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**PARKIN, BLM AND EXO1:
INVOLVEMENT IN POST-NER ACTIVITY
IN NON-REPLICATING CELLS**

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“The universe is the answer, what are the questions?”

(Elon Musk)

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3 TABLE OF ABBREVIATIONS

- 6-4PPS = 6-4 Photoproducts
- AR-JP = Autosomal Recessive Juvenile Parkinsonism
- BER = Base Excision Repair
- BLM = Bloom syndrome RECQ-like helicase
- BPDE= Benzo[a]pyrene Diol Epoxide
- BrdU = Bromodeoxyuridine
- BSyn = Bloom's Syndrome
- ChIP = Chromatin ImmunoPrecipitation
- COLs = Closely-spaced Opposing Lesions
- CPDs = Cyclobutane Pyrimidine Dimers
- CS = Cockayne Syndrome
- DAR = Differentiation Associated Repair / Domain Associated Repair
- DDC = DNA Damage Checkpoint
- DDR = DNA Damage Repair
- DSB = Double-Strand Breaks
- dsDNA = double-strand DNA
- EdU = 5-Ethynyl-2'-deoxyuridine
- EMSA = Electrophoretic Mobility Shift Assay
- EU = 5-ethynyluridine
- EXO1 = Exonuclease 1
- FA = Fanconi anemia
- GG-NER = Global Genome NER
- HR = Homologous Recombination
- IaCL = Intrastrand Crosslink
- ICL = Interstrand Crosslink
- KO = Knock Out
- LUDs = Local UV Damages
- NCA = Neutral Comet Assay
- NER = Nucleotide Excision Repair
- NHEJ = Non-Homologous End Joining
- NIR = Nucleotide Incision Repair
- PCNA = Proliferating Cell Nuclear Antigen
- ROS = Reactive Oxygen Species
- RRS = Recovery of RNA Synthesis
- SSBs = Single-Strand Breaks
- ssDNA = single-strand DNA
- ssDNA = single-strand DNA
- TC-NER = Transcription Coupled NER
- TLS = TransLesion Synthesis
- TTD = TrichoThioDistrophy
- UDS = Unscheduled DNA Synthesis
- UV = ultraviolet (light)
- UVSS = UltraViolet-Sensitive Syndrome
- WRN = Werner's syndrome RecQ-like helicase
- XP = xeroderma pigmentosum
- γH2AX = H2AX phosphorylated at Serine 139

4 ABSTRACT

Non-replicating or terminally differentiated cells constitute the vast majority of the total cells in the human body. As such, granting their survival is fundamental for the correct functioning of every organ they belong. As these cells can be exposed to a variety of DNA insults that could menace their integrity, a complex machinery of DNA Damage Repair (DDR) proteins has evolved, together with associated signaling pathways that coordinate repair activity with other metabolic processes. One specialized branch of DDR is Nucleotide Excision Repair (NER), responsible for the detection and elimination of bulky lesions in the DNA inducible by exposure to ultraviolet (UV) light or compounds such as Benzo[a]pyrene diol epoxide (BPDE), a metabolite of benzo[a]pyrene. In non-replicating cells, data from the literature and previous work in our laboratory has shown that the Exonuclease 1 (EXO1) is recruited on the single-strand DNA (ssDNA) ends generated by the endonucleolytic activity of NER to perform resection of the filament. While in most cases, the repair DNA synthesis activity of the replicative polymerases is faster than the resection activity of EXO1 minimizing the exposure of ssDNA, in a subset of UV lesions, namely the so-called “closely-spaced opposing lesions (COLs)”, proper gap-filling requires the recruitment of Translesion Synthesis (TLS) polymerases via Rad6-Rad18 mediated monoubiquitination of the Proliferating Cell Nuclear Antigen (PCNA). If this process is impeded, long ssDNA generation and exposure trigger the activation of the DNA damage checkpoint signaling cascade and can potentially lead to the generation of toxic Double-Strand Breaks (DSB).

We analyzed the response of murine neurons to the bulky lesions inducing agent BPDE, and we found indeed that the compound was able to trigger the formation of DNA adducts, which resulted in the generation of double strand breaks, checkpoint signaling activation, and overall, an impairment of the survival of these cells. Parkin, an E3 ubiquitin ligase mutated in autosomal recessive juvenile parkinsonism, has been reported to play a role in PCNA ubiquitylation; we investigated its potential role in the response to bulky lesions in non-replicating, neuron-like cells. Although we failed to replicate previously published data and detect a function in COLs-induced PCNA modification, we found that Parkin protected cells from oxidative damage which likely contributed to COLs formation.

To extend our studies, we investigated the possible involvement of the Bloom syndrome RECQ-like helicase (BLM) in the response to COLs, since it is known to work in conjunction with EXO1 helicase in other processes like homologous recombination. Our data show that BLM is recruited to sites of local UV damage with kinetics similar to that of EXO1.

Interestingly, signaling activation has been observed, albeit delayed, also in UV-exposed NER deficient fibroblasts, indicating that in absence of the typical excision machinery, another pathway can generate DNA structures such as exposed ssDNA or DNA DSBs, which are recognizable by apical checkpoint kinases. We thus examined the potential involvement of EXO1 in the generation of NER-independent signaling after UV exposure and reported that this process is mostly dependent on the activity of the exonuclease, as well as other proteins such as SNM1b.

We believe that this project sheds more light on the molecular mechanisms involved in the processing of closely spaced opposing lesions, in different non-cycling cell models. Indeed, we discovered that both Parkin and BLM have a role in COLs processing, although the former indirectly, and EXO1 has a major contribution in NER-independent DDR signaling activation. As the most approved models of the genetic disorders that typically see these proteins involved, namely Parkinson’s disease, Bloom’s syndrome, and NER deficiency models such as xeroderma pigmentosum, did not put particular focus on the involvement of the respective proteins in COL’s processing, we believe that our findings could contribute in the understanding of the

molecular mechanisms laying behind their phenotypes and in the future, aid the development of new therapies.

5 INTRODUCTION

Genetic information is stored in DNA¹, which is an intrinsically fragile molecule as it is susceptible to harm from many different chemical and physical sources. As a highlight, one can think that even water is harmful to DNA as it can lead to its hydrolysis, resulting in breakage in the backbone chain². During evolution, a complex molecular machinery was selected to minimize and repair the number of DNA lesions formed as a consequence of UV exposure. As different lesions require different repair mechanisms, it is logical to think that DNA repair pathways have branched into different sub-pathways (Figure 1). Indeed, today we can identify at least five major pathways, although similarities in the characteristics of different lesions naturally lead to extensive cross-talk and redundancy between different pathways. To provide a more thorough overview of the DNA Damage Repair (DDR) mechanisms, I summarize in these chapters the main kinds of lesions found in DNA and how they can be formed, as well as the corresponding molecular pathways specialized in their repair.

5.1 DNA LESIONS

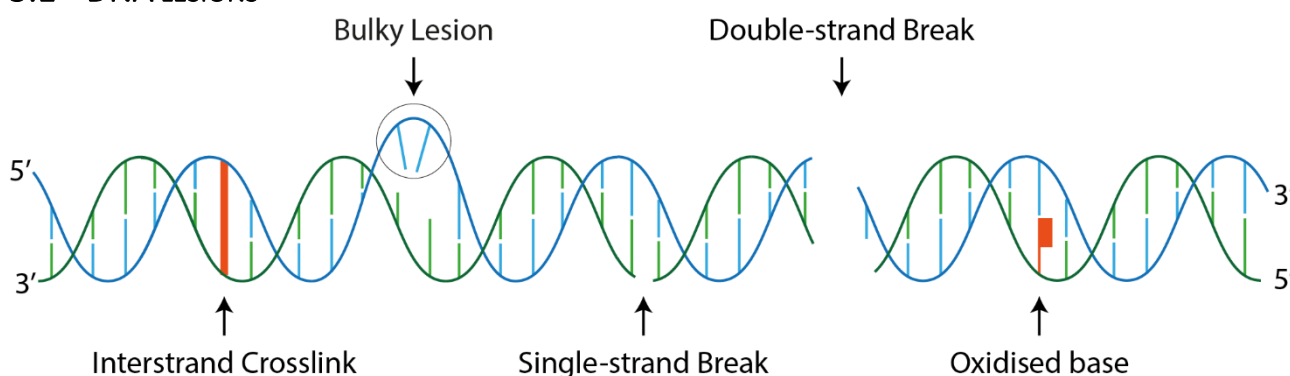


Figure 1: schematic overview of the main types of DNA lesions.

Schematic representation of different lesions that can be present in the DNA, as well as some examples of physical or chemical agents that can induce their formation.

5.1.1 Interstrand crosslinks

The first evidence of interstrand crosslinks (ICLs) in DNA comes from the realization that mustard gas, utilized during the Great War, was highly damaging to hematopoietic cells³. Being a bifunctional alkylator which was found to be much more cytotoxic than monofunctional ones, the mechanisms of action were supposed to lie in the crosslinking of replicating DNA molecules. Indeed, it was later found that the compound can indeed cross-link guanines together⁴. After that, plenty of other compounds have been discovered to have a similar effect and have been extensively used in chemotherapy for their destructive capability on rapidly proliferating cells. Some examples of these include platinum compounds like cisplatin, capable of inducing both interstrand and intrastrand crosslinks, psoralens, and chloroethyl nitrosoureas⁵. Although the chemistry of each adduct highly depends on the specific agent used, it is important to mention that purine nucleotides, and more specifically guanines, tend to be more reactive than pyrimidine nucleotides and as such are more prone to cross-linking⁶. Sources of ICLs can also originate from cell metabolism, making these lesions relevant for all cells even if not exposed to exogenous chemicals. This is the case of different aldehydes, like formaldehyde, acetaldehyde, and malondialdehyde, but also signaling molecules like nitric oxide⁵.

5.1.2 Bulky lesions

Bulky lesions constitute a wide range of DNA lesions characterized by their propensity to distort the DNA double-helix, and because of this feature, they can be recognized by the Nucleotide Excision Repair pathway. The best-studied lesions that are recognized by NER are induced by ultraviolet irradiation of DNA and include 6-4 Photoproducts (6-4PPs) and Cyclobutane pyrimidine dimers (CPDs)⁷. UV light can be classified into three subcategories depending on the wavelength: UV-A spans the 315-400 nm range, UV-B 280-315 nm, and finally UV-C 100-280 nm⁸. While the high energy UV-C is entirely blocked by the presence of the ozone layer, the other two components can still reach the ground, and potentially affect organisms' DNA⁹ (Figure 2A). UV-B photons absorbed by two adjacent pyrimidines in DNA can result in a [2+2] cycloaddition of the C5-C6 double bonds, generating one out of six possible stereochemical configurations of CPDs⁸. Although with less frequency, the [2+2] cycloaddition involving the C5-C6 double bond of a 5'-end pyrimidine and the C4 carbonyl group of a 3'-end thymine results in the formation of an unstable intermediate product, which is then spontaneously rearranged in a pyrimidine (6-4) pyrimidone adduct or 6-4PP⁸ (Figure 2B). Upon subsequent exposure to UV-B light, 6-4PPs can be converted into Dewar valence isomers¹⁰. Interestingly, the specific chemistry of the UV lesion determines their rate of removal: indeed, it is known that 6-4PPs are removed much faster from DNA than CPDs, with the majority of the former removed in a matter of hours compared to the latter being detectable days after irradiation¹¹.

UV-A exposure can generate single-strand breaks, oxidized pyrimidines, oxidized purines, and CPDs in a 1:1:3:10 ratio¹². The generation of these lesions, in contrast to what happens with UV-B light, happens both via direct damage to the bases¹³ or indirectly through absorption of the photons by endogenous photosensitizers, such as vitamins, amino acids, metabolites, proteins, and nucleic acids¹⁴.

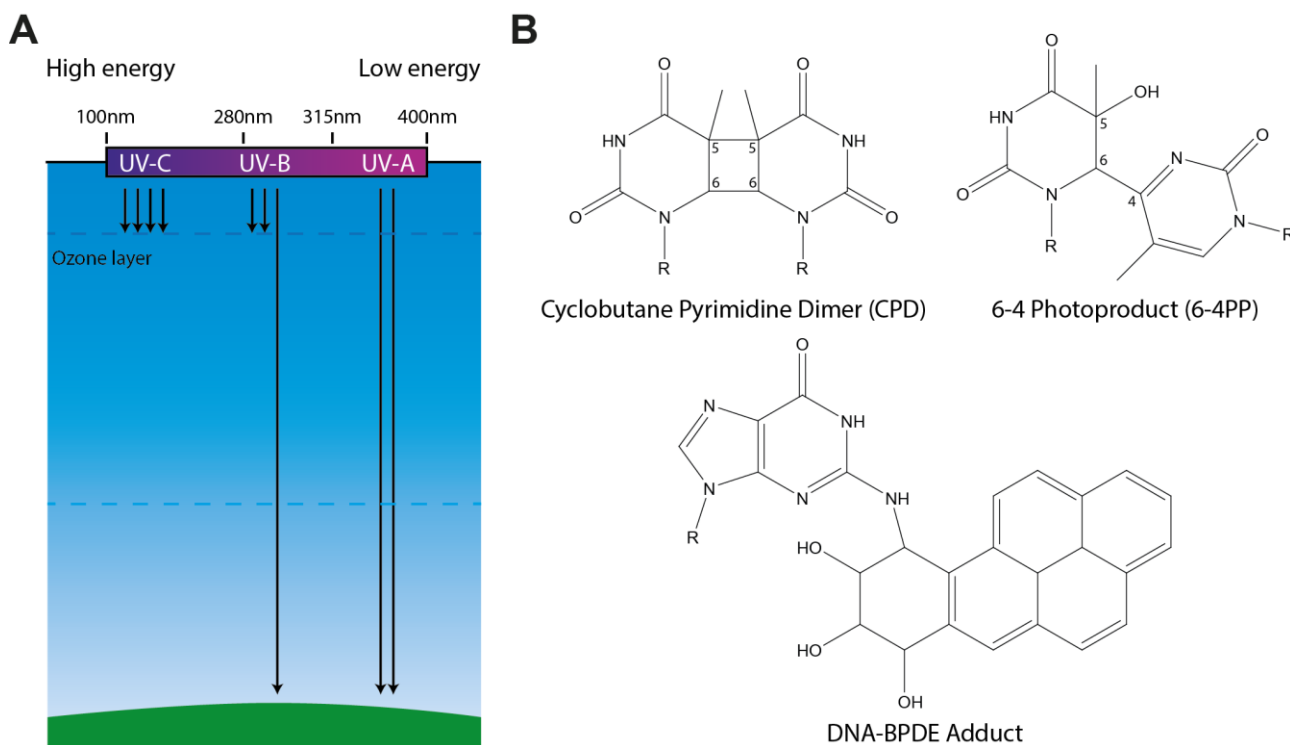


Figure 2: UV light and bulky adducts

(A) graphical representation of different kinds of UV light reaching the biosphere depending on the carried energy. UV-C light is completely blocked by the ozone layer while UV-A can almost entirely pass through. (B) Different kinds of bulky lesions on DNA, including CPDs and 6-4PPs generated by UV light absorbed by adjacent pyrimidines, and a DNA-BPDE adduct formed by the covalent bond of the compound BPDE to a guanine.

One important agent that is able to induce bulky lesions in DNA and that will become relevant in this project is Benzo[a]pyrene-diol-epoxide or BPDE in short. This compound is a product of the cell's metabolism of Benzo[a]pyrene¹⁵, a polycyclic aromatic hydrocarbon and one of the most important pollutants, a byproduct of combustion of organic materials found in industrial wastes, charcoal-cooked foods, diesel exhaust, and cigarette smoke¹⁶, and in natural processes like wildfires and volcanic eruptions¹⁷. If the relevance of Benzo[a]pyrene exposure to the human body was not clear enough, one can think that traces of this compound can be found in the air we breathe, in the food we eat, and even to some extent in the water we drink¹⁸. While Benzo[a]pyrene by itself is not considered to be carcinogenic, its metabolites are. Indeed, BPDE can bind guanines present in DNA, with a particular affinity for sequences that are G-rich in the same strand or both¹⁹. The most common adduct is performed via a nucleophilic attack of the N-2 of guanine upon the C-10 position of the compound²⁰, which results in a DNA double helix distortion, a bulky lesion, recognizable by NER²¹ (Figure 2B).

5.1.3 Oxidative damage

Oxidative damage on DNA originates from exposure of this macromolecule to reactive oxygen species, which can originate from the leakage of electrons to oxygen in the mitochondrial electron transport chain²², or certain enzymatic activities, for example during estrogen metabolism mediated by cytochrome P450²³. In experimental settings, common ways to induce ROS formation include treatment with hydrogen peroxide²⁴ or the more stable compound DMNQ²⁵, but they can also be generated indirectly by disrupting the electron transport chain using CCCP and FCCP²⁶. The highly reactive hydroxyl radical ($\bullet\text{OH}$) can react with DNA by addition to double bonds of DNA bases, leading to the formation of diverse adduct radicals with different redox properties²⁷. Depending on these, as well as the redox environment surrounding them, the bases are then either reduced or oxidized, generating a wide range of base modifications. For example, one of the most common base modifications detectable after ROS exposure is 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is the result of direct oxidation of guanines into 8-oxoG followed by further rearrangement into 8-OHdG²⁸.

To deal with the inevitable generations of free radicals, cells have evolved an intricate network of defense mechanisms which range from the use of direct ROS scavengers such as vitamin C and E, to the presence of specialized enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, which convert the radicals into unarmful compounds²⁹.

Reactive Oxygen Species can also be formed during events outside the normal cellular metabolism, for example in bursts released from phagocytic cells to destroy virus/bacteria infected cells²⁷, upon exposure to alkylating agents and nitric oxide^{30,31}, as well as a result of UV and ionizing radiation^{32,33}.

5.1.4 Single strand breaks

Single strand breaks (SSBs) are discontinuities in the sugar-phosphate backbone of one of the two DNA strands which can occur both due to endogenous factors, like the intrinsic fragility of DNA or the presence of ROS and other electrophilic molecules produced during normal cell metabolism, or by exposure to ionizing radiations and environmental genotoxins³⁴. Importantly, SSBs can be classified in direct lesions, when the damaging agent directly destroys the deoxyribose, or indirect ones when the break originates from the enzymatic removal of damaged bases that occurs during Base Excision Repair³⁴. Additionally, SSBs can also be produced during other physiological processes like DNA replication, transcription, recombination, and chromatin remodeling: for example, during the activity of DNA Topoisomerases, in order to relax supercoiled DNA or decatenate interlocked DNA, they introduce breaks in one of the filaments by using hinged clamps and DNA binding cavities³⁵. This mechanism can be exploited in experimental settings to introduce SSBs in a controlled manner, for example using the compound camptothecin which inhibits the rejoining step of DNA Topoisomerase I induced breaks specifically³⁶.

Although SSBs are generally considered to be less toxic than DSBs, this is mostly due to the higher efficiency with which they are repaired³⁴. Indeed, in situations in which single-strand breaks cannot be repaired, such as in the case of deficiency in the associated repair pathway, the sensitivity of cells to agents that can produce directly or indirectly this type of lesion is drastically increased³⁷. At the same time, mouse models fail to develop beyond day 7.5/8.5³⁸.

5.1.5 Double-strand breaks

Failure to repair single-strand breaks and their accumulation in the genome can easily lead to the formation of DNA double-strand breaks, in which both filaments are disrupted³⁴. Depending on the nature of the damaging agent, the two dsDNA molecules can present blunt ends, or 5'/3' overhangs³⁹. However, DSBs can also originate from a plethora of different mechanisms. For example, ionizing radiation can generate different patterns of DSB formation depending on the energetic characteristics of the damaging particles: photons and β -particles, contained in gamma-rays and x-rays, have high penetration but produce sparse damage, resulting in a random distribution of DSBs; while protons, α -particles and HZE ions contained in cosmic rays or emitted by radioactive decay have low penetration but can produce clustered lesions⁴⁰. The actual formation of the DSB can happen directly, as a result of the impact between the high-energy carrying particle and the DNA strand, or indirectly if these particles hit water molecules, splitting them into hydrogen and hydroxyl free radicals, which then generate SSBs and eventually DSBs⁴¹.

Double-strand breaks are also very commonly produced endogenously during DNA replication in circumstances that lead to the stalling of the replicative DNA polymerase, for example because of the encounter of a SSB, particular secondary structures, bulky lesions, interstrand crosslinks, and others⁴². Additionally, during transcription, the elongation of the RNA molecule can outpace the action of topoisomerases, resulting in the accumulation of a negative superhelical density that delays the re-annealing of DNA, favoring instead the annealing of RNA that just exited the polymerase to its complementary DNA and thus forming what is commonly termed an R-loop⁴³. The formation of these DNA-RNA structures is associated with the insurgency of DSB, and although the exact mechanism remains elusive, it is thought to be linked to the action of DNA and RNA enzymes of the APOBEC family⁴⁴.

A plethora of compounds capable of inducing DSBs exist under the name of "radiomimetics"⁴⁰. One of the most common ones is bleomycin, which is able to generate DSBs directly through its decomposition⁴⁵; other examples include the topoisomerase inhibitors camptothecin and etoposide, which can generate both SSBs as described in the previous chapter, but also DSBs^{46,47}.

Importantly, DSBs can also be generated incidentally during other repair processes that involve the generation of two or more SSBs close to each other and on different strands. This can happen during BER processing lesions of clustered oxidated bases⁴⁸: because this repair mechanism introduces gaps in the DNA strand that require refilling by DNA polymerases, two gaps in proximity to each other can naturally separate the dsDNA in two parts in a manner which is identical to that of a non-blunt DSB.

5.2 DNA DAMAGE AND REPAIR PATHWAYS

5.2.1 Homologous recombination

Lesions that result in the breakage of both DNA strands, called Double double-strand breaks (DSBs) are repaired by the DNA double-strand break repair system. As this results in the generation of free DNA ends, failure in a rapid repair of these lesions can cause loss of heterozygosity, mutations, genomic rearrangements, and even loss of complete chromosomes⁴⁹. Some of the agents that can generate DNA double-strand breaks include ionizing radiations⁵⁰, chemical radicals generated during cellular metabolism⁵¹, and often indirectly by many drugs used in anticancer therapy such as bleomycin⁵². Depending on different

factors, the most prominent being the cell cycle phase, DSB repair can occur via at least two different sub-pathways.

Homologous Recombination (HR) allows for a mutation-free repair, copying the damaged genetic information from a region of homology contained in another filament, like a sister chromatid, or more rarely the homologous chromosome or even homologous regions within the same chromatid⁵³. The first step, often referred to as “pre-synapsis”, consists in the generation of a ssDNA filament with a free 3’OH: as the DNA chemistry of DSB generated DNA ends is not always canonical, this is initially achieved by the Mre11-Rad50-Nbs1 (MRN) complex and its cofactor CtIP, which are able to perform an endonucleolytic incision of DNA releasing a 5’P-ending oligonucleotide. After that, DNA resection is performed by the 5’-3’ exonuclease EXO1 and BLM-TOPOIII α -RMI1-RMI2 complex, together with the DNA2 nuclease by chewing the 5’P end, to extend the exposure of ssDNA (named as DNA end resection)⁵⁴. This strand is then initially recognized by the ssDNA binding protein RPA⁵⁵, which eliminates any secondary DNA structure present⁵⁶. Assisted by mediator proteins, including Rad51B-Rad51C⁵⁷ and BRCA2⁵⁸, the RPA binding is then replaced by binding of Rad51 forming the now termed presynaptic nucleofilament⁵⁹.

The following steps consist of the search for homology and DNA strand invasion, both summarized under the name synapsis, which is catalyzed by the core proteins discussed above. The invasion of the Rad51-covered nucleofilament of the homologous DNA duplex results in the generation of what is called a D-loop intermediate. Here, DNA synthesis can be primed from the 3’OH of the invading strand using the complementary filament of the homologous region as a template. In canonical DSB repair, the second end of the double-strand break is also engaged either by Rad52-assisted annealing or through a second invasion event⁶⁰, thus generating a double Holliday junction (Figure 3). This structure is used as a substrate for dissolution in non-crossover products by BLM-TOPIII α , which migrate the two junctions towards each other⁶¹.

