 2 mM L-Glutamine and 10% FCS.

1.2. 3T3

Cells were maintained on a defined 3-day passage schedule (Todaro & Green, 1963) by plating $3x10^5$ cells in 10 cm dishes. Plating after disaggregation of embryos was considered passage 0, and the first passage 3 days later as passage 1. Cells were counted at each passage, and the total number was calculated prior to redilution.

1.3. Lentivirus production and infection

To produce lentiviral particles expressing shRNA against *Nbn*, 3.5x106 HEK293T cells growing in 100-mm plates were transfected by calcium/phosphate protocol with a mixture composed of 15 μg of either plko.1-puro-Nbs1 (clon TRCN0000012671, SIGMA) or scramble control plKO.1-puro (Addgene #1864), 10 μg of p8.91 (plasmid containing

viral capsid genes) and 5 μg of pVSVG (plasmid containing viral envelope genes). Medium was recovered after 48h and filtered through a 0.45 μm polyvinylidene difluoride (PVDF) filter (SLHV035RS, Millex-HV, Millipore). Then, viral particles wereconcentrated by centrifugation for 1 hour and 30 min at 22000 rpm at 4ºC in a Belckman-Coulter Optima L-100K Ultracentrifuge and resuspended in DMEM and stored at -80ºC.

Tdp2$^{+/+}$ and *Tdp2*$^{-/-}$ primary MEFs growing in 100 mmm dishes were infected with MOI-5. After 5 days, cells were splitted and selected in puromycin (1 μg/ml) for 5 days before assays were performed. For infection, lentiviral particles were added to cells in DMEM supplemented with 4 μg/ml hexadimethrine bromide (H9268, Sigma). For titration U2OS cells were infected with serial dilutions of lentiviral particles. After 72 h, 2x103 cells were seeded in duplicate in 100 mm dishes and selected in puromycine (1 μg/ml) until colonies were formed. Then, plates were stained with Cristal Violet. Multicity of infection (MOI) was calculated based on the amount of puromycine resistant colonies.

1.4. Determining LD20

RPE1-hTERT Cas9-expressing cells were seeded in 150 mm dishes cells ($3x10^6$/plate), each condition was split into technical duplicates. Cells were chronically treated with indicated concentrations of etoposide or IRCF-187 for at least 12 days. Cells were split every 3 days, counted, and $3x10^6$ cells were seeded again for each condition. Proportion of surviving cells from each concentration treatment to untreated cells was calculated to define LD20.

1.5. CRISPR screens

For CRISPR Cas9 screens the second version Toronto KnockOut (TKO)v2 library was used. This library was generated by Jason Moffat's group and it contains ~70,000 sgRNAs cloned into the lentiviral expression vector backbone pLCKO. The library sgRNAs target ~17,500 human genes with approximately four sgRNAs per gene.

RPE1-hTERT Cas9-expressing cells were transduced with the lentiviral TKOv2 library (prepared as previously described in Hart et al., 2015) at a low MOI (~0.2-0.3) and puromycin-containing media (20 µg/ml) was added the next day to select for transductants. Selection was continued until 72 h post transduction, which was considered the initial time point, t0. At this point the transduced cells were split into technical duplicates. During screens, cells were subcultured at day 3 (t3) and at day 6 (t6) each of the two replicates was divided into two populations. One was left untreated and to the other an LD20 dose of etoposide (*P53*$^{-/-}$ *TDP2*$^{-/-}$ RPE1-hTERT: 2nM) or ICRF-187 (*P53*$^{-/-}$ *TDP2*$^{+/+}$ RPE1-hTERT: 25 µM; *P53*$^{-/-}$ *TDP2*$^{-/-}$ RPE1-hTERT: 2 µM). Cells were grown with or without drug until t21 and subcultured every three days. Sample cell pellets were frozen at each time point for genomic DNA (gDNA) isolation. A library coverage of ≥400 cells/sgRNA was maintained at every step, therefore at least $3x10^7$ cells were maintained for each replicate ($3x10^6$ cells/150mm plate).

Genomic DNA from cell pellets was isolated using the QIAamp Blood Maxi Kit (Qiagen) and genome-integrated sgRNA sequences were amplified by PCR using the KAPA HiFi HotStart ReadyMix (Kapa Biosystems). Assuming each diploid genome is 6.5 picograms (pg) 182 µg genomic DNA were needed to achieve 400X coverage (6.5 pg x 28,000,000 = 182 µg). The maximum quantity of template genomic DNA for one 50 µL PCR reaction is 5 µg. Therefore, 37 PCR reactions were prepared per sample in 96-well PCR plates. Primers used were: "Outer_sgRNA_Fwd":AGGGCCTATTTCCCATGATTCCTT and "Outer_sgRNA_Rev": TCAAAAAAGCACCGACTCGG. i5 and i7 multiplexing barcodes were added in a second round of PCR and final gel-purified products were sequenced on Illumina HiSeq2500 or NextSeq500 systems to determine sgRNA representation in each sample. DrugZ (see (Colic et al., 2019) was used to identify gene knockouts, which were depleted from etoposide or ICRF-187-treated t18 populations but not depleted from untreated cells.

1.6. Generation of CRISPR knockout cell lines

P53$^{+/+}$ and *P53*$^{-/-}$ RPE1-hTERT Cas9-expressing cells were a kind gift from Dr. Daniel Durocher's laboratory.

To perform etoposide and ICRF-187 screen we generated *P53*$^{-/-}$ *TDP2*$^{-/-}$ RPE1-hTERT Cas9-expressing cells. To establish this cell line, 0.5x10^{4} cells were seeded in 24-well plates. Cells from two wells for each condition were transfected with either a sgRNA targeting *TDP2* or a non- targeting control using RNAiMAX (Invitrogen) according to manufacture's protocol. 24 hours after transfection, cells were split and seeded in a 60 mm plate. After additional 72 hours, single cells were sorted into 96-well plates on a BD FACSAria™ Cell Sorter instrument and grown until colonies formed. *TDP2*$^{-/-}$ clones were selected on the basis of successful gene editing analysed by TIDE analysis (https://tide-calculator.nki.nl) (Brinkman et al., 2014). Clones from non-targeting gRNA transfection were also isolate so as to they can act as a control. In order to perform subsequent experiments with screen candidates, *P53*$^{+/+}$ *TDP2*$^{-/-}$ RPE1-hTERT Cas9-expressing cells were generated in a similar way. Selected clones were confirmed by immunoblotting.

Once *P53*$^{+/+}$ *TDP2*$^{-/-}$ RPE1-hTERT Cas9-expressing cells and its control were generated, we transfected them as explained above with sgRNAs targeting *ATM, ARTEMIS* or *DNA-PKcs*. Therefore, single *ATM*$^{-/-}$, *ARTEMIS*$^{-/-}$, *DNA-PKcs*$^{-/-}$ and *TDP2*$^{-/-}$ *ATM*$^{-/-}$, *TDP2*$^{-/-}$ *ARTEMIS*$^{-/-}$ *TDP2*$^{-/-}$*DNA-PKcs*$^{-/-}$ double mutants were identified and confirmed by immunoblotting or immunofluorescence and TIDE analysis.

For analysing other combinations between these or others genes, 7x10^{5} RPE1-hTERT Cas9-expressing clones generated where seeded into 60 mm plates and transfected with sgRNA of interest. 48h after transfection, these cells were split and seeded for performing the experiment. Finally, positive clones were selected during immunofluorescence by using an antibody against protein coding for gene targeted by the sgRNA.

2. Molecular biology procedures

2.1. Preparation of sgRNAs

sgRNAs used for transfection were ordered from Integrated DNA Technologies (IDT). crRNAs containing the target-specific sequence for guiding Cas9 protein to a genomic location were annealed with tracrRNA to form a functional sgRNA duplex.

2.2. Western blot

RPE-1 hTERT cells were seeded on coverslips. Once attached, cells were arrested at the G0 phase by serum starvation for 48-72 hours and treated as indicated with F12 media without serum. After 1 hour after treatment, cells were resuspended on ice using a cell scraper and lysed in RIPA buffer supplemented with protease inhibitor cocktail (PIC, Sigma) and phosphatase inhibitor cocktail (PhiC3, Sigma). Protein concentration was determined by the Bradford assay (Applied Biochem). Equivalent amounts of protein (~20 micrograms) were resuspended in Laemli buffer, boiled at 96 °C for 5 min and sonicated. Samples were run in NuPAGE Tris-Acetate Mini gels 3–8% (Novex) for ATM immunoblotting; or 4–20% Mini-PROTEAN Tris-Glycine Precast Protein Gels (BioRad) for Artemis analysis. Then samples were transferred to Immobilon-FL Transfer Membranes (Millipore) o/n at 4ºC, 40mA. Membranes were blocked in Odyssey Blocking Buffer (LI-COR Biosciences) for 1 hour, incubated with primary antibodies for 4 hours at room temperature in Odyssey Blocking Buffer-0.1% Tween20 and washed (three times in TBS-0.1% Tween20). They were then incubated with the corresponding IRDye-conjugated secondary antibodies (1/10000 dilution) in Odyssey Blocking Buffer-0.02% to 0.1% Tween20 and washed (three times in TBS-0.1% Tween20 and 1 × in TBS buffer). Membranes were analyzed in Odyssey CLx with ImageStudio Odyssey CLx Software. Primary antibodies were used at the indicated dilution: ATM (Sigma, MAT3-4G10/8), 1/1,000; α-tubulin (Sigma, T9026), 1/50,000; Artemis (Thermo Fisher, PA5-27112).