5.2.2 Non-homologous end-joining

Since HR relies on the presence of an additional DNA molecule containing a region of homology its feasibility is restricted to certain conditions, commonly when the cells are in the S and G2 phase of the cell cycle when the DNA is being replicated or has been already duplicated. In conditions where this is not possible, Non-Homologous End Joining (NHEJ) is favored instead, which acts by simply ligating together the broken ends in a process that can be mutagenic. The initial event is the binding of Ku70/80 heterodimer to the loose DNA ends, which is critical for the recruitment of all the other NHEJ factors⁶², one of which is DNA-PKcs⁶². Additionally, as very often the ends generated by the DSB are not directly compatible with each other for direct ligation, because of the loss of few nucleotides, nucleases like Artemis can perform a short resection in search of a region of microhomology³⁹. The binding of Artemis to DNA, but also DNA-PKcs binding alone stimulates the kinase activity of the latter, which then phosphorylates different NHEJ and DNA repair factors, including itself⁶³, and brings different proteins together in a synaptic complex including PAXX, XLF, XRCC4 and ultimately DNA ligase IV (LIG4)^{64–67} (Figure 3). These last two proteins are responsible for the direct ligation of the DNA ends⁶⁸. Similarly, to the situation found in HR, as not all DNA end structures generated after DSBs are compatible for DNA ligation, other processing factors like polymerases and nucleases can process the nucleofilament in preparation for LIG4 activity⁶⁹.

Alternative pathways to perform NHEJ also exist and mainly intervene in cases in which the canonical machinery (C-NHEJ) is defective. One of these, simply termed Back-up NHEJ (B-NHEJ), is slower and more error-prone⁷⁰ and involves enzymes such as MRE11, PARP1, the Werner’s syndrome RecQ-like helicase (WRN), the translesion synthesis DNA polymerase θ and Ligase I⁷¹. Another example is Microhomology-Mediated End Joining (MMEJ), which is mainly targeted to 2-25 nt long stretches of ssDNA, even more mutagenic, and relies on BLM/MRN, EXO1 or DNA2 and FEN1 which processes the overhangs: when a region of homology between the two single-stranded filaments is found, the two anneal and Polymerases β , λ or μ

fill the remaining gaps and Ligase I or III concludes the process, while the MisMatch Repair machinery corrects any mismatched base pair⁷¹.

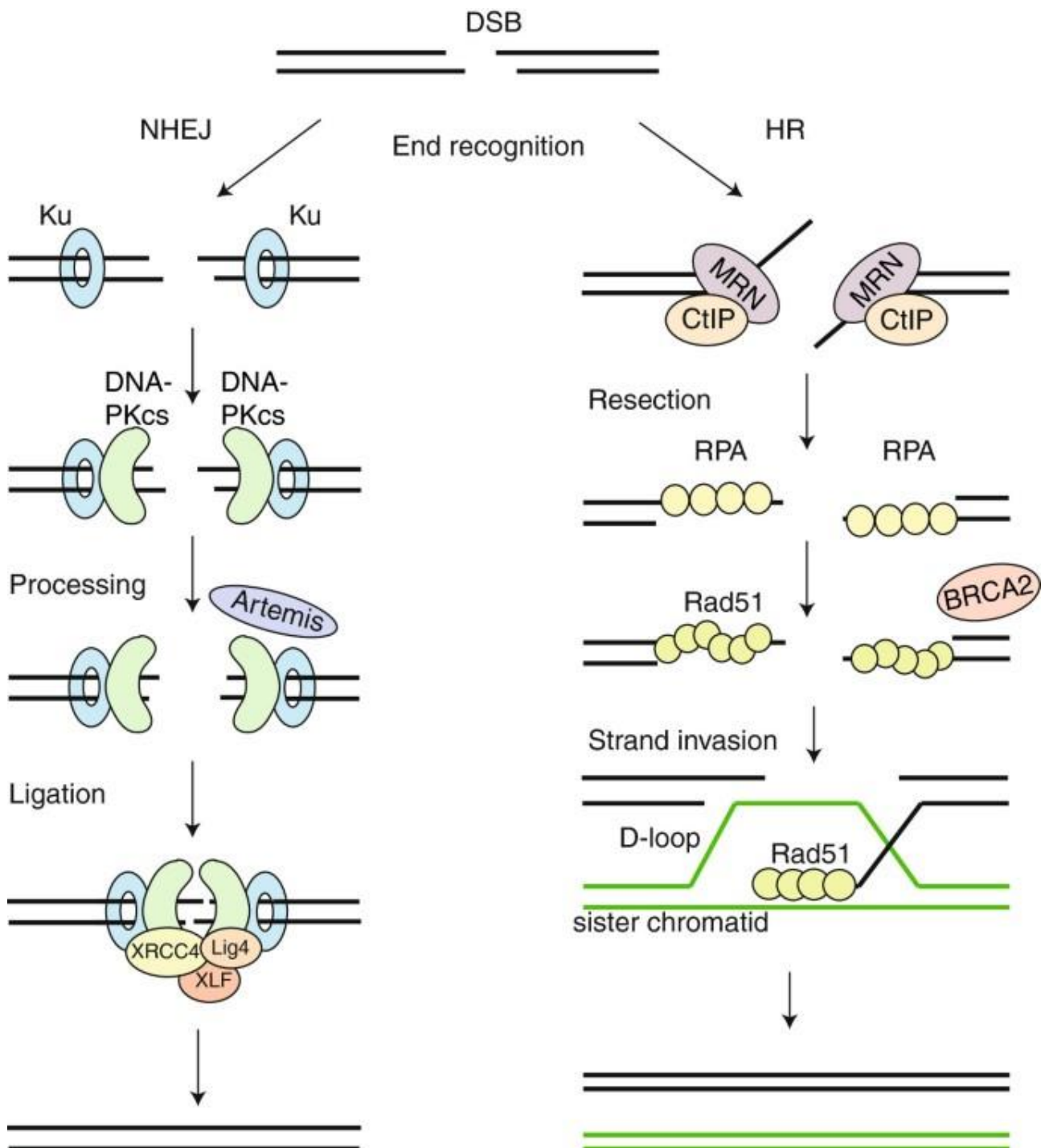


Figure 3: Differences between DSB repair by Homologous Recombination or Non-Homologous End Joining

Schematic representation, taken from Brandsma and van Gent 2012⁷², illustrating the main steps and players of HR and C-NHEJ.

5.2.3 Single strand break repair

Single-strand breaks (SSBs) are repaired by a pathway which has been called Single-strand break repair.. SSBs are first detected by PARP enzymes, which are activated in their presence and transfer ADP-ribose subunits to themselves or other proteins, in a process termed PARylation⁷⁸. Consequences of this PARP activation are chromatin decompaction⁷⁹, transcriptional regulation⁸⁰, and recruitment of the XRCC1 protein complexes which are necessary for proper SSBR⁸¹. Among the many proteins recruited with XRCC1, many are necessary

for end processing, such as PNKP, which promotes the removal of 3'-P residues that are present in the vast majority of direct breaks⁸², and DNA Ligase III which is responsible for the ligation reaction⁸³.

5.2.4 Base excision repair

Chemical modifications of the nucleobases contained in DNA, generated by endogenous or exogenous sources, are repaired by the Base-Excision Repair (BER) pathway. These modifications are mostly induced by exposure of DNA to reactive oxygen species or other oxidizing agents⁸⁴,

Modified bases are first sensed by DNA glycosylases, a protein family which includes at least 11 different members each capable of recognizing a specific set of lesions⁸⁵. They act directly on the modified nucleotides by flipping them outside the DNA double helix and removing the damaged base, leaving an abasic site⁸⁵. Depending on different conditions, including the cell cycle phase, the next steps can be summarized in two processes called short-patch or long-patch BER (Figure 4), the latter being more prevalent in cells that have already initiated replication⁸⁶. Short-patch BER only generates a single nucleotide gap, via the action of the apurinic-apyrimidinic endonuclease (APE1), which cuts the DNA strand 5' of the abasic site, generating 3' OH and 5'dRP ends. This is then repaired by the coordinated activity of DNA Polymerase β , which replaces the damaged nucleotide and removes the 5'dRP through its phosphodiesterase activity, generating a 5'P end that can be used by DNA Ligase I or III to seal the nick^{85,87}. PARP1 and XRCC1 can also be involved in some specific cases, mostly depending on the initial lesion recognized⁸⁸. Long-patch BER instead generates a longer ssDNA gap, which can be 2-10 nucleotides long, through a mechanism that depends upon APE1 activity but also requires a more extensive action by the replicative DNA Polymerases δ/ϵ , PCNA, FEN1, and DNA Ligase I⁸⁹.

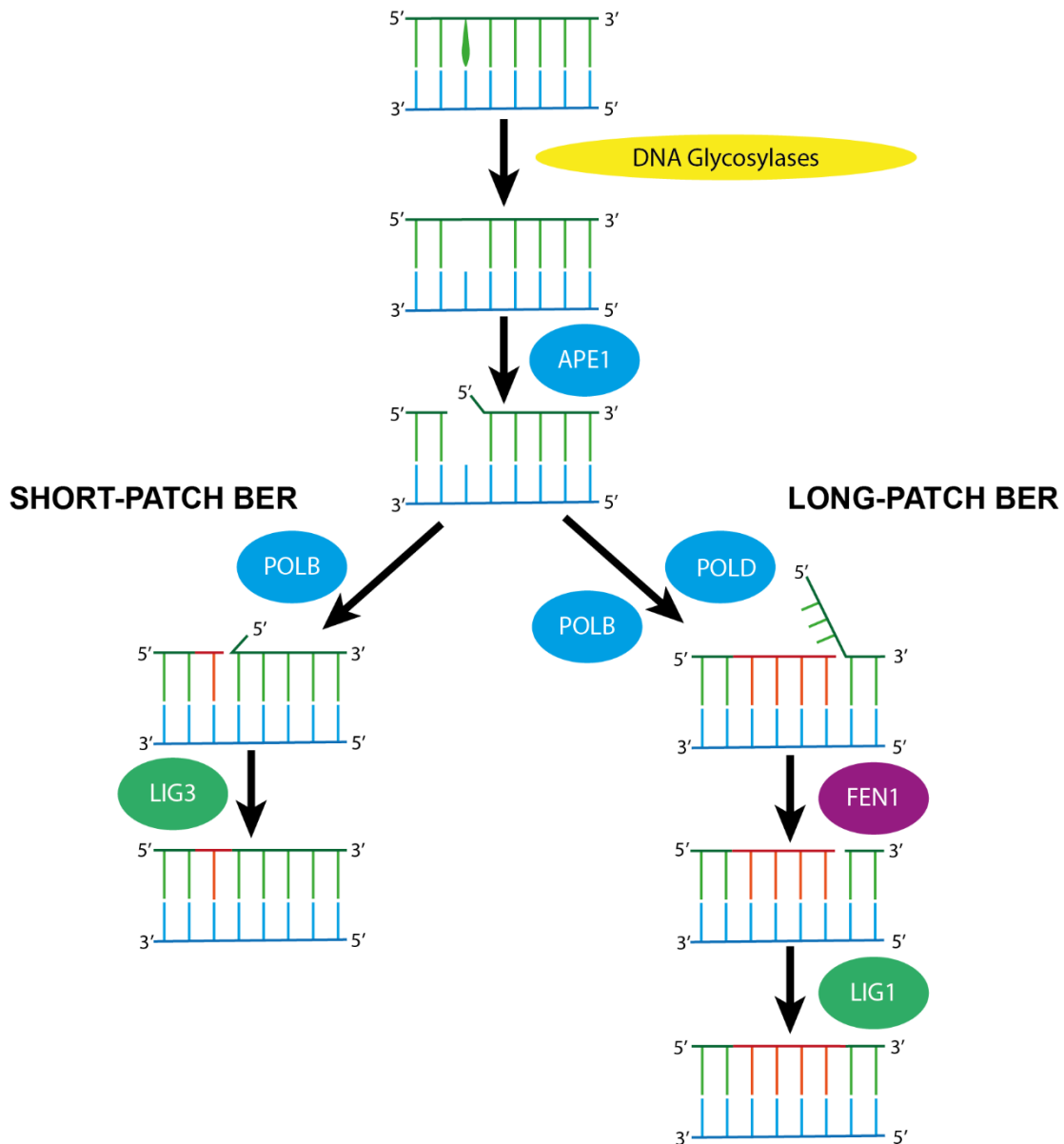


Figure 4: Main branches and players of the Base-Excision Repair pathway

Schematic representation of the main steps of BER. After recognition and removal of the altered base by the action of chemical-structure-specific DNA glycosylases, an abasic site is left on the DNA strand. The endonuclease APE1 performs an incision 5' of this site, leaving a ssDNA gap which will be processed by short-patch BER and long-patch BER depending on several factors. In the first case, Polymerase β fills a single nucleotide and Ligase III seals the dsDNA. In the latter, the action of Pol β or δ fills a larger gap, raising a small flap which will be removed by a second incision performed by FEN1. Ligase I finishes the repair in this case.

5.2.5 Mismatch repair

DNA Polymerases, although very precise when incorporating nucleotides into the nascent DNA strand, can still commit errors by introducing bases that are not complementary. For example, examining the activity of different DNA polymerases in vitro, it was found that DNA polymerases β and γ introduce an error every 3000 and 8000 nucleotides respectively; DNA polymerase α , while more accurate, still introduces an error every 30000 nucleotides⁹⁰. Naturally, if these numbers would reflect the situation in vivo, the mutation frequency of DNA would result incompatible with life as we know it. Instead, rough estimates indicate that the mutation rate in human DNA is around 175 mutations per diploid genome per generation⁹¹. It becomes strikingly clear that to achieve this much lower number, mechanisms that improve the fidelity of DNA polymerases or correct the errors introduced are indispensable.

One of these mechanisms is MisMatch Repair (MMR): it works effectively like a DNA damage repair pathway as it is able to recognize mismatched base pairs and selectively eliminate the newly introduced ones. Notably, although MMR associated with DNA replication is the most studied and likely the most prevalent because of the sheer number of bases that needs to be introduced and the inevitable errors, the pathway seems to be active in other processes that require DNA synthesis, such as during DNA repair and recombination^{92,93}.

MMR has been extensively studied in prokaryotes: the MutS homodimer recognizes mismatched bases, and then recruits the MutL homodimer and the MutH protein forming a single complex⁹⁴. The complex translocates along the dsDNA until it finds a hemi-methylated d(GATC) sequence, and since newly synthesized DNA is unmethylated in these organisms, MutH performs a single incision at the 5' or 3' of the mismatched base in that strand^{95,96}. This single-strand nick provides access for exonucleases and helicases that remove a short ssDNA stretch containing the mismatched base⁹⁷, leaving a gap to be filled by DNA Polymerase III and re-ligated by DNA ligase⁹⁸.

In mammalian cells, homologs of these proteins exist and the fundamental mechanism of MMR remains unchanged: MutS has two homologs called MutS α (heterodimer of MSH2 and MSH6) and MutS β (heterodimer of MSH2 and MSH3); the former primarily binds single mismatched bases or short (up to 3 nucleotides) indels while the latter binds larger indels of up to 13 nucleotides⁹⁸. The mammalian correspondent to MutL is the heterodimer of MLH1 and PMS2, called MutL α ⁹⁹; while no direct equivalent to MutH exists. Instead, the nick on DNA is thought to be performed by MutL α upon activation by PCNA; strand specificity is achieved not by absence of methylation (since in eukaryotes there is no distinction between the mother and daughter strand) but rather by the asymmetry of DNA-bound PCNA^{100,101}. EXO1, recruited and activated by MSH2 and/or MLH1, performs the excision step¹⁰², while gap-filling and ligation is performed by DNA Polymerase δ and DNA Ligase I¹⁰³.

5.2.6 Interstrand crosslink repair

Interstrand crosslinks block DNA replication, and because of this, their repair is tightly connected to the replication machinery. The first mechanism of replication-coupled ICL repair discovered in mammalian cells is the Fanconi anemia (FA) pathway, named after pediatrician Guido Fanconi who in 1927 described three siblings with severe anemia and birth defects^{104,105}. Cells derived from FA patients have been later found to be particularly sensitive to bifunctional alkylating agents, indicating that they probably lacked an effective repair mechanism¹⁰⁶. These discoveries led to the identification of 22 genes, named FANC proteins (although many of them share a different name as part of other cellular mechanisms), that have been classified into four functional groups⁵. The first group, referred to as the FANCM module, includes the DNA translocase FANCM and its interactors FAAP24, and histone fold proteins MHF1 and MHF2¹⁰⁷. The FANCM complex is able to recognize stressed replication forks and recruit the members of group 2, forming the FA core complex. The second group includes FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FAAP20, and FAAP100 forming a large E3 ubiquitin ligase complex, and also includes the E2 ubiquitin-conjugating enzyme FANCT alias UBE2T⁵. The objective of these proteins is to monoubiquitinate the members of group three, the ID complex, containing FANCI and FANCD2⁵. Notably, while ubiquitination of the latter is essential for ICL repair, FANCI ubiquitination only serves a regulatory role¹⁰⁸. Finally, group 4 includes different proteins involved in other DNA repair and DNA damage bypass pathways⁵.

The FA pathway is a complex mechanism that involves different players and steps depending on the following general parameters: the specific chemistry of the ICL, and in particular how much they distort the DNA double helix, the cell cycle phase, and whether or not they are found in highly transcribed genes⁵. In S phase, repair of ICLs is typically started by the convergence of two replication forks onto the ICL^{108,109}. In this case, CMG gets ubiquitinated by the E3 ubiquitin ligase TRAP110 and subsequently unloaded from the chromatin by the p97 ATPase¹¹¹. This event allows for one of the forks to undergo reversal¹¹² and sets the necessary conditions for the ubiquitination and DNA binding of the ID complex¹¹³. The complex then recruits the scaffolding protein

FANCP, alias SLX4, which in turn recruits FANCD1, which is the endonuclease XPF that also plays a critical role in Nucleotide Excision Repair, and as such performs a nucleolytic cleavage on the 5' side of the ICL¹¹⁴. The protein responsible for the second cut, at the 3' of the lesion, is to this date unknown, although its genetic epistasis in ICL repair with XPF¹¹⁵, high correlation with FANCD1 genes in CRISPR screenings¹¹⁶ and moderate sensitivity to cross-linking agents when depleted¹¹⁷ make SNM1 a perfect candidate. These double incision events result in the so-called “unhooking” of the DNA as the two filaments that are being replicated are free from each other; however, as the corresponding complementary DNA strands are already present on both sides of the lesion this situation inevitably generates a DSB⁵. To resolve this, Homologous Recombination is performed using the Translesion Synthesis DNA Polymerases ζ and REV1 to bypass the presence of the unhooked ICL on the template strand¹¹⁸. This still present lesion is later removed by either Base Excision repair or Nucleotide Excision Repair based on the chemistry of the adducted nucleotide¹¹⁹.

Interestingly, other mechanisms of ICL repair have been observed over the years. To cite a few examples, one of these is replication traverse, which allows the cell to resume replication prior to the unhooking event. This is achieved by ATR-dependent phosphorylation of FANCD1 and the subsequent displacement of the GINS subunit from the CMG complex¹²⁰. Although it is still to be demonstrated, the more realistic model is that this dissociation allows for the ring of CMG to open enough to bypass the ICL and continue unwinding after the lesion, coupled with a repriming event on the leading strand performed by PRIMPOL so that replication can continue on the other side⁵. The two dsDNAs could be later unhooked by a similar mechanism as the one described during fork convergence¹²¹. It is worth mentioning that, based on DNA combing assays performed in cells, lesion traverse is performed on roughly 60% of the ICLs¹²¹. Another pathway, termed the NEIL3 pathway, was documented to be performed on psoralen-induced ICLs specifically. Upon replication fork convergence, TRAP1-dependent CMG ubiquitination occurs as described before; however, this does not result in CMG dissociation but rather the recruitment of the glycosylase NEIL3 to the complex¹²². NEIL3 performs its unhooking action by cleavage of one of the two N-glycosyl bonds in the ICL, forming an abasic site on one side and a psoralen adduct on the other, which are both bypassed by REV1/Pol ζ ¹²³. Another example is the acetaldehyde pathway, which also requires fork convergence but does not produce abasic sites and therefore does not involve the action of glycosylases. Instead, the unhooking step is performed by enzymatic reversal of the ICL by an unknown protein, leaving an undamaged dG on one side and a propanoguanine adduct on the other strand, which is bypassed as described before¹²⁴.

ICL repair can also occur outside of S-phase on DNA which is not being replicated. In this case, the presence of the ICL is sensed by the NER protein XPC and shares a lot of similarities with the NER pathway¹²⁵. One major difference however is that, while in NER the two incisions on DNA are performed on the 5' and 3' side of the lesion (resulting in its excision), here both incisions are performed on 5' of the ICL, one of them being performed by XPF and the other by an unknown protein¹²⁵. The thus generated 22-28 nt long gap might allow the recruitment of an exonuclease, such as SNM1¹²⁶ and FANCD1¹²⁷, degrading DNA past the ICL and performing effectively the unhooking step¹²⁵.

5.3 NUCLEOTIDE EXCISION REPAIR

The Nucleotide Excision Repair pathway, or NER, represents the molecular tools that the cells use to remove and repair bulky lesions formed on DNA. Interestingly, NER participates also to the repair of intrastrand crosslinks which can be induced by certain chemotherapeutic drugs such as cisplatin, and to some extent Reactive Oxygen Species (ROS) generated lesions such as cyclopurines¹²⁸. Although the machinery is overall quite complex, the general mechanism of action can be divided into a few steps: recognition of the damage, assembly of the core components of the pre-incision complex, excision of the lesion containing DNA fragment and finally filling of the thus generated gap in the strand.

5.3.1 NER diseases

Just like many other molecular pathways, also the discovery of the existence and mode of action of Nucleotide Excision Repair is tightly linked to research on patients carrying genetic disorders. Indeed, mutations in NER factors give rise to xeroderma pigmentosum (XP), Cockayne syndrome (CS), Trichothiodystrophy (TTD), and UV sensitive syndrome (UVSS); patients with these diseases are classified into different complementation groups depending on the specific protein mutated. Xeroderma pigmentosum was first discovered in 1874 by dermatologist Moritz Kaposi, who noted a hereditary sensitivity to light in his patients¹²⁹. Another identifiable symptom is the increased predisposition to skin cancers. The molecular bases of the disorder were however identified only 94 years later by James Cleaver, who first identified defects in the processing of unscheduled DNA synthesis, the final step of NER, in skin biopsies derived from XP patients¹³⁰. Trichothiodystrophy, as the Latin name suggests, is characterized by a low content of sulfur-rich proteins in the nails and hair of its patients, which results in brittleness and coloration with a pattern of light and dark bands referred to as “tiger tail”¹³¹. Cockayne syndrome is mainly characterized by developmental and neurological symptoms like dwarfism, microcephaly, intracranial calcification, choroidoretinitis, and mental retardation¹³². Importantly, neurological defects are not exclusive to CS but also appear in some TTD patients as well as some complementation groups of XP¹³³.