3. Cell biology procedures

3.1. γH2AX foci analysis and immunofluorescence

RPE-1 hTERT cells expressing Cas9 cells were seeded on coverslips. Once attached, cells were arrested at the G0 phase by serum starvation for 48-72 hours and treated as indicated with F12 media without serum. Primary MEFs were grown on coverslips for 7 days until confluency arrest and treated as indicated. Then, cells were fixed 10 min in ice-cold methanol or 4% PFA-PBS. Cells were then permeabilized (5 min in PBS-0.2% Triton X-100), blocked (30 min in PBS-5% BSA) and incubated with the required primary antibodies (1–3 h in PBS-1% BSA). Cells were then washed (three times in PBS-0.1% Tween 20), incubated for 30 min with the corresponding AlexaFluor-conjugated secondary antibodies (1/1,000 dilution in 1% BSA-PBS) and washed again as described above. Finally, they were counterstained with DAPI (Sigma) and mounted in Vectashield (Vector Labs). Primary antibodies were used at the indicated dilution: γH2AX (Millipore, 05-636), 1/5000 for RPE-1 hTERT cells and 1/1,000 for primary MEFs; Artemis (Novus Biologicals, NBP2-56362) 1/200; MRE11 (Novus Biologicals, NB100-142); DNA-PKcs (Santa Cruz, H163 sc-9051) 1/500; 53BP1 (Santa Cruz, sc-22760) 1/1000; RIF1 (Santa Cruz, N-20 sc-55979) 1/200.

3.2. Micronuclei assay

RPE-1 hTERT cells were seeded on coverslips. Once attached, cells were arrested at the G0 phase by serum starvation for 48-72 hours and treated as indicated with F12 media without serum. Cells were let for repair 24 hours. After repair, serum was added for 48 hours so as to cells can restart cell cycle. Then, cells were fixed 10 min in methanol at -20ºC, and immunofluorescence was performed as previously described using antibody γH2AX and stained with DAPI.

3.3. Microscopy analysis

Standard immunofluorescence and micronuclei assays were visualized using Leica DM6000 microscope and images were acquired using a Leica AF6000 image processing station. For DSB repair kinetic, γH2AX foci were manually counted (double-blind) in 40 cells preselected by DAPI staining for showing regular and standard nuclear morphology from each experimental condition. For micronuclei assay more than 500 cells were manually scored for the presence of micronuclei by DAPI visualization. At least three independent experiments were performed.

4. Animal procedures

4.1. Ethics statement

All animal procedures were performed in accordance with European Union legislation and with the approval of the Ethical Committee for Animal Experimentation of the University of Seville and local Committee of Cabimer.

4.2. Animal maintenance

The mouse colony was maintained in an outbred 129Ola, CD1 and C57BL/6 background under standard housing conditions, at 21±1°C with a photoperiod of 12:12 h (lights on at 8:00). They were housed in isolated cages with controlled ventilation trough HEPA-filters and were in flow cabins. Sterile food pellets and water were available ad libitum. Breeding pairs between heterozygotes were set to obtain wild-type (*Tdp2*$^{+/+}$) and knock-out (*Tdp2*$^{-/-}$) littermates for analysis. Mice were genotyped with Phire Animal Tissue Direct PCR Kit (Thermo) following manufacturer instructions and using primers 5′-CCTTCATTACTTCTCGTAGGTTCTGGGTC-3′, 5′-ACCCGCTCTTCACGCTGCTTCC-3′ and 5′-TACACCGTGCCATAATGACCAAC-3′. This results in amplification of a 429 bp fragment from the wild-type allele or 561 bp fragment from the mutant allele.

4.3. Lifespan and tumor incidence analysis after chemotherapeutical etoposide doses

Minimum 10 mice per experimental condition were included in the analysis. *Tdp2*$^{+/+}$ and *Tdp2*$^{-/-}$ mice were treated with relevant doses for chemotherapy. 8 weeks old mice were injected daily with 4mg/kg etoposide or dmso during 5 days and left to analysed survival and tumor incidence. Weight and general health status was monitored weekly. Animals were sacrificed by cervical dislocation and dissected for histopathological analysis in the case of showing a 20% loss of the maximum weight, the presence of a detectable tumor or signs of evident pain.

5. Stadistical analyisis

Stadistical analysis was performed using GraphPad Prism software (GraphPad. San Diego, CA, USA).