5.3.2 Global Genome NER

The first step of NER is the recognition of the damage, which can be achieved through two different sub-pathways, termed Global Genome-NER (GG-NER) and Transcription Coupled-NER (TC-NER). GG-NER is mediated by the XPC protein, which is able to recognize the thermodynamic destabilization and distortion of the DNA double helix, now forming a bubble-like structure, regardless of whether it actually contains a lesion or not¹³⁴. The proteins Rad23B, and to a lesser extent, Rad23A, in complex with XPC, stabilize it and assist its binding to DNA^{135,136}, then dissociate from it just after damage recognition¹³⁷. Participating in the complex is also a third protein, the calcium-binding protein centrin-2 (CETN2); however, while it is known that it generally stimulates NER activity, its exact role still remains to be determined¹³⁶. As some lesions that do not significantly distort DNA, like CPDs, are not recognized by the XPC-Rad23A/B complex, other proteins are involved in their recognition, called UV-DDB1 and UV-DDB2 (XPE)¹³⁸ (Figure 5). DDB2 is indeed able to accommodate both 6-4 Photoproducts and CPDs in its binding pocket¹³⁹ and it is part of a bigger complex containing also the CUL4-ROC1 ubiquitin ligase, responsible for the ubiquitination of histone H2A and DDB2 itself, targeting them for degradation¹⁴⁰. XPC gets also ubiquitinated, but instead of being degraded its DNA binding properties are enhanced¹⁴¹.

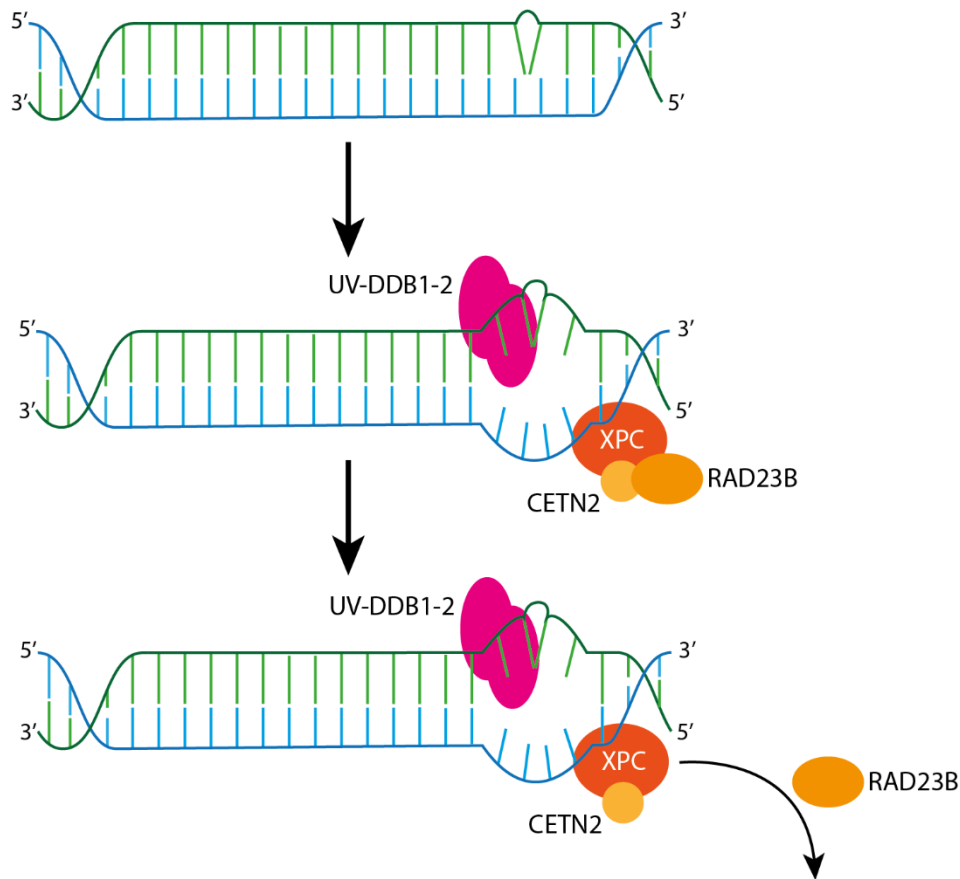


Figure 5: Steps of damage recognition by Global Genome NER

The bulky lesion, however the nature, when found in a non-actively transcribed strand is recognized by UV-DDB1-2, XPC, Centrin-2 (CETN2), and Rad23B. The latter protein leaves the damaged site before the assembly of the pre-incision complex.

5.3.3 Transcription-coupled NER

In TC-NER, on the other hand, the recognition step is mediated by the stalling of RNA Polymerases when it encounters a bulky lesion during transcription^{142,143}. While the exact mechanisms that connect this event with the recruitment of the other component necessary for NER are poorly understood, it is known that two additional proteins, CSA and CSB, are involved; interestingly XPC and UV-DDB are dispensable in this case¹⁴⁴. In short, the stalling of RNA Pol II allows the binding of CSB, which in turn assists the recruitment of CSA and UVSSA¹⁴⁵ (Figure 6). The CSA protein is recognized by the DDB1-CUL4A-RBX1 ubiquitin ligase complex (CLR4), which gets activated and ubiquitinates CSA, CSB, and RNA Pol II¹⁴⁶. The binding of all of these factors to stalled RNA polymerases is highly cooperative, and the recruitment of UVSSA is what determines the association of the TFIIH complex and the displacement of the transcriptional apparatus¹⁴⁷.

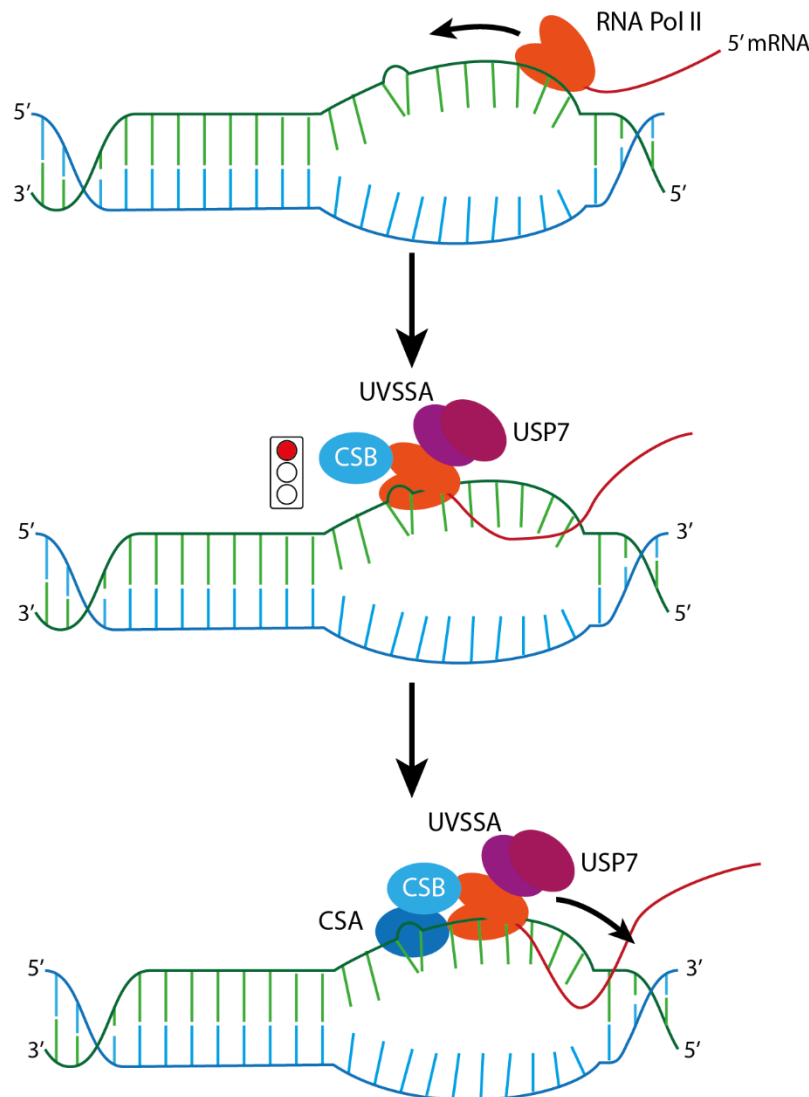


Figure 6: Steps of damage recognition in Transcription-coupled NER

5.3.4 Assembly of the pre-incision complex

Whether recruited by interaction with XPC-Rad23B¹⁴⁸ or by TC-NER, the next player to join and lay the foundations for the assembly of the pre-incision complex is TFIIH. This protein complex consists of 10 subunits, that can be divided into two major sub-complexes bridged by the protein XPD helicase. The core complex contains the XPB helicase, p52, p8, p62, p34 and p44. The CAK complex, standing for cyclin-activated kinase, contains CDK7, cyclin H and MAT1. While having a major role in transcriptional regulation, the CAK complex is not necessary for NER function and it actually dissociates from the TFIIH once XPA is recruited^{149,150}. Both the helicases involved, XPB and XPD, have a major role in opening the DNA around the lesion¹⁵¹: XPB, assisted by p52, is important to facilitate the loading of the entire TFIIH itself, prying open the DNA double helix with its ATPase activity and through a conformational change¹⁵². XPD, now fitting in the DNA bubble, is stimulated by p44, opens the DNA and translocates along it using both its helicase and ATPase activity¹⁵³. This activity also serves as a damage verification step, as the translocation is stopped once the protein encounters bulky lesions¹⁵⁴. XPD is released, together with the rest of the TFIIH complex, while still associated to the excised fragment¹⁵⁵. In the whole process, p8 also seems to play an important role as it is essential for the opening of the DNA helix and the recruitment of subsequent factors; the gene (TTD-A) is mutated in trichothiodystrophy patients¹⁵⁶.

With the recruitment of XPD, the pre-incision complex can be completed. XPA, the endonuclease XPG, and the ssDNA binding protein RPA are recruited next, independently of each other, while XPC-Rad23B dissociates from the complex¹⁵⁷ (Figure 7). XPA acts as the very center of the entire NER machinery, as it interacts with TFIIH, XPC-Rad23B, DDB2, and RPA but also with the endonucleases ERCC1-XPF and XPG, which cut the DNA strand 5' and 3' to, the lesion, respectively and also with PCNA^{158–164}. XPA function is crucial, as it ensures that all NER factors are in the right place before the incision of DNA; and indeed, in its absence NER activity is completely abolished¹⁶⁵. Mechanistically, it was proposed that XPA binds closer to the 5' side of the bubble, where it finds itself in an ideal position to interact with both RPA and ERCC1-XPF, coordinating the further steps¹⁶⁶. Both RPA and XPA cooperate for ssDNA interaction, with RPA binding a 30 nucleotides long region¹⁶⁷ (more or less the size of the DNA fragment that will be excised, implying that only one RPA complex is able to fit in the bubble at this time), favoring the non-damaged strand and positioning the endonucleases on the correct lesion-containing strand¹⁶⁸. The physical interaction between XPA and RPA, in particular subunits RPA70 and RPA32 with XPA, is fundamental for optimal NER activity¹⁶⁶.

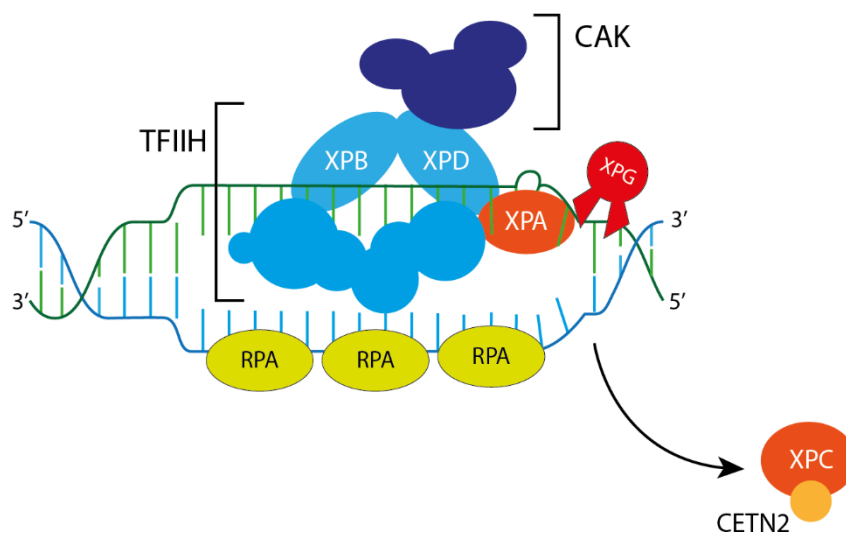


Figure 7: The assembled pre-incision complex

Together with the dissociation of XPC and CETN2, multiple factors allow the assembly of the pre-incision complex. This series of events leads to the recruitment of TFIIH and CAK complexes, together with XPA and XPG. As a bubble starts to form via the action of DNA helicases, the non-damaged ssDNA gets covered by RPA.

5.3.5 Excision of the damaged oligonucleotide

With the endonucleases in place, the actual excision process can start. Interestingly, while the presence of XPG is required for ERCC1-XPF recruitment and activity, its actual endonucleolytic activity is not, supporting the notion that ERCC1-XPF cut first. The ERCC1-XPF incision generates a free 3'OH group that can be extended by DNA polymerases; indeed, in XPG catalytic mutants the gap-filling activity starts and proceeds through the ssDNA gap while the lesion-containing DNA flap is still attached to the rest of the strand¹⁶⁹. The exact mechanisms that coordinate the order of these 2-step reactions are however still poorly characterized. When XPG cuts, the ~30 nucleotide-long lesion-containing DNA fragment is excised and actively evicted from the repair site through the translocase HLT¹⁷⁰, with TFIIH still bound (Figure 8). The latter will only be released via ATP binding. At this point, and after a small nucleolytic degradation that brings its size closer to 18-20 nts¹⁷¹, the oligonucleotide is bound by RPA and eventually totally degraded by nucleases such as TREX1^{155,172}. Interestingly, what happens to the actual damaged nucleotides is still not completely clear¹⁷³.

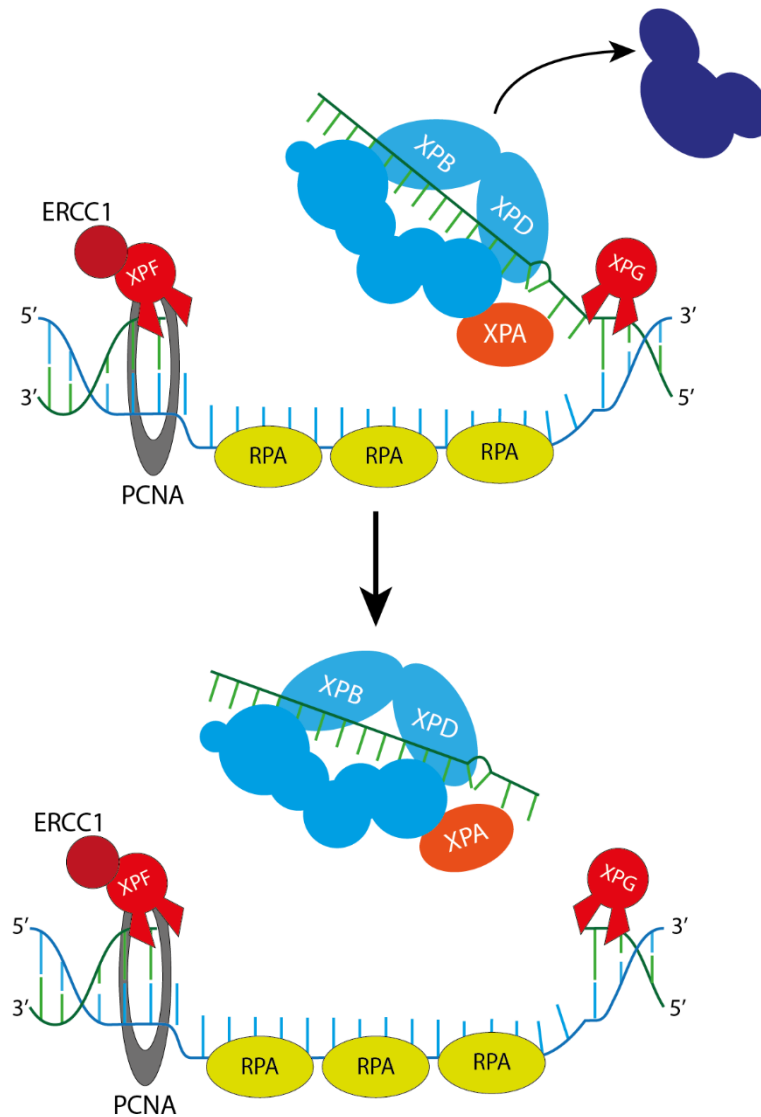


Figure 8: Excision of the damaged oligonucleotides by double incision of XPF and XPG

Two incisions at the 5' and 3' sides of the recognized bulky lesion allow for the dissociation of the damaged ssDNA from the genome, together with many of the NER factors involved thus far. PCNA is recruited to prepare for the following gap-filling step.

5.3.6 Gap filling and ligation

The last step of Nucleotide Excision Repair is the filling of the ssDNA gap, which is usually performed by the replicative DNA polymerases δ and ϵ and to some extent by Translesion Synthesis (TLS) DNA Polymerase κ ¹⁷⁴. Refilling requires RFC-PCNA¹⁷⁵ and is usually referred to as Unscheduled DNA Synthesis (UDS). Some exceptions to this mechanism exist and will be explored in detail in the chapter about Closely-Spaced opposing lesions. Finally, the nick left after UDS is repaired by specific DNA ligases¹⁷⁶.

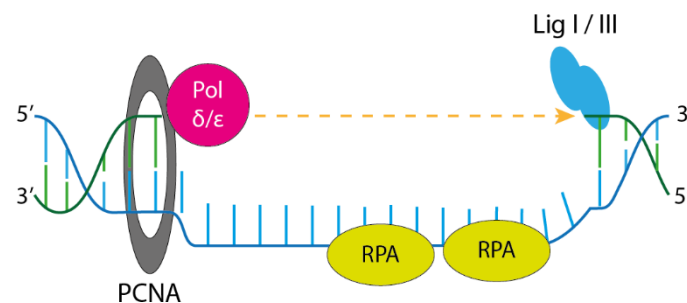


Figure 9: canonical gap-filling and ligation steps of NER

In the most described model, gap-filling step is mainly performed by DNA polymerases δ and ϵ , while ligation occurs via Ligase I or III.

5.3.7 Methodologies to study NER

At the time of writing this thesis, sixty years have passed since the first publications describing NER^{177–179}: during this period, different assays and techniques to study each of the steps of this mechanism have been developed.

Starting, from the actual measurement of bulky lesions in DNA, the most specific and at the same time accurate approach is to perform mass spectrometry; however, in order to have quantitative data, for example, to know the exact number of damaged nucleotides in one example, it requires the use of an isotopically labeled internal standard to account for the formation and loss of lesions during sample processing^{180,181}. These internal standards are often very expensive and not always available and therefore represent the main limitation of the technique¹⁸². Other methods characterized by high sensitivity of detection are ones based on radioactive labeling, but they are lacking in the specificity for bulky lesions¹⁸³. Similarly, single-gel electrophoresis, often called comet assay, when performed in alkaline conditions can help identify the presence of bulky lesions in DNA, but it recognizes also single and double-strand breaks and a plethora of other kinds of DNA damages¹⁸⁴. To achieve high levels of specificity, and distinguish between different kinds of bulky lesions, approaches that exploit lesions-specific antibodies have been developed: there are most often done via immunofluorescence, dot/slot-blot, or ELISA¹⁸⁵. Promising new techniques exploit an appositely engineered DNA polymerase to perform Polymerase Chain Reaction (PCR) over DNA adducts, incorporating an artificial nucleotide whenever one is encountered, allowing for genomic locus-specific probing of DNA damage¹⁸⁶.

Another step of NER that is often extremely useful to assay is the gap filling. This can be achieved by providing cells with excess quantities of radioactively or fluorescently labeled nucleotides that get incorporated into the nascent strand of DNA during Unscheduled DNA Synthesis (UDS)¹⁸⁷. Examples of this, are the BrdU or EdU incorporation assays, in which bromodeoxyuridine or 5-ethynyl-2'-deoxyuridine are incorporated in DNA and later on detected by using specific antibodies, in the first case, or CLICK chemistry reactions that covalently bind the nucleotide to a fluorescent molecule, in the second¹⁸⁸. The main limitation of these techniques is buried in the fact that any cells that are in S-phase and therefore replicating DNA will also incorporate the modified nucleotides, impairing the possibility to distinguish actual UDS from replication. While these approaches allow for spatial analysis as they can be performed *in vivo*, Host Reactivation Assays serve a similar role but without this advantage: they are instead based on the transfection of damaged exogenous DNA in cells which, if repaired, transcribe a reporter gene¹⁸⁹.

Finally, to specifically probe TC-NER, the Recovery of RNA-Synthesis (RRS) and Recovery of Protein-Synthesis (RPS) assays are used. The former, similarly to the above-mentioned approaches to study UDS, exploits the incorporation of ribonucleotide analogs that are incorporated in nascent RNA and can be detected with CLICK chemistry, such as in the case of 5-ethynyluridine (EU). The latter exploits the expression in cells of a construct carrying a reporter gene, like GFP, linked to a degradation tag: before induction of damage, the protein is degraded bringing the signal of the reporter to zero; this way, the recovery of the signal can be used as a way to monitor the translation and indirectly the transcription after NER activity. The main advantage of this technique compared to the first one is adaptability to different assay formats and the possibility for live monitoring¹⁹⁰.

5.3.8 Crosstalk with different pathways

Many of the factors involved in NER also take part in other cellular pathways. For example, the TFIIH complex takes a fundamental role in transcriptional regulation, and indeed it is part of the pre-initiation complex that controls the activity of RNA Polymerase II¹⁹¹. Additionally, the XPB and XPD subunits also serve a protective role against retroviral insertion in the host genome through degradation of the viral cDNA¹⁹², and are also

potentially involved in G-quadruplexes formation as they have been observed to bind many putative G4 forming regions in the genome¹⁹³. The CAK subunits regulate cell cycle progression through activation of several cyclin-dependent kinases, including CDK1, CDK2, CDK4, and CDK6¹⁹⁴.

As previously mentioned, extensive crosstalk between NER and ICL repair exists; in fact, ERCC4/XPF takes part in both pathways. Repair of ICLs via the FA pathway in the genome requires the use of either NER or HR, largely depending on the cell cycle phase. NER also often interacts with the BER machinery and their functions can be overlapping. For example, lesions that were thought to be exclusively repaired by BER, like 8-oxo-dG and thymine glycol, were found to be repaired even in the presence of NER alone: the latter mechanism acting probably as a slower backup system in cells¹⁹⁵. Furthermore, XPC is capable of improving the OGG1 glycosylase-dependent removal of 8-oxo-dG in the genome¹⁹⁶ and physically interacts with APE1 in oxidative stress conditions¹⁹⁷.

Some evidence also has shown potential interactions between NER and HR. Indeed, repair of protein-DNA crosslinks involves both pathways depending on the size of the protein to remove: less than 14 kDa ones mainly involve NER while larger ones require HR, presumably due to the need for repair of DSBs generated by stalling of replication forks in the latter case¹⁹⁸.

5.4 DNA DAMAGE AND CHECKPOINT SIGNALING

One critical aspect of DNA damage repair is that in most conditions it is happening at the same time as other important cellular processes that often require DNA to be intact for their correct functioning, including DNA, replication, transcription, and cell cycle progression. To coordinate these numerous mechanisms, complex pathways (DNA damage checkpoint; DDC) of protein-protein signaling evolved, which in multicellular organisms can also serve to trigger senescence and apoptosis that are critical for the survival of the whole organism. For the most part, DDC pathways are based on the activity of different proteins implicated in a complex signal transduction cascade, involving principally different posttranslational modifications events, which amplify and transmit the signals to various effectors. In such a way, the signal due to a single DNA double-strand break or a single-strand break can generate a global cellular response. Importantly, the same signaling cascade can also be triggered during the execution of different DDR pathways, including NER, as ssDNAs are intermediate products of such mechanisms¹⁹⁹. Several protein kinases have been identified as key players in DDC. Traditionally, these proteins have been classified based on their position in the signaling cascade in three major categories: apical kinases, which function as the primary sensor of the damage; mediators, which receive the signal from the apical factors, amplify it and relay it to effectors; which are directly interacting with downstream proteins to regulate their activities²⁰⁰.

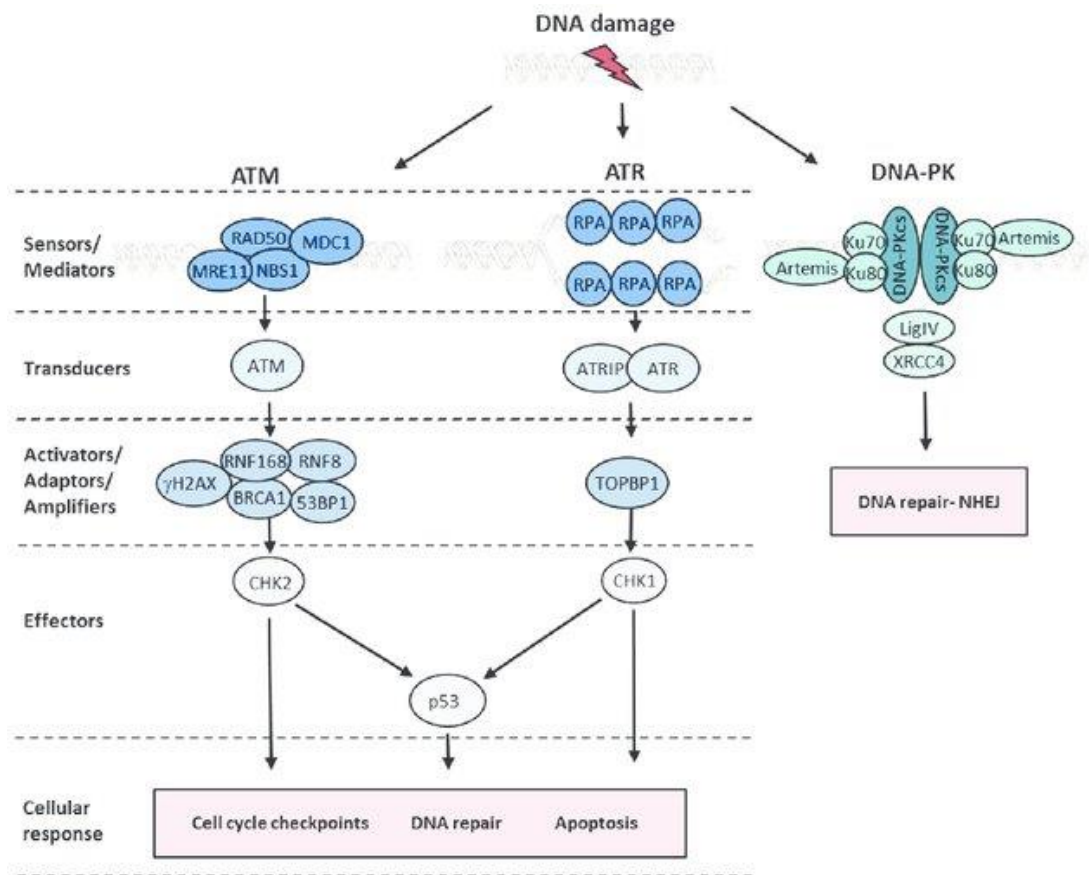


Figure 10: signalling cascade and crosstalks between DNA apical kinases

Schematic representation, taken from Ryan et al 2016²⁰¹, illustrating some of the major targets of ATM, ATR, and DNA-PKcs; as well as the corresponding signalling cascade and the effects that this produce on the cell

In human cells, the apical kinases are ATM, ATR, and DNA-PKcs. They belong to the PIKKs family and share similar characteristics with each other. From C- to N- terminus they all present a FATC motif, a kinase domain, a FAT domain, and multiple helical solenoid HEAT-repeats domains regulating protein-protein interactions^{202,203} (Figure 11). All of them target for phosphorylation serine or threonine residues followed by a glutamine (S/T-Q motif), which interestingly are also present in their own sequences²⁰⁴.

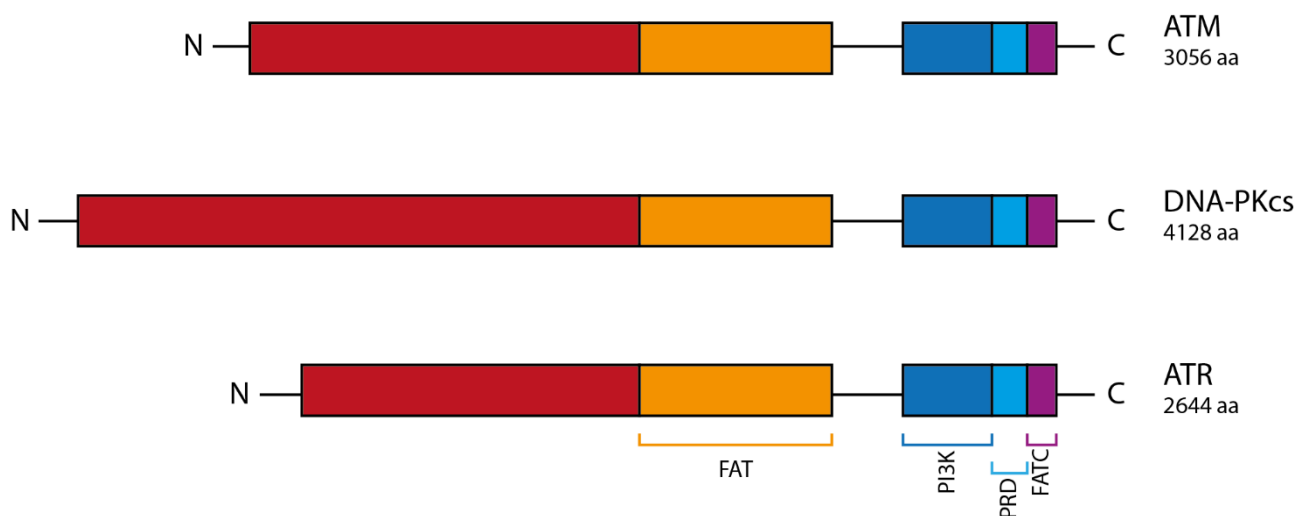


Figure 11: Structural similarities between the apical kinases

Schematic representation of ATM, DNA-PKcs, and ATR domain configurations. Notably, the N terminal domain is the one presenting the highest variability between the three.

5.4.1 DNA-PK

Indeed, DNA-PKcs, once recruited to double strand breaks by its cofactor Ku80²⁰⁵, is able to phosphorylate itself at Serine 2056 and Threonines 2609 and 3950: This event is a marker of DNA-PKcs activation and is also critical in DNA repair, for example by promoting dissociation of the kinase from DSBs sites to allow DNA-ends ligation²⁰⁶. To this day, the role of DNA-PKcs kinase on other targets is not entirely clear, however it is known that activation of the protein is necessary for the recruitment of factors to DSBs, like the protein SNM1C/Artemis which promotes DNA-end processing necessary for ligation of some DNA ends²⁰⁷. Additionally, it can phosphorylate the ssDNA binding protein RPA, on residues 4 and 8²⁰⁸, and histone H2AX on Serine 139, both directly or via the Akt/GSK3 β pathway²⁰⁹.

5.4.2 ATM

Also, ATM, after its recruitment at DSBs by the MRN complex²¹⁰, can self-activate by autophosphorylation on Serine 1981. This event marks the transition from an inactive kinase dimer to active monomers²¹¹. ATM was thought to mainly stimulate Homologous Recombination as its activation leads to the stimulation of DNA-end resection to generate the pre-synaptic filament²¹². However, it was found that repair of a minor subset of lesions in G1 via Non-Homologous End Joining (NHEJ) also require activation of ATM and the MRN complex, indicating that ATM is involved in NHEJ as well as HR²¹³. The targets of active ATM are literally thousands, but most importantly for this thesis is that it can phosphorylate mediators such as Chk2²¹⁴ and effectors such as p53²¹⁵. Phosphorylation of p53 at Serine 15 has been particularly studied and it has been shown to contribute to a rapid stabilization of the protein and its activation in response to DSBs, but also after UV-treatment^{174,216,217}. Another key ATM target is histone H2AX, which when phosphorylated at Serine 139 is termed γ H2AX. This single modification is responsible for an entire set of chromatin-based signaling events involving not only phosphorylation but also ubiquitylation and other post-translational modifications²¹⁸. Importantly, γ H2AX is read by MDC1 through a specific binding mediated by its BRCT domains; MDC1 recruits more MRN complex that in turn recruits more ATM, which phosphorylates more H2AX. Through this positive feedback loop, the major players involved in DNA damage signaling are spread in a vast area surrounding the DSB²¹⁹. As chromatin containing γ H2AX is transcriptionally inactive, this signaling also serves the purpose of avoiding collision of DNA repair factors with the transcriptional machinery and counteracting the formation of DNA-RNA hybrids called R-loops^{220,221}. Additionally, H2AX phosphorylation also participates in the liquid-demixing surrounding double strand breaks, which serve the purpose of concentrating DNA repair factors to enhance their efficiency²²².

5.4.3 ATR

ATR, the third apical kinase, responds to a much wider variety of DNA damages, including UV-induced lesions²²³. Indeed, ATR and its partner ATRIP are recruited to long stretches of RPA-covered ssDNAs that, as described above, are intermediates of different DNA repair pathways²²⁴. ATR activation also requires its interaction with different activator proteins, including TopBP1²²⁵ and ETAA1²²⁶, and like the other apical kinases results in its autophosphorylation on Serine 1964²²⁷. Similarly, to ATM and DNA-PKcs, also ATR has many targets: major targets of ATR dependent signaling are the mediator Chk1, which promotes proteasomal degradation of the CDC25A phosphatase leading to cell cycle arrest²²⁸, and p53-Serine 15 in non-cycling cells²²⁹. Other important targets of ATR are RPA32, which is phosphorylated at Serine 33²³⁰, and once again H2AX. This latter modification implies that γ H2AX foci can be observed also in non-double strand breaks.

5.5 THE CLOSELY-SPACED OPPOSING LESIONS MODEL

Since none of the apical kinases are able to directly identify UV lesions and are instead activated by the presence of either ssDNA regions or DNA double strand breaks, and since the rapid activation of the DDC

after UV irradiation requires NER activity²³¹, we have to hypothesize that the DDC is activated after the initial processing of UV lesions.

The involvement of Translesion Synthesis DNA polymerases such as Pol κ in the NER process, as well as the detection of non-S phase PCNA ubiquitylation that is a marker for the switch to TLS polymerases²³², and the involvement of EXO1 in UV-dependent DDC activation in non-proliferating cells²³³, were all indications that at least some UV-induced lesions were subjected to a more elaborate processing, following NER incision events.

A paradigm of these 'special' lesions is the so-called "closely-spaced opposing lesions" (COLs), which consist of a bulky lesion on one DNA strand, and another polymerase-blocking DNA lesion in close proximity but in the opposite strand. When the filament containing the bulky lesion is processed by NER nucleases, a 3'OH end and a 5'P end are produced. Unscheduled DNA synthesis starts from the 3'OH, but it is stalled by the presence of the second lesion on the template strand that blocks the activity of DNA polymerases δ and ϵ . In parallel, the free 5'P end can be attacked by EXO1 which would enlarge the gap generated by XPG and XPF/ERCC1²³⁴ (Figure 12). This is supported by data produced by our lab showing accumulation at local UV damage sites (LUDs) of both human isoforms of EXO1, which requires XPF activity and XPG presence (but not necessarily its catalytic activity) and that is fundamental for proper activation of the DDC²³³. This was also confirmed in vitro by the Sancar lab²³⁵. Generally, gap enlarging by EXO1 is counteracted by gap refilling by DNA polymerases. At COLs, when UDS is blocked, EXO1 activity persists and gap-filling can only be accomplished through the recruitment of TLS polymerases that can bypass the blocking lesion in the template strand. Indeed, PCNA is monoubiquitinated at Lysine 164 by the E3 Ubiquitin ligase Rad18 in complex with Rad6²³⁶ to recruit TLS polymerases; if UDS is inhibited by preventing PCNA ubiquitination, by silencing TLS polymerases or by poisoning DNA synthesis with AraC, DNA double-strand breaks are generated and the DDC response is overall increased²³⁷. All Y family TLS polymerases, including Pol κ , Pol ι , Pol η , and REV1, are recruited at LUDs and are involved in gap filling at COLs, although the first shows a much greater contribution to UDS in general compared to all the others²³⁷.

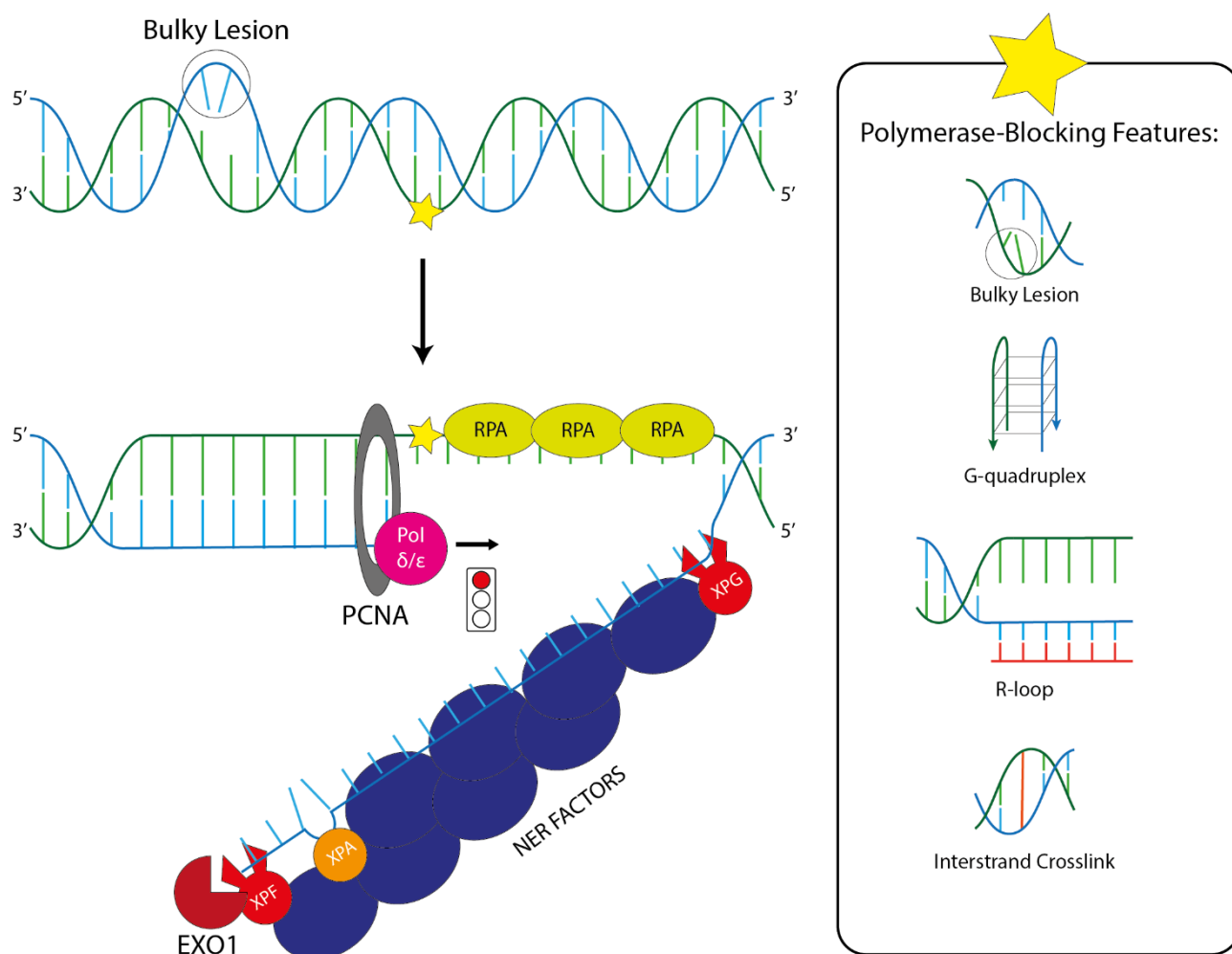


Figure 12: Closely-spaced opposing lesions and problematics during their repair

Representation of a COL, formed by a bulky lesion on the 5'-3' strand and a generic polymerase blocking feature (indicated here with a star symbol) on the opposite. Examples of the latter include other bulky lesions including the ones generated by ROS exposure like cyclopurines, and ongoing transcription forming R-loops, but also features binding different strands together like G-quadruplexes and interstrand crosslinks. As per their description, these indeed stall the replicative DNA Polymerases δ/ϵ during the process of gap-filling.

While according to rough estimates, only between 0.0002%-0.02% of the human genome is damaged²³⁸, and therefore the likelihood of COLs generation seems low, one must consider the following facts. First is that in principle, COLs can derive from a wide range of DNA lesions or structures: the requirement is any kind of bulky lesion on one strand and DNA polymerase-blocking lesions on the other. Different DNA lesions can block polymerase activities, including of course bulky lesions, intrastrand/interstrand crosslinks, and some oxidative lesions like Thymine glycol and cyclopurines^{239,240}. Additionally, stable DNA structures can also hinder DNA polymerases: for example, R-loops generated by collisions with the transcriptional machinery²⁴¹ and G-quadruplexes²⁴². Second, as bulky lesions induce a significant distortion of the DNA double helix and as a consequence a local weakening of Watson-Crick interactions, their presence reduces compaction of the chromatin thus exposing the surrounding DNA to additional damages, increasing the risk of clustered lesions^{243,244}.

5.6 DNA REPAIR IN NON-REPLICATING CELLS

Cells that are actively replicating constitute only a very small portion of the total amount of cells that are present in the human body, mostly located in self-renewing tissues like epithelia and bone marrow²⁴⁵. The

vast majority of our body is instead composed of cells that have either terminally differentiated, like neurons and myocytes, or have gone into a quiescent state and are as such stuck into the G₀ phase of the cell cycle²⁴⁶. While most of these cells can be steadily replaced thanks to a pool of stem cells, cells such as neurons are for the most part irreplaceable²⁴⁷. It is therefore immediately apparent why preserving genome integrity is especially crucial for these cell types, and how research on the mechanisms used by these cells to regulate their DNA damage response has important clinical relevance, in particular for developmental and neurodegenerative diseases. For these reasons, I will highlight in this chapter some features of neurons, which often can be extended to most non-cycling cells.

The field of DNA repair in neurons is an emergent one, with many discoveries that are still to be made. Because of this, data about neuronal-specific DNA repair or the correspondent DDC signaling is largely lacking. It was proposed that the entire GG-NER machinery is inactive in neurons²⁴⁸, as well as in other terminally differentiated cells such as myoblasts²⁴⁹, macrophages²⁵⁰ and keratinocytes²⁵¹: that made sense as, since they don't need to replicate, DNA repair needed to be only performed on actively transcribed genes. However, TC-NER activity requires the integrity of the non-transcribed strand which is used as template, so how would that be kept free of bulky lesions in the absence of global genome repair? In reality, it was found that lesions on both strands of active genes are repaired in these cell types, using a variant form of NER initially named "Differentiation-Associated Repair"²⁵², but that was later reported also in non-differentiated but quiescent cell types²⁵³. After further analysis, it was suggested that DAR is due to the persistence of GG-NER in restricted compartments of the nucleus characterized by active transcription; this led to the change of its denomination to "Domain-Associated Repair"²⁵⁴. It is still unclear how the downregulation of GG-NER in the same areas of the genome and its persistence in other genomic regions is controlled; however, it seems to rely on the differential phosphorylation of the E1 ubiquitin activating enzyme²⁵⁵.

The signaling cascade also has particular features in neuronal cells. First of all, while ATR is essential in mitotic cells, ATR deletion in postmitotic neurons does not compromise their survival; instead, it enhances neuronal electrical activity, resulting in the aberrant firing of the action potential, hyperexcitability, and epilepsy, independently of its role in DDC²⁵⁶. In other cell types, such as non-cycling lymphocytes, the ATR-p53 pathway is entirely suppressed²⁵⁷. Additionally, in response to DNA damage neurons can undergo senescence²⁵⁸, but most interestingly they can also re-enter the cell cycle, although this is usually followed by the induction of programmed cell death²⁵⁸. While cell cycle re-entry was generally thought to result in higher exposure of neurons to the possibility of apoptosis, it was also found that it can have protective roles, for example against cytotoxicity caused by amyloid- β exposure²⁵⁹. The exact mechanisms and biological significance of this process are still to be elucidated.

For their biology, neurons are obviously susceptible to potential closely-spaced opposing lesions. While never directly exposed to UV light, other compounds can induce bulky lesions in these cells. For example, the commonly diffused compound benzo[a]pyrene, which was introduced above, crosses the blood-brain barrier, which normally acts as an impenetrable protection against many other harming agents²⁶⁰. The chemotherapeutic drug cisplatin can also cross this defense, under certain conditions, and cause NER target lesions^{261,262}. Neurons, like myocytes, are some of the highest energy-demanding cells that rely on oxidative phosphorylation performed in mitochondria for their correct functioning²⁶³. As during the aging process, the function of mitochondria declines resulting in reduced ATP production and ROS leakage²⁶⁴, it is thus expected that these cells exhibit high loads of oxidative damage, a fraction of which would be polymerase blocking. R-loops and G-quadruplexes are also incredibly dynamic in neurons as they are used as transcriptional regulatory tools for the transient silencing and activation of genes involved in learning and memory²⁶⁴. Indeed, being able to rapidly change transcription profiles is one of the fundamental ways in which plasticity is achieved in these cells²⁶⁵.

5.7 CANONICAL AND NOVEL FUNCTIONS OF THE E3 UBIQUITIN LIGASE PARKIN

The polymerase switch triggered by PCNA monoubiquitination has been extensively characterized in several conditions and models; however, we still have little knowledge of how this process is regulated. Some papers in the literature suggest a potential involvement of the Parkin E3-Ubiquitin ligase. Before delving into this topic, it is important to highlight some of the more traditional aspects related to this protein.

The gene, now called PARK2, was first isolated in 1998 as a part of the long arm of chromosome 6 carrying frequent deletions in autosomal recessive juvenile parkinsonism (AR-JP), and at the time it was the second largest gene after dystrophin²⁶⁶. The gene, spanning 12 exons along 500 kilobases, encodes for a 465 amino acids long protein composed of an N-terminal ubiquitin-like UBL domain, and four zinc-coordinating RING-like domains called RING0, RING1, IBR, and RING2, the latter containing the catalytic cysteine in position 431²⁶⁷. Interestingly, while the RING-like domains are important in manipulating the ubiquitin protein, the UBL domain acts as a regulatory domain which normally keeps the protein inactive²⁶⁸. While the localization of this protein is mainly cytoplasmic²⁶⁹, it has also been observed in the nucleus in physiological conditions²⁷⁰; the nuclear localization is altered by some pathogenic missense mutation mainly but not exclusively located in the RING1 region^{271,272}. As of today, it is still unclear how exactly the loss of Parkin leads to the neuronal death typical of AR-JP; however, multiple speculations have been formulated.

One of the earliest found and probably the widest known functions of Parkin is as an E3 ligase that targets protein for proteasomal degradation via their Lysine 48-linked polyubiquitination. This happens for example during the process of mitophagy. Briefly, in healthy mitochondria the protein kinase PINK1 gets translocated to mitochondria and processed via multiple steps^{273,274}. The mature protein is dissociated from the mitochondria and rapidly degraded by the proteasomal system, resulting in very low levels of detectability of the protein in normal cells²⁷⁵. In damaged mitochondria, where the membrane potential is severely reduced, the translocation of PINK1 is no longer possible; the protein homodimerizes and auto-phosphorylates at Serine 228 and Serine 402, becoming a highly active protein kinase²⁷⁶. Major targets are Parkin, which gets phosphorylated at Serine 65 in the ubiquitin-like domain²⁷⁷, and ubiquitin itself which is also phosphorylated at the same residue²⁷⁸. When phosphorylated Parkin is bound to phosphorylated ubiquitin, it becomes maximally active as an E3 ubiquitin ligase²⁷⁹. Strong ubiquitylation of Mitofusin by Parkin leads to its degradation²⁸⁰ and results in a deficiency in mitochondrial fusion and elimination of the defective. Mitochondria by their incapsulation in autophagosomes followed by fusion with lysosomes and degradation²⁸¹. Loss of Parkin activity therefore disrupts effective mitophagy, leading to the accumulation of damaged mitochondria and neuronal toxicity²⁸².