6. Tables of materials

Cell name	Species	Origin
Primary MEFs	*Mus musculus*	Embryonic fibroblasts
HEK293T	*Homo sapiens*	Embryonic kidney transformed cell line
U2OS	*Homo sapiens*	Osteosarcoma cell line
hTERT RPE-1 expressing Cas9	*Homo sapiens*	Retina pigmented epithelium cells immortalized with hTERT

Table 1: **Cells used in this thesis**

Gene Targeted	Sequence
TDP2	CTTGCTGAGTATCTTCAGAT
ATM	TTGTTTCAGGATCTCGAATC
ARTEMIS	TTCCTGTCCCACTGCCACAA
DNA-PKcs	TACAAGCAAACCGAAATCTC
MRE11	GTTGCAACAGATATTCATCT
RNF168	CCAGTGAAGAATACATACAG
53BP1	TCCAATCCTGAACAAACAGC
RIF1	GTGCTGCTCTACAAGCCCTG

Table 2: Sequences (5'-3') of DNA templates used for sgRNA production.

Name	Sequence	From
TDP2_fw	CAAAGTGCAGCGTGATTAATG	Sigma
TDP2_rv	TAACAGTTCAAGGTTGTGGGC	Sigma
ATM_fw	TCTGAAATTGTGAACCATGAG	Sigma
ATM_rv	CCAAATTCATATGCAAGG	Sigma
ARTEMIS_fw	CCCTCCTGTCCTCTCTCCAG	Sigma
ARTEMIS_rv	ACTGCACCTCCACAGACATG	Sigma
DNA-PKcs_fw	ATAAGAAAATCATTGCAACCC	Sigma
DNA-PKcs_rv	AGCCCACTTCATTTGTAACAC	Sigma
MRE11_fw	ACTCACTTCATTTTCCTGGGCA	Sigma
MRE11_rv	TTGGGCCTGGGTTACATGAG	Sigma
RNF168_fw	AGGCTGAGATGGGACAGCTA	Sigma
RNF168_rv	ATTTGTGGGATGCAGAACCT	Sigma
53BP1_fw	CTCCAGACGCACAAAG	Sigma
53BP1_rv	GCAAAGGGGACAGATAGC	Sigma
RIF1_fw	TACTCAGTGGAAGGGCCTTG	Sigma
RIF1_rv	CCACACCCAGCCATTCTTTC	Sigma
TDP2_seq	CAAAGTGCAGCGTGATTAATG	Sigma
ATM_seq	CCAAATTCATATGCAAGG	Sigma
ARTEMIS_seq	AGTGGCGGCGCGGTCAGGGCT	Sigma
DNA-PKcs_seq	AGCCCACTTCATTTGTAACAC	Sigma
MRE11_seq	TTGGGCCTGGGTTACATGAG	Sigma
RNF168_seq	ATTTGTGGGATGCAGAACCT	Sigma
53BP1_seq	CTCCAGACGCACAAAG	Sigma
RIF1_seq	CCACACCCAGCCATTCTTTC	Sigma

Table 3: DNA oligonucleotides used for diagnostic PCR and sequencing of CRISPR-Cas9 gene-editing events.

Primary antibodies	Dilution	Obtained from	Species	Application
ATM	1/1000	Sigma, MAT3_4G10/8	Mouse	WB
ARTEMIS	1/200	Novus Biologicals, NBP2-56362	Rabbit	IF
ARTEMIS	1/1000	Thermo Fisher, PA5-27112	Rabbit	WB
MRE11	1/200	Novus Biologicals, NB100-142	Rabbit	IF
DNA-PKcs	1/500	Santa Cruz, H163 sc-9051	Rabbit	IF
53BP1	1/1000	Santa Cruz, sc-22760	Rabbit	IF
RIF1	1/200	Santa Cruz, (N20) sc-55979	Goat	IF
gH2AX	1/1000-1/5000	Millipore, 05-636	Mouse	IF
a Tubulin	1/5000	Sigma, T9026	Mouse	WB

Secondary antibodies	Dilution	Obtained from	Species	Application
IRDye 680RD goat anti-mouse IgG (H+L)	1/10000	LI-COR, 926-68070	Goat	WB
IRDye 800RD goat anti-rabbit IgG (H+L)	1/10000	LI-COR, 926-32211	Goat	WB
Goat anti-mouse-AlexaFluor488	1/1000	Jackson	Goat	IF
Goat anti-mouse-AlexaFluor594	1/1000	Jackson	Goat	IF
Donkey anti-mouse-AlexaFluor488	1/1000	Jackson	Donkey	IF
Donkey anti-goat-Cy3	1/1000	Jackson	Donkey	IF

Table 4: Antibodies used in this thesis.

VII. BIBLIOGRAPHY

VII. BIBLIOGRAFY

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