Since Parkin is also responsible for the degradation of several other targets, other mechanisms may contribute to neuronal death in AR-JP patients. To cite a few relevant examples, it was proposed that Parkin would target α -Synuclein, a protein frequently found aggregated in Lewy bodies in Parkinson's Disease²⁸³. Furthermore, Parkin is also able to target unfolded proteins, preventing unfolded-protein stress-induced cell death²⁸⁴.

Parkin was also reported to be involved in cell cycle progression, connecting it also to cancer. Indeed, Parkin regulates the stability of important mitotic regulators including Plk1, Cyclin B1, Aurora A, Aurora B, and Nek2A via the interaction with Cdc20 and Cdh1, two co-activators of the Anaphase Promoting Complex (APC/C). In this model, Parkin could substitute for some of the functions that are canonically attributed to the APC/C itself²⁸⁵.

Aside from its function as an E3 ubiquitin ligase, Parkin seems to also have roles in other cellular processes such as transcriptional regulation in ways that are independent from ubiquitination. For example, chromatin immunoprecipitation (ChIP) and Electrophoretic Mobility Shift Assay (EMSA) assays have demonstrated the physical interaction of Parkin with the promoter region of TP53²⁸⁶, presenilin-1, and presenilin-2²⁸⁷. The DNA-

binding capacity of Parkin was attributed mainly to its RING1 finger domain, which binds specific oligonucleotides *in vitro*²⁸⁸. Subsequent analyses led to the identification and validation of a Parkin-responsive element in the loci mentioned above²⁸⁷.

Finally, a possible role of Parkin in the regulation of the DNA damage response was suggested in 2009 by two papers from Shyan-Yuan Kao^{289,290}. In these works, the author observed that induction of DNA damage with UV light, hydrogen peroxide, or the double-strand break-inducing agent etoposide triggered the translocation of Parkin from the cytoplasm to the nucleus, where its total levels increased to 2-fold. Additionally, Parkin was reported to interact with chromatin in both UV-treated cells and in samples from aging human brains that showed increased oxidative damage. Moreover, the expression of Parkin in human cells increased the repair of UV-induced cyclobutene-pyrimidine dimers or the repair of UV-treated exogenous DNA in a host cell reaction assay. Interestingly, endogenous Parkin was also able to interact with PCNA as observed via an immunoprecipitation experiment, in a process that depends on the region containing the first RING domain, and on a putative PCNA-interacting protein-box (PIP). Expression of the PIP region alone, which interacts with PCNA with no physiological function, reduced the repair of DNA damage, suggesting that the interaction of Parkin with PCNA is indeed important for DNA repair. A 2017 paper²⁹¹ showed that Parkin overexpression increases a Rad18- and UV- dependent post-translational modification of PCNA that the authors propose to be ubiquitinated at Lysine 164, while the knockout produces the opposite effect. These data suggest that Parkin is implicated in PCNA monoubiquitination. Parkin knockout resulted in a drastic reduction of RPA and PCNA recruitment to damaged chromatin and a severe lack of Unscheduled DNA Synthesis consistently with the halving of TLS DNA polymerase η recruitment.

5.8 DNA HELICASE BLOOM AND FUNCTIONS IN DDR

Mutations in the BLM gene give rise to Bloom syndrome (BSyn). This autosomal recessive genetic disorder is characterized by severe pre and postnatal growth deficiency, abnormalities in the immune system, defects in fertility, and insulin resistance. Curiously, patients with BSyn often show sensitivity to UV light in a manner similar to XP patients, implying a potential role of the BLM protein in the repair of UV-induced lesions²⁹². Another characteristic of this syndrome is an increase in tumor development, with a frequency that is at least 150 times higher, and an anticipation in the age of tumor insurgency²⁹³.

The BLM gene is located on chromosome 15 at 15q26.1 and contains 21 coding exons, resulting in the production of a 1417 aminoacids-long protein with a molecular weight of 159 kDa²⁹⁴. Most disease-inducing mutations result in the production of a truncated, non-functional form of the protein, while a smaller percentage of cases, roughly 20%, result in the loss of its helicase activity^{295,296}.

The BLM protein, is part of the RecQ helicases family, and it plays a fundamental role in processes such as DNA replication, transcription, recombination, and repair; like all RecQ helicases, it is considered a “genome caretaker”²⁹⁷. BLM, similarly to WRN and RecQ4 has a very well-characterized 3’-5’ ATP-dependent helicase activity²⁹⁸, while WRN is the only one that also presents 3’-5’ exonuclease activity²⁹⁹. All members of this subfamily, when mutated give rise to genetic disorders which share the increased predisposition to cancer development.

In physiological conditions, most of the BLM molecules are confined in PLM nuclear bodies, punctate nuclear structures that serve as storage locations for different proteins involved in genomic stability³⁰⁰. It is additionally found in the nucleolus, at telomeres³⁰¹ and also at meiotic and replication foci^{301,302}. The levels of the protein are very low during the G1 phase of the cell cycle and in G0³⁰⁰, but increase sharply during S phase, G2, and in mitosis³⁰³, after which they return low via a process dependent on its ubiquitination by Fbw7a³⁰⁴.

As helicases rarely operate alone, BLM is able to interact with a multitude of other proteins using its RQC domain³⁰⁵, including Topoisomerases I, II α and III α , P53, ATR, 53BP1, and RPA^{306–311}. Importantly, the interaction with the latter leads to a strong activation of its helicase activity³¹². Furthermore, BLM is also part of a supercomplex of BRCA1-associated proteins, which also includes ATM, the MRN complex, and the DNA replication factor C (RPC)³¹³.

The physiological roles of BLM span from replication, recombination, telomere maintenance to transcription³¹⁰. Curiously, BLM was shown also to have a role in G-quadruplexes stability as it is able to unwind the G4s³¹⁴. The function that is most relevant to this thesis is in DNA double-strand break repair, in particular during Homologous Recombination. Here BLM is recruited to the sites of lesions in a process dependent on its HRDC domain, which is also responsible for a conformational change^{315,316}. Active BLM forms an oligomeric ring structure of assembled hexamers³⁰⁹ and stimulates the exonuclease activity of EXO1 and the helicase/endo-exonuclease activity of DNA2. This process is dependent on the MRN complex and promotes 5' resection of the DNA ends generated from the double strand break³¹⁷. Interestingly, EXO1 stimulation is independent of BLM helicase activity and is instead mediated by physical interaction between the two proteins³¹⁸. In later steps, BLM is also responsible for deciding whether to promote recombination or not. Indeed, in high sequence homology conditions, BLM is SUMOylated³¹⁹, promoting the strand exchange activity of the Rad51-covered DNA strand and resolution of the D-loop once DNA synthesis is finished³²⁰. However, in low sequence homology conditions, BLM SUMOylation is lacking, and to avoid illegitimate recombination it disrupts the RAD51-ssDNA filament and dissociates from the D-loop³²¹.

The functional interaction between BLM and EXO1 is what piqued our interest and stimulated part of the research reported in this thesis.

5.9 NER-INDEPENDENT SIGNALING ACTIVATION

As described in the first part of this Introduction, NER activity is a prerequisite for the rapid activation of the DDC following UV irradiation. A NER-independent mechanism, acting at later time points after UV exposure has also been reported and its role in neuronal cells has been investigated as part of this thesis.

A paper dating back to 2001³²² reported a late P53 hyperphosphorylation in UV-irradiated cells lacking functional NER that was suggested to be due to a blockage in transcription elongation. Indeed, while cells deficient in TC-NER showed an accumulation of such post-translational modification at relatively late time points, in cells deficient in GG-NER but with a functional TC-NER this response was completely absent. The result was strengthened using transcription-blocking compounds, such as Actinomycin D, which had very similar effects to TC-NER deficiency. Therefore, it is possible that one potential structure that triggers checkpoint signaling could be stalled RNA polymerases, which expose RPA-coated ssDNA in the transcription bubble to recognition by ATR. Indeed, similar chromatin remodeling events, inducible by incubation of cells with hypotonic buffers or with chromatin-modifying drugs, have been proposed to result in signaling activation³²³. Other DDC markers, such as phosphorylation of histone H2AX, as well as ATM, NBS1, and Chk1, were shown to be induced in cells completely lacking NER activity (XP-A and XP-B), or even in cells defective in global genome-NER (XP-C) but proficient in TC-NER, although to a considerably less extent^{235,324,325}.

Works performed in yeast^{326,327} have shown phosphorylation of Rad53, the yeast functional equivalent of human Chk2, in a NER-independent manner at late time points after UV exposure and was suggested to be due to DNA breakage.

Evidence of DNA breaks induced after UV exposure in a NER-independent manner was found also in human fibroblasts deficient in GG-NER, detected by alkaline comet assay³²⁸. This kind of analysis is capable of detecting both SSBs and DSBs and therefore gives very little information about the nature of the lesions. However, the authors of the paper also performed pulsed-field gel electrophoresis, which is generally more

sensitive to double-strand breaks, and were not capable of detecting any. While due to the inability of the assay to detect low frequencies DSBs it is impossible to completely negate their existence, it is nonetheless reasonable to assume that the majority of the breaks detected in the alkaline comet assay were in fact single strand breaks. Importantly, these DNA breaks were not detectable in cells that were only deficient in TC-NER, which seems to weaken the hypothesis of them originating from the stalling of transcription bubbles. Instead, the authors proposed the involvement of the endonuclease Ape1, as they found that silencing the protein caused a considerable reduction in both H2AX phosphorylation, PCNA recruitment, and DNA breaks. Furthermore, an *in vitro* experiment showed how the protein was truly capable of performing an incision on an oligonucleotide containing a single 6-4PP, or to a considerably lesser extent, a CPD.

While being a key component of Base Excision Repair, the apurinic/apyrimidinic endonuclease Ape1 was found to be involved also in less canonical processes, one of which was termed Nucleotide Incision Repair (NIR). In NIR, the action of DNA glycosylases is completely skipped and it is Ape1 that directly recognizes the oxidated bases, with a particular affinity to 8OH-dG. Ape1 then performs an incision 5' of the damaged base without generating an apurinic/apyrimidinic site, followed by 3' to 5' exonuclease degradation³²⁹. Because of the evidence that Ape1 is able to incise UV lesions containing DNA strands, one can imagine that the NIR machinery could be at work also if the NER machinery is not functional. However, to this day, data about *in vivo* Ape1-dependent incisions near UV lesions is still lacking.

Another player potentially involved in the NER-independent processing of UV lesions is the DNA glycosylase MUTYH. A high-throughput chemical screening aimed at identifying compounds able to alleviate the UV sensitivity of NER-deficient cells identified acetohexamide as a hit. The compound, originally approved as an anti-diabetic drug, exerted its action by promoting the proteasomal degradation of MUTYH³³⁰. This finding hinted at the possibility of MUTYH being responsible for the toxicity of UV light in NER-deficient cells. Indeed, knockout of this protein greatly improved the viability of XPA-deficient cells after UV treatment. The protein colocalized with XPC at local UV damages, suggesting that it may recognize UV lesions, and the authors did in fact provide an *in silico* model of MUTYH stably bound a CPD and flipping it outside the DNA double helix, in a manner similar to that of other MutY family glycosylases removing mispaired adenines. The increased sensitivity to UV light caused by the presence of MUTYH in normal NER deficient cells was speculated to be caused by its induction of single-strand breaks in front of UV lesions, similarly to its activity on 8-OHdG³³¹. These nicks may be used as starting points for other processes already mentioned in this introduction, including resection by exonucleases.

6 MATERIALS AND METHODS

6.1 CELL LINES AND CULTURES

The following cell lines have been used:

- SH-SY5Y human neuroblastoma cell line (from ATCC)
- U2-OS human osteosarcoma cell line (from ATCC)
- HEK 293T human embryonic kidney-derived cells (from ATCC)

These cells were cultured in complete DMEM High Glucose GlutaMAX Supplement (Gibco™)

- RPE_hTERT immortalized retinal pigment epithelial cell line (kindly gifted by Nicoletta Landsberger, Dipartimento di Biotechnologie Mediche e Medicina Traslazionale UNIMI)

These cells were cultured in complete DMEM/F-12 High Glucose GlutaMAX Supplement (Gibco™)

- Primary neurons from mouse hippocampus (explanted at Istituto di Ricerche Farmacologiche Mario Negri)
- C3PV (ctr), XP12RO (XP-A) primary fibroblasts (kindly gifted by Miria Stefanini, Istituto di Genetica Molecolare CNR)

Every cell line was kept at 37°C in a humidified atmosphere with 5% CO₂. All non-primary cells were cultured in media supplemented with 10% Fetal Bovine Serum (Gibco™), previously heat inactivated for 30 minutes at 56°C, 1% Penicillin/Streptomycin solution (Gibco™), 0.5% Sodium Pyruvate Solution (Lonza™ BioWhittaker) and 0.1% Plasmocin® prophylactic (InvivoGen).

Primary neurons were isolated from postnatal day 2 mice and grown in Neurobasal™ serum-free medium (Gibco™), supplemented with B-27™ (Gibco™), for 10 days.

Primary fibroblasts were cultured in DMEM 15% FBS and subcultured for a maximum of 20 passages.

6.2 CELL TREATMENTS

Compounds used include:

- Benzo[a]pyrene Diol Epoxide (BPDE) (Toronto Research Chemicals)
- Cytosine β-D-arabinofuranoside hydrochloride (Sigma-Aldrich®)
- Retinoic Acid (Sigma-Aldrich®)
- Brain-Derived Neurotrophic human Factor (BDNF) (Sigma-Aldrich®)
- NU-7441 (DNA-PK inhibitor)
- AZD-6738 (ATR inhibitor)
- KU-55933 (ATM inhibitor)
- Hexadimethrine bromide (Polybrene) (Sigma-Aldrich®)
- H₂O₂ (Sigma-Aldrich®)

Concentration and exposure times of these compounds varies depending on the cell line used and the specific experiment, and both are therefore reported in the corresponding figures.

UV treatments were performed with a UV lamp at 254nm wavelength. Local UV damage was performed by putting an Isopore™ 5.0µm PC membrane on top of the cells-covered slides.

Differentiation of SH-SY5Y was performed by a gradual decrease of serum in the media and treatments with 15µM retinoic acid and 10ng/ml BDNF. In detail, the cells were plated on day 0 in complete DMEM 10% FBS. One day after, on Day 1, media was replaced with DMEM 10% FBS supplemented with retinoic acid. On day 4 media was replaced with DMEM 3% FBS with retinoic acid, and on day 5 changed again with DMEM 0.5% FBS supplemented with BDNF. On day 7 cells were considered differentiated.

Starvation of RPE_hTERT cells was performed by plating cells at high density and changing the medium with DMEM/F12 0.25% FBS for a minimum of 48 hours.

6.3 ANTIBODIES

The following primary antibodies have been used:

Antibody	Description	Dilution
Anti-Vinculin, V9131	Mouse monoclonal, Sigma-Aldrich®	1:40.000 in Milk 3% PBST 0.1%
Anti-GAPDH, 0411	Mouse monoclonal, Santa Cruz Biotechnology	1:1000 in Milk 3% PBST 0.1%

Anti-Parkin, PRK8	Mouse monoclonal, Santa Cruz Biotechnology	1:300 in Milk 3% PBST 0.1%
Anti-pRPA32 Ser4-8	Rabbit polyclonal, Bethyl Laboratories	1:1000 in BSA 3% TBST 0.1%
Anti-RPA32/RPA2, 9H8	Mouse monoclonal, Abcam	1:1000 in Milk 3% PBST 0.1%
Anti-pP53 Ser15	Rabbit polyclonal, Cell Signaling Technology	1:1000 in BSA 3% TBST 0.1%
Anti-P53, DO1	Mouse monoclonal, GeneSpin	1:1000 in Milk 3% PBST 0.1%
Anti-γH2AX, JBW301	Mouse monoclonal, Sigma-Aldrich	1:2000 in BSA 3% TBST 0.1%
Anti-H2AX, D17A3	Rabbit monoclonal, Cell Signaling Technology	1:1000 in Milk 3% PBST 0.1%
Anti-PCNA mUb K164	Rabbit polyclonal, Cell Signaling Technology	1:1000 in BSA 3% TBST 0.1%
Anti-PCNA, PC10	Mouse monoclonal, Santa Cruz Biotechnology	1:1000 in Milk 3% PBST 0.1%
Anti-eGFP	Rabbit polyclonal, CLONTECH Laboratories	1:500 in Milk 3% PBST 0.1%
Anti-XPA, 12F5	Mouse monoclonal, kindly provided by Richard D. Wood, University of Texas MD Anderson Cancer Center	1:1000 in Milk 3% PBST 0.1%
Anti-Cleaved Caspase 3	Rabbit polyclonal, Cell Signaling Technology	1:500 in Milk 3% PBST 0.1%
Anti-Actin	Rabbit polyclonal, Sigma-Aldrich	1:10.000 in Milk 3% PBST 0.1%
Anti-8OHdG, 15A3	Mouse monoclonal, Santa Cruz Biotechnology	1:3000 in 3% BSA in PBS
Anti-DNA-BPDE	Mouse monoclonal, Santa Cruz Biotechnology	1:200 in 0.1% PBST
Anti-Bloom helicase, BL4	Mouse monoclonal, Santa Cruz Biotechnology	1:200 in 0.1% PBST
Anti-RPA70	Rabbit polyclonal, Cell Signaling Technology	1:100 in 0.1% PBST
Anti-pDNA-PK Ser2056	Rabbit polyclonal, Abcam	1:500 in 0.1% PBST
Anti-TUJ1	Mouse monoclonal, Covance (from Lab DeBiase)	1:1000 in 0.1% PBST

The following secondary antibodies have been used:

Antibody	Description	Dilution
Anti-Mouse IgG (H+L) HRP-conjugated	Goat polyclonal, ThermoFisher Scientific	1:5000 in Milk 3% PBST 0.1%
Anti-Rabbit IgG (H+L) HRP-conjugated	Goat polyclonal, ThermoFisher Scientific	1:5000 in Milk 3% PBST 0.1%
Anti-Mouse IgG (H+L) Alexa Fluor 488	Goat polyclonal, ThermoFisher Scientific	1:1000 in 0.1% PBST
Anti-Mouse IgG (H+L) Alexa Fluor 594	Goat polyclonal, ThermoFisher Scientific	1:1000 in 0.1% PBST
Anti-Mouse IgG (H+L) Alexa Fluor 647	Goat polyclonal, ThermoFisher Scientific	1:1000 in 0.1% PBST

Anti-Rabbit IgG (H+L) Alexa Fluor 488	Goat polyclonal, ThermoFisher Scientific	1:1000 in 0.1% PBST
Anti-Rabbit IgG (H+L) Alexa Fluor 594	Goat polyclonal, ThermoFisher Scientific	1:1000 in 0.1% PBST
Anti-Rabbit IgG (H+L) Alexa Fluor 647	Goat polyclonal, ThermoFisher Scientific	1:1000 in 0.1% PBST

6.4 PLASMIDS AND RNA INTERFERENCE

The following plasmids have been used:

- eGFP-C3 (Addgene)
- pEGFP-parkin WT (Addgene #45875)
- pCMV-VSV-G (Addgene #8454)
- psPAX2 (Addgene #12260)
- pLKO.1 puro (Addgene #8453)

The following siRNA and shRNA have been used for RNA-interference experiments:

- shSCR: 5' – CAACAAGATGAAGAGCACCAA – 3'
- shPRK#1: 5' – CCTAAGGTTAAGTCGCCCTCG – 3'
- shPRK#2: 5' – CGCAACAAATAGTCGGAACAT – 3'
- siLUC: 5' – CGUACGCGGAUACUUCGA – 3'
- siPRK#1: 5' – GAGCTCCATCACTTCAGGA – 3'
- siXPA: 5' – GUCAAGAAGCAUUAGAAGA – 3'
- siEXO1: 5' – GCACGUAAUUCAAGUGAUG – 3'
- siSNM1b: 5' – ACCCUAUC AUGUGAGUAUA – 3'

Small interfering RNAs were delivered to SH-SY5Y using Lipofectamine™ RNAiMAX (ThermoFisher Scientific), and to RPE-hTERT and primary fibroblasts using HiPerfect Transfection Reagent (Qiagen), according to the respective kits' instructions.

Plasmids for overexpression were delivered to SH-SY5Y prior to differentiation using the Lonza™ Nucleofector 2b device and Amaxa® Cell Line Nucleofector® Kit V, specific to SH-SY5Y using the A-023 nucleofection program.

Short hairpin RNAs were delivered via lentiviral infection. Briefly, lentiviral particles were prepared by transfecting HEK293T with 9µg VSVg, 20µg psPAX2, and 12µg pLKO containing the shRNA sequence, using CaPO4 transfection. The day after, the cell medium was changed and its volume decreased to concentrate the particles. Finally, 48 hours after transfection media containing the lentiviral particles was collected, filtered using a 45µm filter, and aliquoted in one-use vials stored at -80°C. Different amounts of infection medium were tested on SH-SY5Y to assay toxicity, and the highest non-toxic dose was used for infection experiments. Cells were incubated for 16 hours with lentiviral-containing media in the presence of 10µg/ml polybrene. Three days after infection, cells were selected using 2µg/ml Puromycin (Sigma-Aldrich) for at least three days to select stable shRNA-expressing cells.

6.5 IMMUNOBLOTTING

Cells were scraped off the plates on ice and incubated in RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP-40 substitute, 0.5% Sodium Deoxycholate, 0.1% SDS, 1mM EDTA, 10mM NaF with protease and phosphatase inhibitors) for 10 minutes. Then samples were sonicated at 40% amplitude for 10 seconds on

ice. After 20 minutes, samples were clarified by spinning at 8000rcf for 10 minutes at 4°C. To load a known amount of proteins in polyacrylamide gels, the protein concentration was measured using BioRad DC Protein Assay using a standard BSA curve as reference. Samples ready for loading were then prepared using Laemmli buffer (350mM Tris-HCl pH 6.8, 10% SDS, 30% glycerol, 600mM DTT, 0.012 w/v bromophenol blue) as running buffer, and then boiled for 5 minutes at 95°C. At least 10µg of protein were loaded in each experiment.

6.6 IMMUNOFLUORESCENCE AND IMAGING

Coverslips were fixed in 3.7% Formaldehyde in PBS for 15 minutes, then washed 3 times in PBS. Cells were permeabilized using 0.1% Triton X-100 in PBS for 5 minutes, then incubated with 10% BSA in PBS for blocking. Primary antibodies were diluted in PBST 0.1% and incubated with the cells for 2 hours at room temperature. After 3 washes in PBST 0.1%, the slides were incubated for 1 hour with a solution containing the secondary antibodies and DAPI 1µg/ml, washed again 3 times in PBS, and mounted with Prolong Glass Antifade Mountant (ThermoFisher Scientific) on microscopy slides.

For the detection of 8OHdG, a protocol adapted from “Bilge Debelec-Butuner, Aykut Bostanci et al.”³³² was used. Briefly, cells were fixed for 30 minutes in ice-cold methanol. After 3 washes in PBS, DNA was denatured by treatment with 2M HCl for 20 minutes. The acidity was then neutralized with 0.1M pH 8.5 Sodium Borate for 2 minutes. After one wash in PBS, cells were permeabilized for 5 minutes in 0.2% Triton X-100 in PBS. After an additional wash, the slides were blocked with 3% BSA in PBS for 5 minutes, washed again, and then incubated overnight with the primary antibody solution (3% BSA in PBS) at 4°C. Three washes later, cells were exposed to the secondary antibody solution (3% BSA in PBS) with DAPI for 1 hour at room temperature. Finally, after 3 washes, cells were dried with one-minute sequential incubation with ice-cold 70% ethanol and 100% ethanol, then air dried and mounted as described before.

For the detection of DNA-BPDE adducts, a protocol from “J. M. F. Fischer et al.”³³³ was used. Briefly, cells were fixed with 1:1 Methanol/Acetone for 20 minutes at -20°C. After three washes in PBS, RNA was degraded by treatment with 100 µg/ml RNase A in 150 mM NaCl, 1 mM Na-Citrate for 1 hour at 37°C. After three washes in PBS, the cells were exposed to increasing concentrations of ethanol for 3 minutes each (35%, 50%, and finally 75%), immediately followed by a denaturation using 150mM NaOH in 70% ethanol for 4 minutes. After 2 quick washes in PBS, the cells were rehydrated using 50% and 35% ethanol for 2 minutes each, and then incubated for 10 minutes at 37°C with a solution containing 10 µg/ml Proteinase K, 20mM Tris-HCl pH 7.5, 1mM EDTA to get rid of interfering proteins. Blocking, incubation with primary and secondary antibodies, and nuclear staining were performed as described in the first paragraph.

Images were captured with either a Nikon A1R confocal microscope using a 40x silicon oil immersion objective or with a Nikon CSU-W1 SoRa using a 60x oil immersion objective. Images were then analyzed using ImageJ software.

6.7 SENSITIVITY ASSAYS

Cells were incubated with 5 mg/ml solution of MTT diluted 1:10 in medium for 2h at 37°C. After that, media was removed and after confirmation of the formation of formazan crystals, these were dissolved in 0.1 N HCl diluted in anhydrous isopropanol. The absorbance of the sample solutions was read at the 540nm wavelength using a PerkinElmer EnSight plate reader.

6.8 TOTAL RNA EXTRACTION, RETROTRANSCRIPTION, AND QRT-PCR ASSAY

Total RNA was isolated using a RNeasy Mini kit (Qiagen s.r.l., Milano, Italy) and 1 µg was retrotranscribed using iScript Reverse Transcription Supermix (Bio-Rad laboratories s.r.l., Milano, Italy) according to the manufacturer’s protocol. cDNA was diluted 1:100 and 5 µg were used as a template for real-time PCR (2x

Mastermix Sybr Green from Genespin s.r.l. was used, Milano, Italy). The following oligonucleotides were used:

Name	Sequence	Target
MAP2_For	CATGGGTCACAGGGCACCTATTC	hMAP2
MAP2_Rev	GGTGGAGAAGGAGGCAGATTAGCTG	hMAP2
RARbeta_For	TCGATGCCAATACTGTCGACTCCA	hRAR β
RARbeta_Rev	AGCTGGCAGAGTGAAGGGAAAGT	hRAR β
GAPDH_For	ATGGGTGTGAACCATGAGAAG	hGAPDH
GAPDH_Rev	AGTAGAGGCAGGGATGATGT	hGAPDH

6.9 QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of the local UV experiments was performed using the Arivis Vision 4D software to generate regions of interest around nuclei and LUDs with a parent/child relationship, coupled with an R script to calculate the ratio between the mean fluorescence signal in the two related compartments. All statistical analysis was performed in GraphPad Prism using One-way ANOVA as the main statistical test.

7 RESULTS

7.1 AIM 1: CHARACTERIZATION OF THE NEURONAL RESPONSE TO BPDE EXPOSURE AND PARKIN'S INVOLVEMENT IN DNA DAMAGE REPAIR

Considering the already existing data in the literature about the potential involvement of the E3 Ubiquitin ligase Parkin in the DNA damage signaling response to bulky lesions, and its obvious relevance in neurodegenerative disorders such as Autosomal Recessive Juvenile Parkinsonism, the first part of this project was focused on investigating Parkin's role in DDR in neuron-like cells. To strengthen the clinical relevance of our work, the compound BPDE was used to induce bulky lesions in these cells, as the chemical is known to trigger NER activity and cross the blood-brain barrier that would otherwise protect neurons of the central nervous system from its exposure.

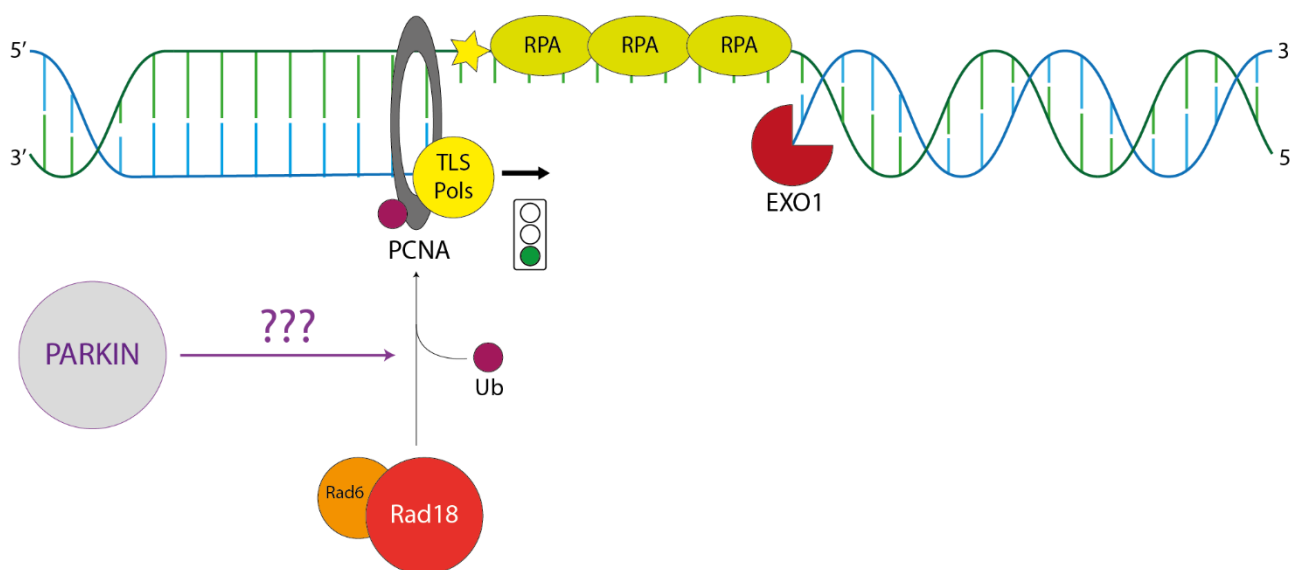


Figure 13: Parkin's potential involvement in COLs repair

Starting hypothesis for Parkin's contribution on COLs repair, acting on assisting the Rad6-Rad18 dependent PCNA monoubiquitination necessary for proper lesion bypass in the gap-filling step of NER.

7.1.1 BPDE induces a DNA Damage response in primary neurons

Despite Benzo[a]pyrene Diol Epoxide is well known to induce a NER-dependent DNA damage response, and it has been used in neurons in other works, a DNA-damage tailored analysis of the response of these cells to BPDE exposure was never fully characterized. For this reason, as a first step of this project, we treated primary neurons from hippocampi of mice with relatively long exposures to this genotoxin.

In Figure 14A, we investigated the activation of DNA-damage markers such as the phosphorylation of H2AX at serine 139 (γ H2AX), and phosphorylation of RPA at serine 4 and 8 at different time points after exposure to different concentrations of BPDE. Consistently with the notion that DNA-BPDE adducts are processed by the NER machinery and also with the model of closely-spaced opposing lesions, we found that increasing doses of the drug trigger a proportional activation of γ H2AX, and RPA phosphorylation (as it can be clearly observed comparing the 2.5 μ M and 5 μ M dose), which stands as an indirect indication of the presence of DNA double-strand breaks. It is indeed expected that a higher drug dose would enhance the chances of generating a COL, especially in cells where other DNA polymerase-blocking lesions/structures (oxidated bases, G-quadruplexes, and strong transcriptional activity) are abundant. Interestingly, while γ H2AX seems to always increase over time, RPA phosphorylation seems to either remain constant or even decrease at the 48h mark. The 10 μ M dose is likely too high for the cells to repair properly.

Next, using inhibitors, we investigated which of the apical checkpoint kinases (ATM, ATR and DNA-PKcs) is mainly responsible for BPDE-induced H2AX phosphorylation in neurons. As shown in Figure 14B, only DNA-PKcs inhibition results in a reduction of the γ H2AX levels after BPDE exposure. This suggests that this phosphorylation is probably due to the formation of DSBs. Since neurons are blocked in G₀ the only pathway available for repair would be Non-Homologous End Joining.

To confirm the presence of DSBs in BPDE-treated neurons, we checked the presence of these lesions using Neutral Comet Assay (NCA). As shown in Figure 14C, even low doses of BPDE are sufficient to induce DNA double-strand breaks after a relatively long 24-hour exposure to the carcinogen. This also results in a dose-dependent reduction in neuron viability over time (Figure 14D).

Finally, to investigate the actual removal over time of the NER-processed lesions in neurons we used DNA-BPDE adducts-directed immunofluorescence. While different concentrations of BPDE were tested, each one was normalized over an internal condition and because of this it is only possible to evaluate the differences in quantity of adducts over time, but not the relative amount of adducts comparing different doses. Although the variability was very high, which is intrinsic in primary neurons in general, the trend observable in Figure 14E is a reduction in the amount of DNA adducts. While impossible to determine in this experiment alone, based on what is known in literature, this repair is likely mainly due to transcription-coupled NER with a lesser contribution of Domain-Associated Repair²⁵⁴.

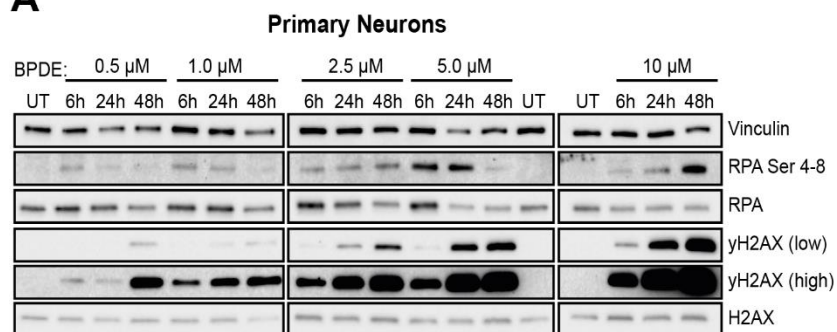
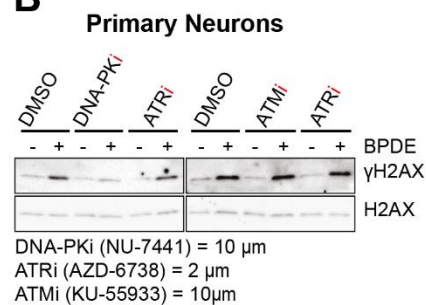
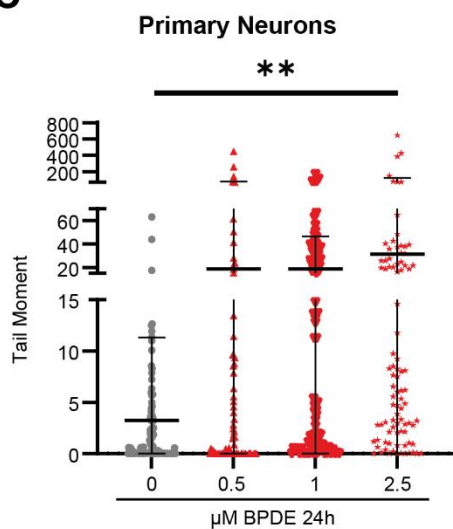
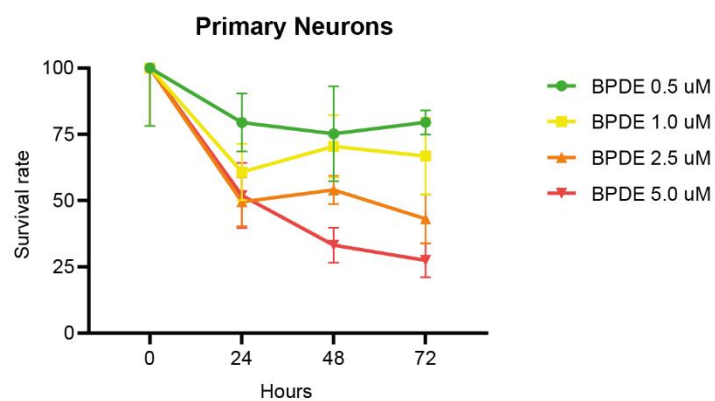
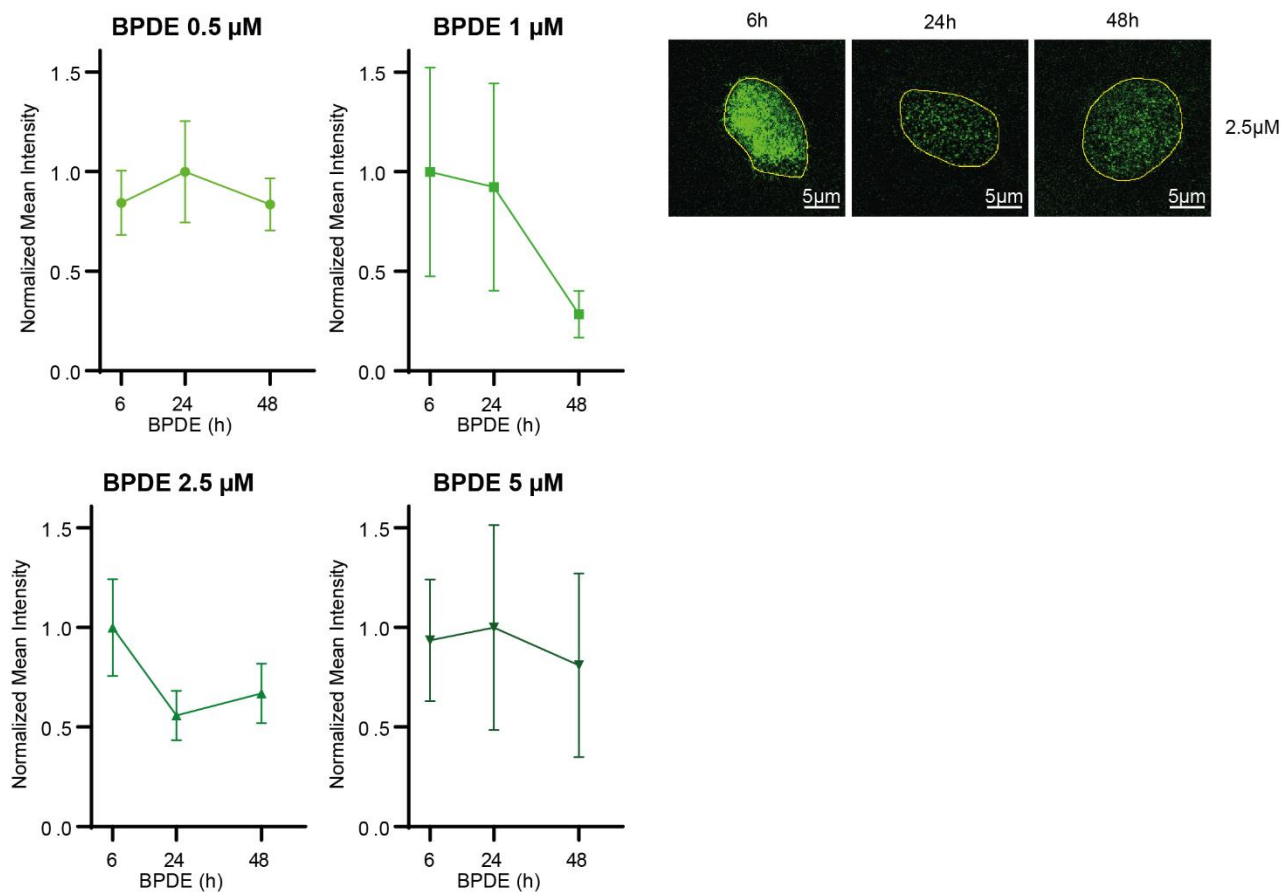
A**B****C****D****E**

Figure 14: BPDE leads to DSBs and triggers a DNA damage response in primary neurons

(A) Western blot analysis of DNA damage markers phosphorylated RPA and H2AX in response to different doses and exposure times to BPDE. (B) Effect of apical kinase inhibition on BPDE-dependent activation of γ H2AX. Neurons were pre-exposed for 1h to the inhibitors before BPDE was added to the media, then treated with 1 μ M BPDE (C) Neutral comet assay showing the generation of DNA double-strand breaks in BPDE-treated neurons. (D) MTT assay showing the sensitivity of neurons to incremental concentrations and exposure to BPDE. (E) Quantification of DNA-BPDE adducts found in neurons exposed to BPDE showing the reduction of bulky lesions over time, normalized on the highest signal measured. Each BPDE concentration represents an independent experiment and thus cannot be compared to each other. Images of a representative dose of BPDE are shown as an example.

7.1.2 Parkin has a protective role in BPDE-Induced DNA damage

Previous data reported in the literature, suggest that Parkin may play a function in the response to DNA damage^{289–291}. In order to investigate the involvement of the Parkin protein in the repair of BPDE-induced lesions in neuron-like cells, we performed silencing experiments using differentiated SH-SY5Y neuroblastoma cells as a model for post-mitotic neurons, and we checked different markers of DNA damage. The correct functioning of the differentiation protocol used was tested by expression and localization of neuronal-specific markers (TUJ1) and by qPCR analysis of markers such as MAP2 and RAR β , as shown in Figure 15A and Figure 15B respectively. BPDE treatment was performed by exposing the cells for 1 hour to a BPDE-containing medium, followed by a medium change and a recovery time of 5 hours to simulate the same conditions of UV treatment²³⁷.

As shown in Figure 15C, cells lacking Parkin show higher activation of the DNA damage response as seen by the activation of general DNA damage markers such as γ H2AX (which was also reproducible with a more modest reduction in Parkin levels after siRNAs treatment, as summarized in Figure 15D) and P53 phosphorylated at serine 15, but also the DNA-PKcs specific RPA phosphorylation at serine 4 and 8. Despite our predictions based on the Parkin overexpression experiment presented in “Zhu et al, 2017”³³⁴, in which an increase of a Rad18-dependent UV-induced PCNA post-translational modification that was proposed to be ubiquitination at Lysine 164 was shown in U2-OS cells overexpressing Parkin, in our condition we also observed the same increased levels of PCNA monoubiquitination. The same experiment was also performed on SH-SY5Y cells while still cycling, and except for PCNA monoubiquitination we did not observe similar results for the other markers; however, we did observe a strong apoptotic response as shown by the cleavage of Caspase-3 which was however independent of Parkin silencing (Figure 15E). In contrast to the data present in literature and mentioned in the introduction, the lack of Parkin did not result in any changes in cell cycle progression (Figure 15F)

To test whether the DNA damage signaling activation observed following Parkin depletion was due to the exposure of DNA double-strand breaks, we performed neutral comet assay in the same conditions. Indeed, in Figure 16A, we observed a clear increase in the amount of BPDE-induced DSBs in cells lacking Parkin.

To strengthen the results obtained by the silencing experiments, we also overexpressed a Parkin-GFP construct in our cells using nucleofection to circumvent the SH-SY5Y notoriously low overexpression efficiency. In these conditions and accordingly to the data already presented and in contrast to what was reported by Zhu et al 2017²⁹¹, the overexpression of Parkin reduced both PCNA monoubiquitination and H2AX phosphorylation after BPDE treatment, as shown in Figure 16B.

To elucidate the differences observed in the experiments so far presented and the data published in Zhu et al 2017²⁹¹, we performed overexpression experiments with the same conditions used in the paper. We exposed U2-OS cells to a dose of 15 J/m² of UV light and collected protein extracts after 3 hours. In addition to that, we also exposed cells to BPDE to test whether any difference could have been due to the mechanism of actions of the two genotoxic agents. In Figure 16C however we can observe that in our hands, overexpression of Parkin in U2-OS cells had no significant effect on PCNA monoubiquitination, as well as phosphorylation of histone H2AX, in neither UV nor BPDE exposed cells. We propose two possible explanations for the different outcomes in the two experiments: the first hypothesis is based on the fact that in our experiment, a Parkin eGFP construct was used. The presence of the additional eGFP protein might hinder whatever function Parkin has on PCNA monoubiquitination. However, this would not explain the contradictory data that we collected in experiments in which the endogenous Parkin was silenced instead. The second explanation is based on the different methods used to observe PCNA monoubiquitination: in fact, in Zhu et al 2017²⁹¹ the data showed that Parkin increased a PCNA post-translational modification, detected using a monoclonal antibody against PCNA, that was suggested to be monoubiquitination based on the molecular weight of the band and its dependency on UV and the presence of the E3-ubiquitin ligase Rad18.

In our experiments instead, we directly used an antibody that was validated to specifically recognize PCNA monoubiquitinated at Lysine 164. It's therefore possible that what Zhu observed was either a different modification or a modification at a different residue. To confirm this latter hypothesis however, additional experiments are required, starting from the detection of the band using the PCNA monoclonal antibody as it was performed in the paper.

7.1.3 Parkin effects are dependent on NER

In order to demonstrate that the Parkin dependent DNA damage signaling activation that we observed in differentiated SH-SY5Y was actually due to the processing of BPDE adducts by the nucleotide excision repair machinery, we tested the response in cells in where the NER core protein XPA was silenced¹⁶⁵.

In this experiment, presented in Figure 16D, the clear increase in H2AX phosphorylation that is observable in Parkin lacking cells is completely abrogated by depleting XPA. Indeed, in these cells γ H2AX reaches levels that are even lower to that of mock-silenced cells. This is consistent with what was demonstrated in Sertic et al 2018²³⁷, since at this time point the signaling activation observable in non-replicating cells is largely dependent upon the processing activity of the Nucleotide Excision Repair pathway. This therefore clearly demonstrates that the increased DNA damage signaling activation present in Parkin silenced cells is triggered by NER processing. Unfortunately, in this particular experiment not many conclusions can be taken regarding PCNA monoubiquitination as its levels do not seem to particularly change between any of the BPDE treated samples.

7.1.4 Parkin protects cells from BPDE-Induced oxidative damage on DNA

Extensive data are present in the scientific literature about Parkin's role on the process of mitophagy, and how damaged mitochondria are prone to increased ROS leakage. One straightforward explanation for the outcome of the experiments presented so far is therefore that Parkin lacking cells are more exposed to oxidative damage. This may generate DNA polymerase blocking lesions that could be involved in Closely-spaced Opposing Lesions. Thus, Parkin depletion may lead to an increased incidence of COLs along the DNA which would results in increased DNA double strand breaks and a corresponding increase in checkpoint activation.

As anticipated in the introduction, BPDE also has a tendency to accumulate in mitochondria due to its highly lipophilic nature. Because of this, it is possible that BPDE exposure would contribute to damaging the mitochondria, which in Parkin lacking cells would not be properly dealt with.

In order to test this hypothesis, we performed an immunofluorescence to detect the presence of 8-oxo-guanines (8OH-dG) in the DNA of differentiated SH-SY5Y. Although this kind of base modification is reported to only pause the gap-filling activity of DNA polymerases δ and ϵ , instead of completely blocking it, this pause might still trigger an opportunity for EXO1 to generate long ssDNA gaps and trigger PCNA-dependent polymerase switch³³⁵. Furthermore, 8OH-dG base modifications being the most abundant product of oxidative damage to DNA, is a proxy for detecting oxidation of DNA in general.

As shown in Figure 16E, an increase in 8OH-dG foci in the nuclei of the cells was indeed observable, reaching the highest point in BPDE treated, Parkin silenced conditions. Interestingly, Parkin silencing alone was already sufficient to induce a mild, but statistically significant, increase in the presence of these lesions. This fits with the model reported in the literature where neurons and neuron-like cells are more reliant on oxidative metabolism for energy production and with Parkin's role in mitochondrial maintenance. In line with what is known about BPDE, also the treatment in non-silenced cells was sufficient to produce an increase in the levels of 8OH-dG.

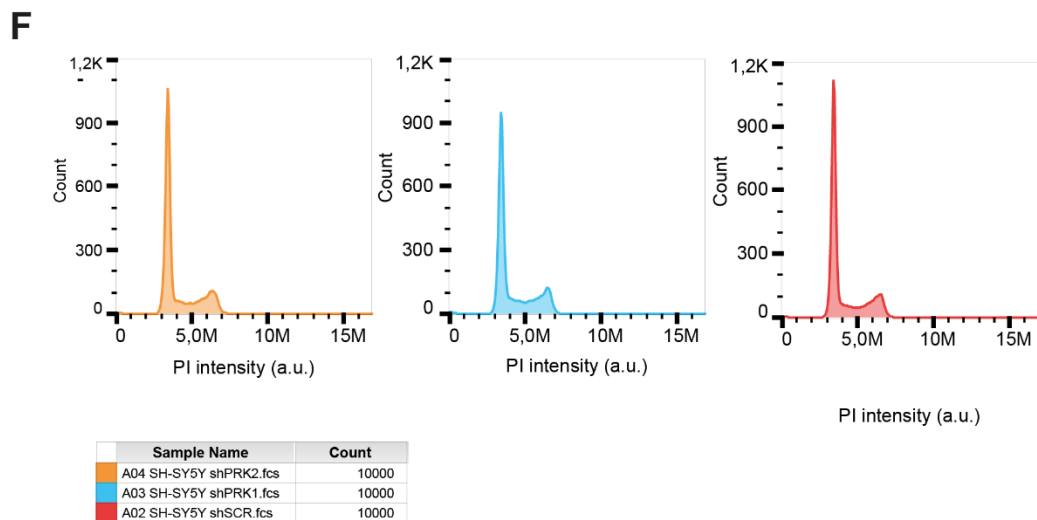
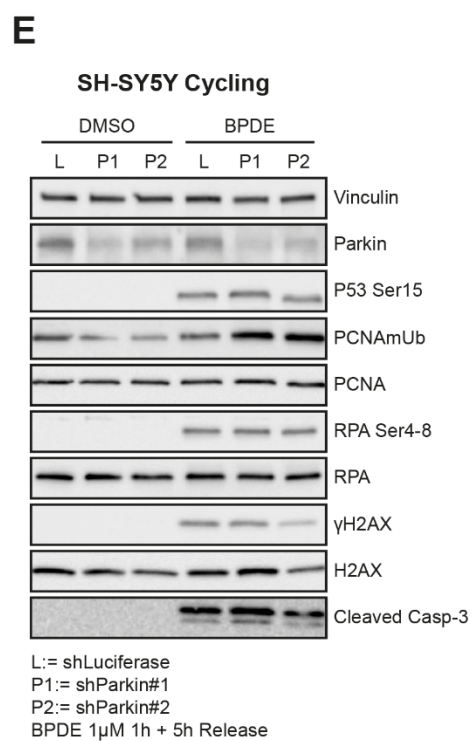
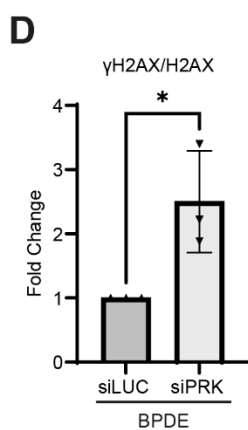
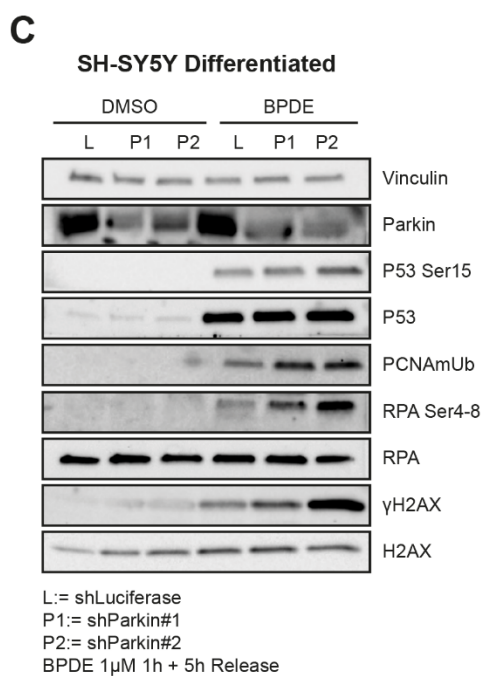
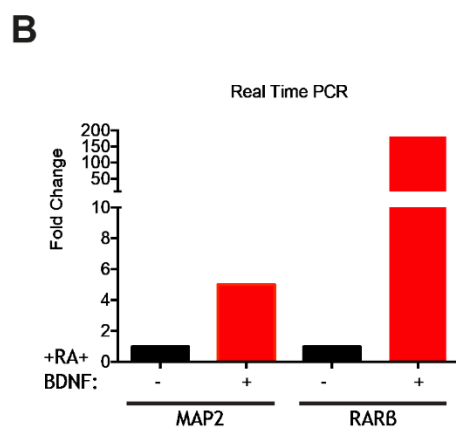
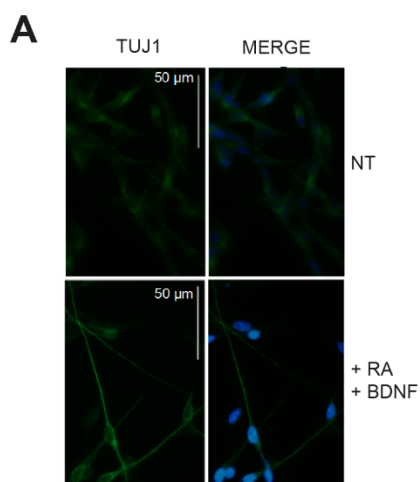
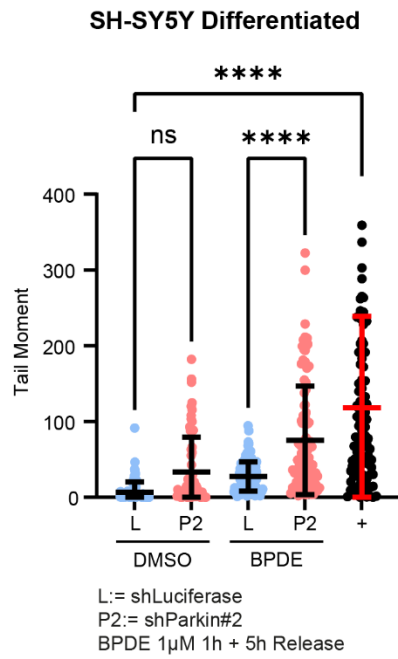


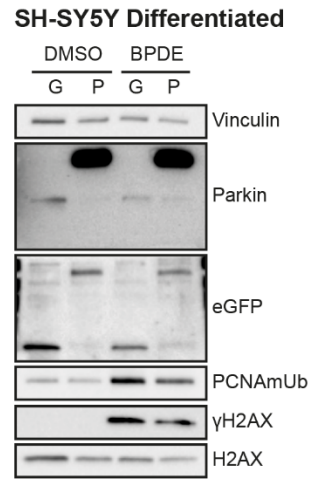
Figure 15: Parkin has a cell-cycle independent protective role against BPDE-induced DNA damage

(A) Immunofluorescence with staining against TUJ1 and DAPI of SH-SY5Y untreated, or differentiated using a one-week protocol involving retinoic acid (RA) and BDNF (exposure) coupled with a progressive decrease in serum concentration. Structures that resemble neurites and axons can be observed in the treated condition. (B) qPCR analysis of expression of neuronal markers MAP2 and RAR β in SH-SY5Y untreated or differentiated. (C-D) Western blot or relative quantification of differentiated SH-SY5Y stably expressing shRNAs (C) or transiently transfected with siRNAs (D) to silence Parkin, exposed to BPDE for 1h, followed by a release in fresh medium for 5h. (E) Western blot of cycling SH-SY5Y stably expressing shRNAs to silence Parkin, exposed to the same conditions of BPDE just described. (F) Cell cycle analysis with propidium iodide incorporation of the same SH-SY5Y cells without differentiation protocol. 10.000 single cells were read per each condition.

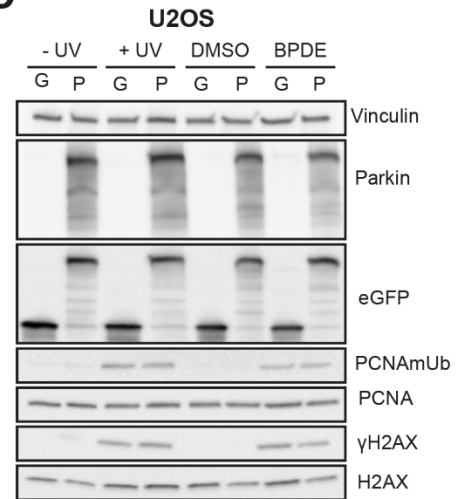
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B

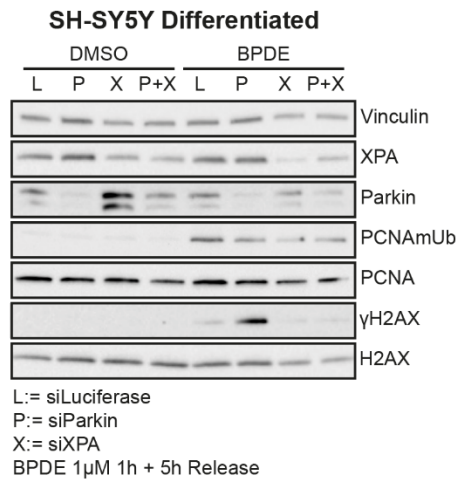


C



G:= eGFP overexpressed
P:= Parkin-eGFP overexpressed
BPDE 1μM 1h + 3h Release
UV 15J/m² + 3h

D



L:= siLuciferase
P:= siParkin
X:= siXPA
BPDE 1μM 1h + 5h Release

E

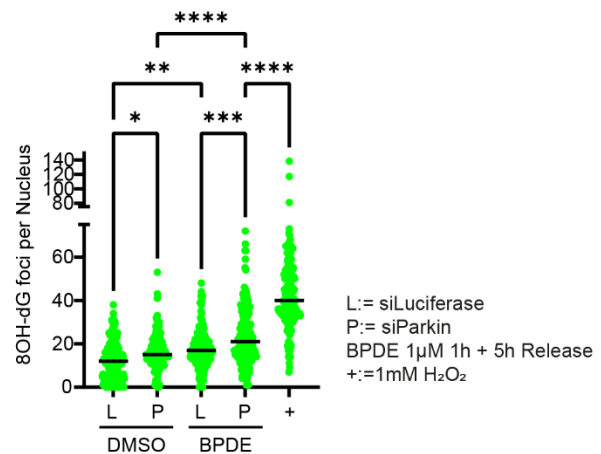
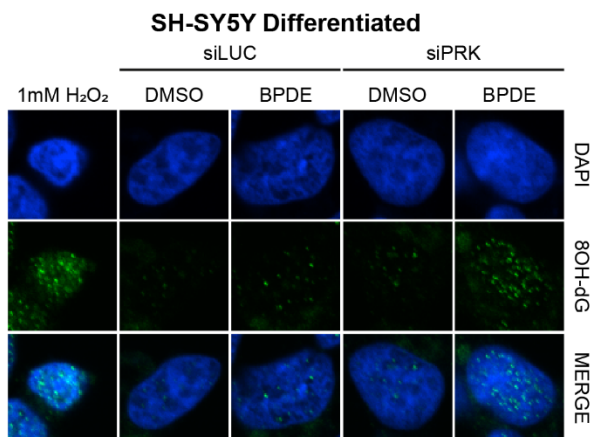


Figure 16: Parkin protects cells from DSB and 8OHdG, and the signalling associated with BPDE exposure is dependent on NER activity.

(A) Quantification of the tail moment of comets from multiple Neutral Comet Assay experiments in differentiated SH-SY5Y stably expressing shRNAs to silence Parkin. 10 μ M treatment of Bleocin for 2h was used as a positive control of double strand break induction. (B-C) Western blots of Differentiated SH-SY5Y or cycling U2OS cells, overexpressing a Parkin-eGFP construct or GFP as a control, while being treated with BPDE or UV light and releases in fresh medium for 3 hours. (D) Western blot of differentiated SH-SY5Y silenced transiently with siRNAs targeting Parkin or XPA, and treated with BPDE for 1 hour followed by 5 hours of damage release. (E) Immunofluorescence using an antibody targeting 8OH-dG residues in DNA in differentiated SH-SY5Y silenced transiently with siRNAs targeting Parkin, after BPDE exposure, and quantification of the number of maxima point in the nucleus in each condition. H2O2 treatment was used as a control of 8OH-dG induction.

7.2 AIM2: CHARACTERIZATION OF BLOOM HELICASE INVOLVEMENT IN POST-NER PROCESSING OF ssDNA ENDS

The Bloom helicase is known to be involved in EXO1 dependent resection after particular types of DNA damage, for example during the process of Homologous Recombination³¹⁰, and work in our lab has demonstrated the importance of the EXO1 nuclease in post-NER processing in non-cycling cells²³³. The aim of this second part of this PhD project was to investigate whether the BLM helicase is also involved in the response to bulky lesions.

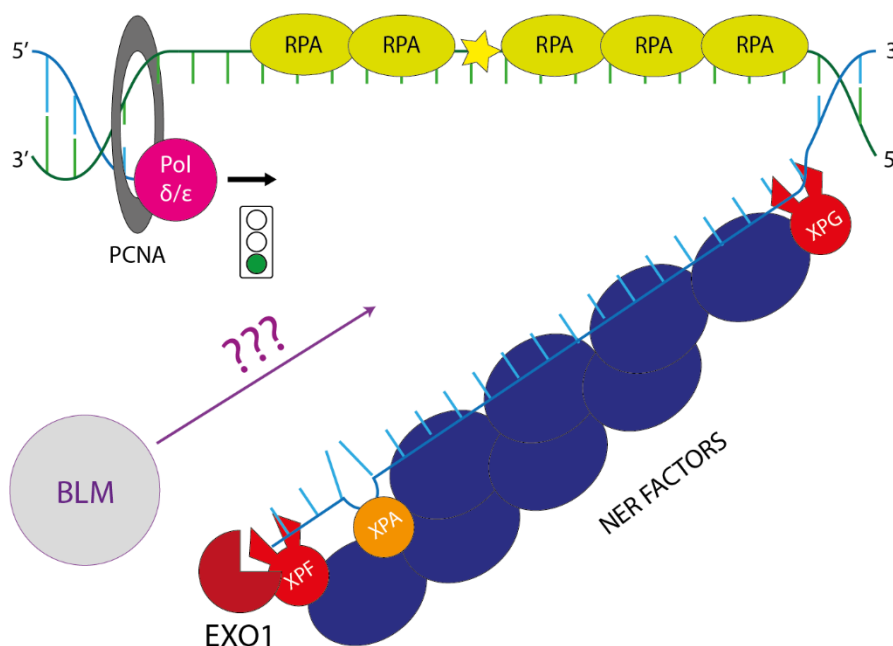


Figure 17: Bloom's helicase potential involvement in COLs repair

Schematic representation of Bloom's helicase contribution in COLs repair, possibly acting in conjunction with EXO1 in a manner similar to HR repair of double-stranded breaks.

7.2.1 Bloom helicase localizes at sites of Local UV Damage

In order to investigate the possible involvement of Bloom helicase (BLM) in post NER processing of bulky lesions, we treated non replicating RPE-hTERT cells with a UV-C lamp to reach 40 J/m² through an Isopore filter to generate local UV damages (LUDs). We then monitored the localization of the endogenous BLM protein using immunofluorescence. Importantly, the cells were serum starved for 48-72 hours to minimize the amount of cycling cells in the samples. Additionally, any S-phase cell still present after starvation was excluded from the analysis based on EdU incorporation, as the cells were treated for at least 180 minutes prior to fixation with

EdU. The local incorporation of EdU was also used to mark the LUD of interest. For shorter time points the signal of the endogenous XPA was used instead.

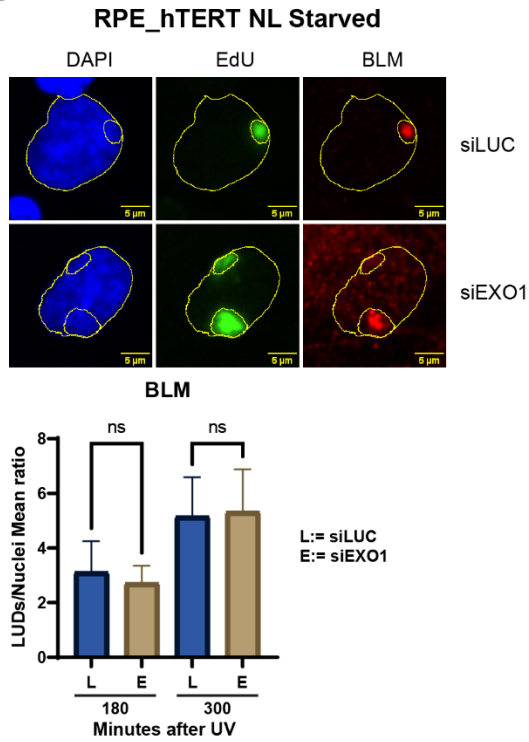
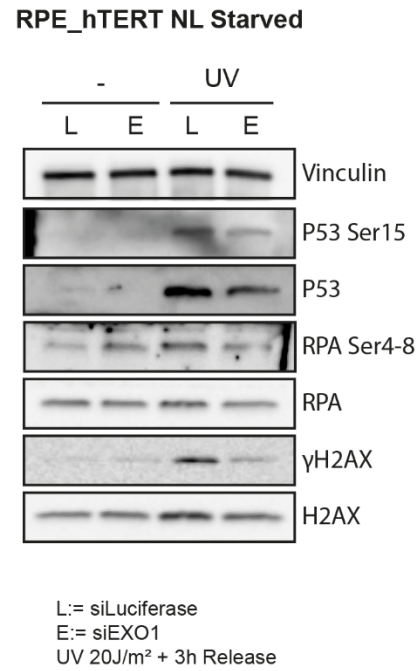
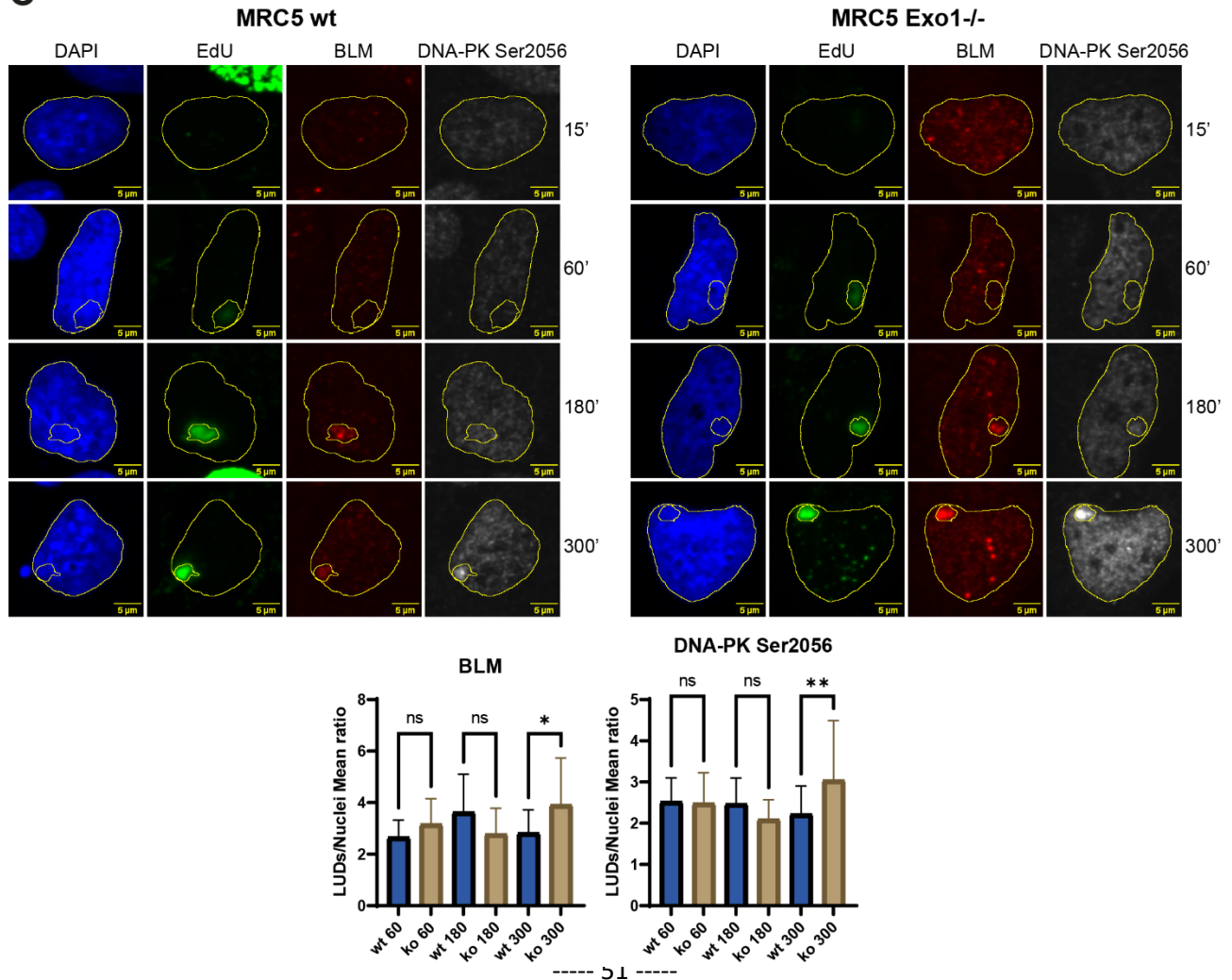
As shown in Figure 18A, Bloom helicase (BLM) is found at LUDs 60 minutes after damage and reaches high levels of accumulation at 180 and 300 minutes. At 15 minutes, when XPA is clearly already localized in the region of interest, no Bloom helicase can be detected. Interestingly, while at 180 minutes RPA70 is at levels below detection, at the later time points it is clearly localized, which is consistent with the generation of long ssDNA stretches typical of COLs.

7.2.2 Bloom helicase localization is independent of Unscheduled DNA Synthesis

Blocking repair DNA synthesis can be used to simulate a situation of impairment of the NER dependent gap-filling activity, effectively converting all UV-induced lesions to COL-like situations. If the Bloom helicase is directly involved in COLs processing following DNA polymerase stalling, one would expect it to accumulate at LUDs upon UDS blockage. To test this hypothesis, we treated cells with the chain terminator compound AraC that was added immediately after LUDs induction, together with EdU, and we tested the localization of Bloom helicase after 180 minutes (Figure 18B). Some EdU incorporation could still be detected in LUDs, because it was incorporated before the chain terminator. We show that, at the 40 J/m² UV dose, Bloom helicase is recruited at local UV lesions and it is further enriched in AraC treated cells. Interestingly, this difference was not so evident at 100 J/m², where instead DNA-PKcs autophosphorylation started to appear. Curiously, at this short time points, Ara-C did not increase DNA-PKcs autophosphorylation, suggesting that DNA double strand breaks may not be induced yet. This was a great indication that Bloom helicase has indeed a role in post-NER processing that is independent by its role in DNA double strand break repair.

7.2.3 Bloom helicase localization is independent of Exo1

Finally, to test whether Bloom helicase recruitment is dependent on the presence and/or activity of EXO1, we performed knock down experiments where the latter was silenced using RNA interference. In both timepoints tested, shown in Figure 19A, there was no statistically significant difference in Bloom helicase's enrichment. In this particular experiment, to make sure of proper EXO1 silencing, we tested for DDR signaling activation after UV irradiation and observed the same reduction in EXO1 silenced samples that was reported in previous publications from the lab²³³ (Figure 19B). Furthermore, using MRC5 EXO1 knock out cells that were generated in our laboratory for a previous publication²³⁷, the only significant difference between any of the time points tested was at 300 minutes; however, it is possible that this enrichment could be connected to the increases of DNA-PK autophosphorylation, hinting at the possibility of an effect that is dependent on DNA DSB repair (Figure 19C). The general lack of an effect of EXO1 silencing could be expected when thinking about a potential role of Bloom protein as a helicase which is facilitating EXO1 recruitment and resection activity. It would be interesting to test whether Bloom helicase silencing has any effect in these two processes; however, this is yet to be performed.

A**B****C**

----- 51 -----

Figure 19: Bloom helicase recruitment to Local UV Damage sites is independent of EXO1

(A) Immunofluorescence analysis and EdU incorporation detection in serum-starved RPE_hTERT cells that have been silenced with EXO1 targeting siRNAs. Signal quantification was performed as previously described. (B) Western blot of cells silenced parallelly to the ones in panel A, showing reduction in the phosphorylation of DDR markers P53 and H2AX, which was demonstrated in a previous publication from the lab to be Exo1 dependent. The experiment was therefore used here as an indirect silencing control. (C) Immunofluorescence against BLM and auto-phosphorylated DNA-PKcs of MRC5 wild type (wt) or knocked out for EXO1, treated with Local UV damages, with relative quantification plotted in the graph.

AIM3: CHARACTERIZATION OF EXO1'S ROLE IN THE NER-INDEPENDENT DNA DAMAGE SIGNALING ACTIVATION

As the final sub-project, having previously linked checkpoint signaling activation after UV exposure to ssDNAs generated by EXO1 activity^{336,337}, and aware of the many questions surrounding the topic of NER-independent checkpoint signaling activation, we aimed to investigate whether EXO1 and other factors identified through yeast genetic screenings performed in the lab could be involved in this process.

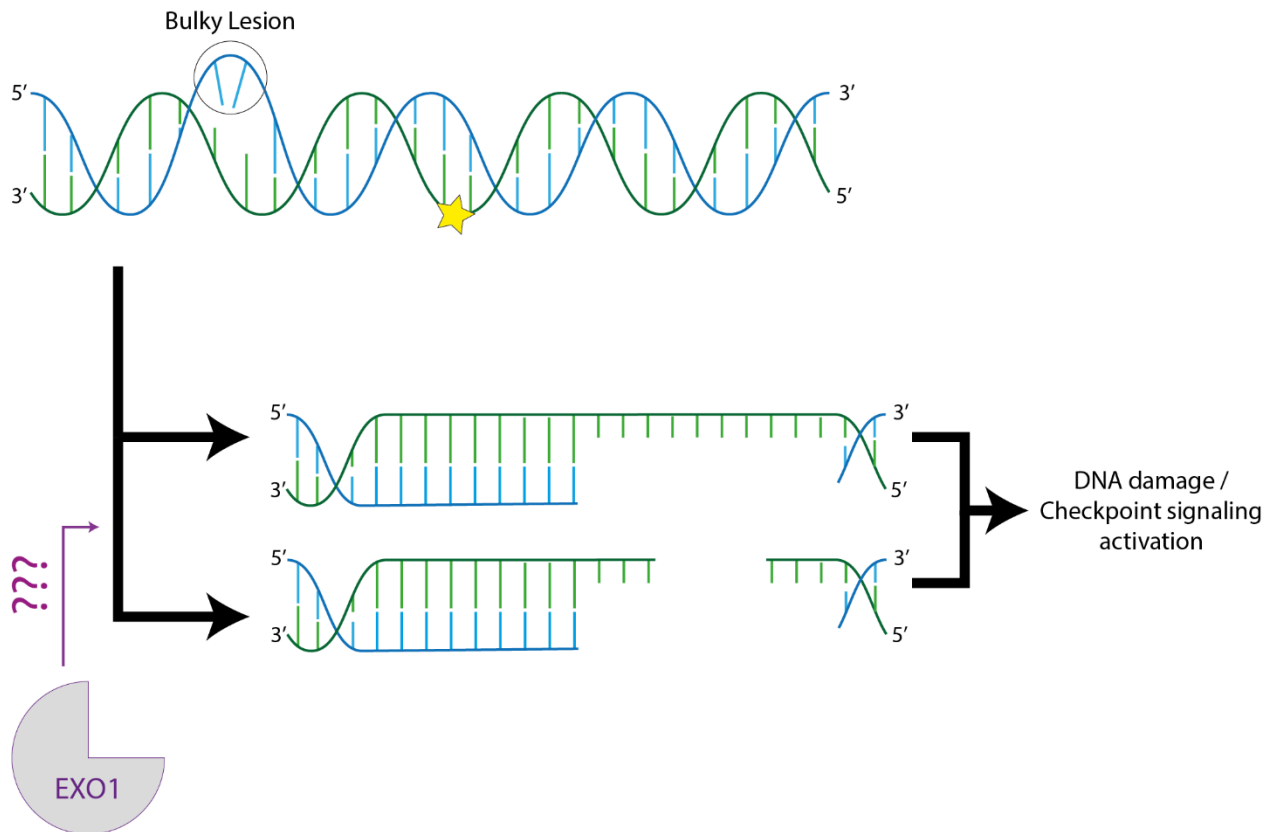


Figure 20: potential DNA lesions associated with DNA damage/checkpoint signaling activation in NER deficiency, and potential contribution of EXO1

Single-strand or double-strand breaks originating from NER independent processing of bulky lesions in DNA, leading to DNA damage/checkpoint signaling activation. The process, already described in literature, might involve also the action of EXO1 as well as members of the SNM1 family.

7.2.4 DNA damage and checkpoint signaling is active in NER deficient cells at long time points

DNA damage checkpoint activation observable shortly after UV or BPDE treatment is induced by NER and EXO1 activities, as described above. A second form of late checkpoint activation that is NER-independent was reported in literature^{326,327}. As this process is likely activated by ssDNA exposure or the presence of DSBs, we wondered whether EXO1 or other nucleases may be involved. Genetic screening performed in yeast in our laboratory have shown that the UV induced DNA damage checkpoint signaling is also partially dependent on another protein, Pso2. We wondered whether Pso2 homologs in human, namely proteins of the SNM1 family, could participate to the process as well.

We initially tested that in our experimental conditions it was possible to observe a NER independent checkpoint signaling. We UV irradiated both immortalized fibroblasts, 48BR_hTERT, serum starved to reduce to a minimum the amount of cycling cells (Figure 21A), and primary fibroblasts from XPA-proficient and XPA-deficient patients (As highlighted in Figure 21B, which is an extract from Figure 21C), and investigated different damage and checkpoint markers at various timepoints. Notably, in both cases, we could observe that after 24 hours, H2AX was indeed phosphorylated as well as P53 also in a NER-defective situation;

indicating that indeed the DNA damage checkpoint can be triggered, at late timepoints, even though NER activity is reduced or absent.

7.2.5 NER independent signaling activation is partially dependent on Exo1

Using RNA interference, we tested the dependence of this process on EXO1. In primary fibroblasts (Figure 21C), EXO1 knock down completely abolished the H2AX phosphorylation after 24 hours, while the phosphorylation of p53 was greatly reduced, implying that the exonuclease is indeed involved, but likely not the only player in this process. Interestingly, in serum starved retinal cells (Figure 21D), depletion of EXO1 in siXPA cells caused an increase in H2AX phosphorylation while it did not seem to affect p53 phosphorylation at 24 h. On the contrary, RPA phosphorylation at Serine 4 and 8 was completely abrogated when EXO1 was silenced, suggesting the absence of DSBs. The source of signal leading to the increase in gammaH2AX needs further investigation.

7.2.6 NER independent signaling activation is not affected by UDS blockage

To test whether a mechanism similar to the one reported above for COLs could be involved in the process, we treated serum starved RPE-hTERT cells with the chain terminator AraC. As shown in Figure 21E, AraC treatment increased every checkpoint marker tested in NER proficient conditions but had absolutely no effect in NER deficient samples. This means that the generation of the checkpoint signal does not involve repair DNA polymerases.

7.2.7 NER independent signaling activation is partially dependent on SNM1b

Finally, we tested the involvement of Pso2 homologs, starting from SNM1b/Apollo (Figure 21F). With knock down experiments, we observed that H2AX phosphorylation after 24 hours was reduced, while the phosphorylation of p53 was barely affected. These findings suggest that SNM1b is at least partially involved in the signaling. Its mechanistic relationship to EXO1 remains to be defined.

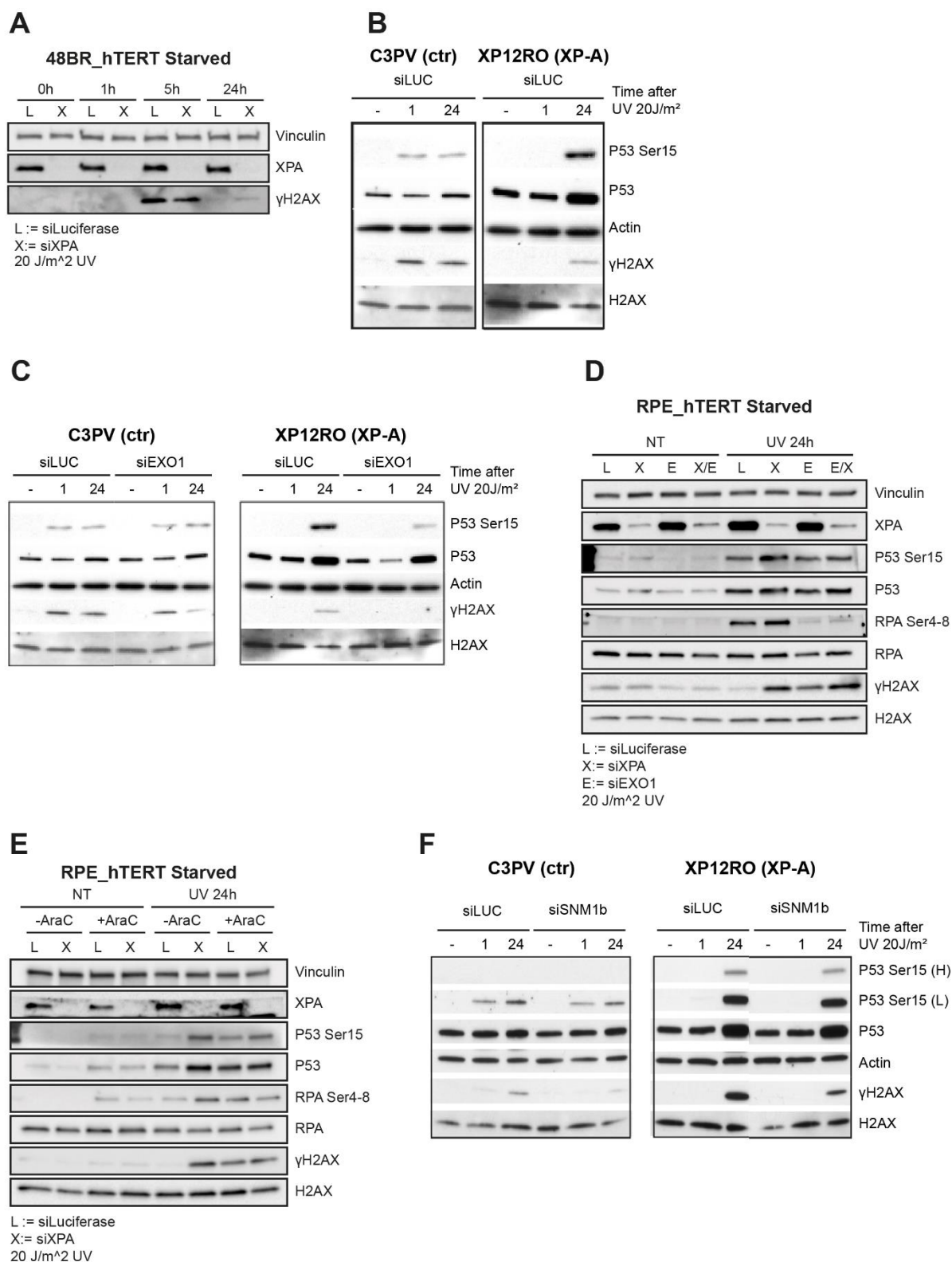


Figure 21: a NER-independent DNA damage signalling activation exists in human cells and depends on EXO1 and partially SNM1b

(A) Western blot analysis of DNA damage signalling markers after UV treatment in serum starved 48BR_hTERT rendered NER deficient by silencing XPA. Similar analysis repeated in primary fibroblasts “XP-A” genetically deficient in NER and additionally silenced for EXO1 (C) or SNM1b (F) via RNA interference. An extract of the first image highlighting the existence of a NER independent signaling activation is presented in (B). Similar treatments have been performed also in serum starved RPE_hTERT cells, rendered NER deficient with XPA targeting siRNAs, and with additional knock down of EXO1 (D) or treatment with DNA synthesis inhibitor chain terminator AraC (E).

8 DISCUSSION

Understanding the molecular mechanisms laying behind the repair of bulky lesions in non-replicative cells has enormous clinical potentials, as many diseases involve them. Neurons in particular represent some of the least understood cells but nevertheless some of the most important since they are for the most part irreplaceable, and considering that neurodegenerative disorders currently affect more or less 15% of the worldwide population³³⁸. Because of these considerations, the initial part of our research had a very specific neuronal tailoring.

PROJECT 1: Using primary neurons explanted from mouse hippocampi, we demonstrated that treatment with the benzo[a]pyrene metabolite BPDE elicited a specific DNA damage and signaling response, which correlated with the generation of DNA double strand breaks, likely generated some hours after the formation of DNA-BPDE adducts. Importantly, the DSBs presence correlated with a decrease in these cells' survival rate, potentially connecting neurodegeneration with the organism's exposure to pollutants. Although experimentally we did not provide evidence of a specific mechanism connecting bulky lesions in DNA with formation of DNA double strand breaks in this cell type, the results collected fit the model of the Closely-spaced opposing lesions described in our previous papers^{237,336}. Considering data reported in literature showing that both TC-NER and GG-NER activity are retained in neurons, but are confined to specific nuclear domains characterized by high transcriptional activity²⁵⁴, it is entirely possible that some of the DNA-BPDE adducts that we detected could be found in close proximity and on the opposite strand of each other. Other types of polymerases blocking lesions/structures that could contribute to generating the checkpoint signal including active transcription itself, G-quadruplexes that are implicated in transcriptional regulation required to preserve neuronal plasticity, and oxidative damage induced by the enormous mitochondrial load that neurons intrinsically possess.

Indeed, we managed to find evidence of reliance of neuronal like differentiated SH-SY5Y cells on mitochondrial activity as we saw that silencing Parkin in these cells increases 8OH-dG lesions on nuclear DNA. Although not directly capable of stalling DNA polymerases, these lesions act as a canary in coalmines suggesting the presence of yet other oxidative lesions that may be able to block DNA synthesis, such as cyclopurines and thymine glycol^{239,240}. Considering that BPDE induced signaling activation, observable in Parkin lacking cells, was almost entirely dependent on the presence of a functional NER machinery, it is possible that in the short term these lesions do not represent a problem for the cell, but their NER dependent processing does. It is therefore entirely possible that some of the toxicity observable in autosomal recessive juvenile parkinsonism, a genetic disorder caused by mutations in the Parkin gene, could be attributed to genotoxic oxidative damage exacerbated by the exposure to bulky lesions inducing chemicals like BPDE, cisplatin or even many other sources. Because of time-constraints, we could not assess the dependency of the increased harming effect of BPDE in Parkin deficiency to the presence of EXO1, which would serve as a validating argument for the COLs model as we observed in previous publications on non-S phase cells^{237,336}. Additionally, to demonstrate that mitochondrial generated ROS contribute the second lesion in COLs, one potential experiment would be to treat BPDE exposed, Parkin silenced cells with ROS absorbing compounds such as antioxidants or redox modulators, for example glutathione³³⁹. In these conditions, if the Parkin silencing effects are to be attributed to just mitochondrial instability, we would expect similar signaling activity and DSB formation to that of non-silenced cells. An alternative approach could be instead to completely uncouple the mitochondrial contribution to DNA damage by repeating the experiments shown in SH-SY5Y cells depleted of mitochondrial DNA, generating ρ^0 cells³⁴⁰. These cells lack critical components of the respiratory chain, and although they still contain small mitochondria, these are insufficient to perform oxidative phosphorylation, they are unable to maintain a redox potential and most importantly they are incapable of generating reactive oxygen species. If our model stands correct, in these cells we would expect that a lack of Parkin does not significantly change the DNA damage signaling after BPDE or UV insult.

One important point to be raised is that the data that we collected so far does not exclude a direct role for Parkin protein in the regulation of the DNA damage response, that was the starting point of this project. While in our hands we saw no differences in PCNA when Parkin is silenced, the data that we collected experimentally, referring in particular to DSB generation and increased signaling, seem in line with a potential role of the protein in regulating TLS DNA polymerases recruitment. Indeed, in previous works we demonstrated that inhibiting UDS, which is what would happen in the case in Parkin lacking conditions, greatly enhances the formation of double-strand breaks and its correspondent DNA signaling pathway²³⁷. Regarding all the Parkin knockdown experiments, we encountered great variability in the response observed depending on the specific siRNA/shRNA sequence used (data not shown). A potential explanation is that different sequences may be recognizing different Parkin isoforms, resulting in variable levels of remaining Parkin which might affect the results. In the literature the exact number and sequence of Parkin isoforms present in the cells, and in neuronal cells specifically, is not clear with some papers reporting the canonical three³⁴¹ and some up to 21³⁴². It is therefore entirely possible that different isoforms may have different roles in the cells, explaining the diversity of phenotypes that was encountered. For example, as the mechanisms laying behind Parkin's localization into the nucleus are not fully known, it's possible that some isoforms have a mainly nuclear localization, and thus would be responsible for the effects on transcriptional regulation, cell cycle control and potentially regulation of DNA damage repair, while others might be strictly cytoplasmatic and regulate mitophagy. We believe that investigating the specific roles of different Parkin protein sequences in specific pathways, perhaps through experiments involving both RNA-Seq and proteomics approaches like mass spectrometry, would be greatly beneficial to the scientific community and to better connect different Parkin mutations to the observed symptoms in AR-JP. An alternative approach could be to completely knock out the Parkin gene, preferably by substitution of the entire gene with a non-coding sequence or a reporter gene using CRISPR-Cas9 combined with a recombination (rather than relying on the Cas9 cut and random insertion of indels to produce a frameshift causing premature termination of the protein synthesis) in order to avoid the expression of even the smallest isoforms, and express instead artificial mutant constructs: for example, a Parkin protein fused with a nuclear localization signal would be beneficial to investigate the effects of Parkin that are exclusively happening in the nucleus.

PROJECT 2: In this part we demonstrated the involvement of the DNA helicase BLM, in post-NER processing in a mechanism likely cooperative with EXO1. The involvement of BLM in this process is, to our knowledge, novel. The protein is accumulated at time points that correlate with EXO1 recruitment, based on data published before in the lab^{237,336}; direct evidence in these experimental conditions has not been obtained yet but is in the works. Furthermore, we observed an increase in BLM recruitment when UDS was inhibited, supporting the idea of BLM and EXO1 working together. While removal of EXO1 did not seem to affect BLM recruitment, it is entirely possible that the helicase localizes independently of EXO1 function since BLM is expected to operate close to the ssDNA-dsDNA junction to unwind DNA while the exonuclease may operate on the displaced ssDNA filament. It would be interesting, to assess whether the recruitment of the latter is possible in absence of BLM, or if this affects its resection activity at all. In vitro work has indeed shown that BLM, but not other RecQ helicases, is capable of stimulating EXO1's resection of dsDNA³⁴³. Additionally, it could be worthwhile to assess the response of non-replicating cells to UV in absence of BLM, potentially linking the protein's activity in post-NER processing with the light sensitivity of Bloom's syndrome patients.

In this project there are a lot of questions that still need to be answered, most of them mainly involving the structural mechanics of Bloom's loading on the DNA after NER processing of the lesion. During Homologous Recombination, a physical interaction between BLM and EXO1 stimulates the latter exonuclease activity, in a process that does not depend on the helicase activity but probably just by mechanically increasing its tethering to dsDNA resulting in an increase of processivity³⁴³. Because of the structural differences between dsDNAs being repaired by HR or NER, mainly the fact that in HR both strands present extremities and the

tighter ssDNA region generated just after NER double incision, as highlighted in Figure 22, it could be interesting to investigate whether this applies also in the latter case.

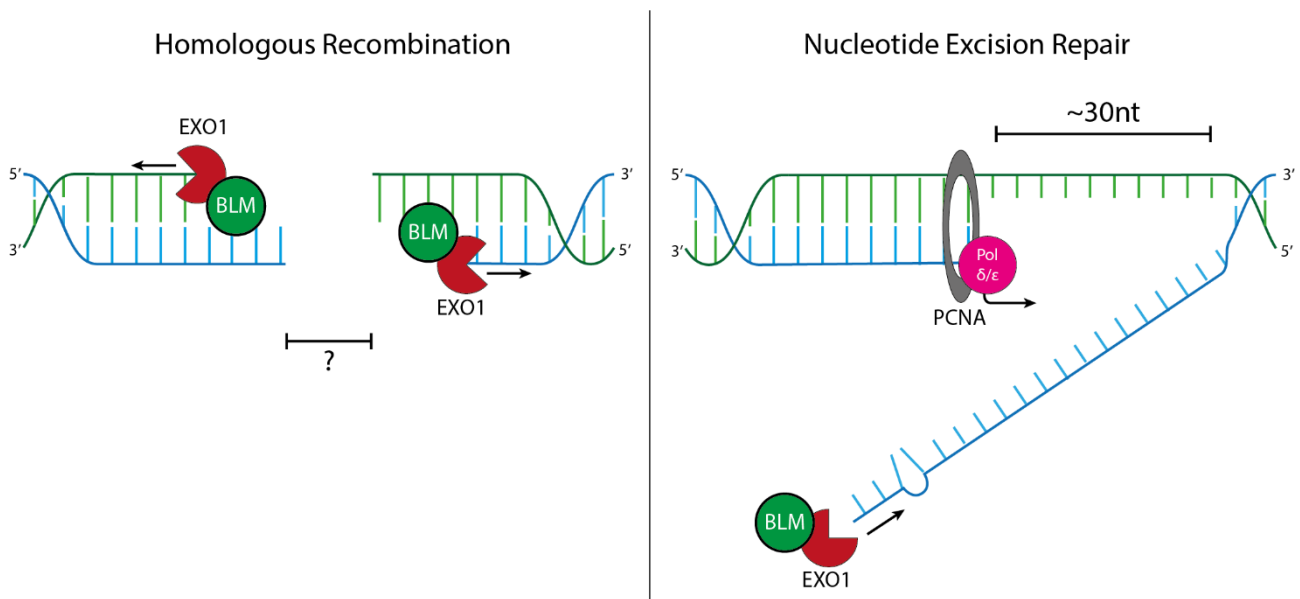


Figure 22: BLM's helicase loading on dsDNA in Homologous Recombination and Nucleotide Excision Repair

In Homologous Recombination, both DNA strands present free extremities that are available for protein access. Furthermore, a relatively large gap can remain between the two dsDNA molecules. In strike contrast, during NER it's possible that EXO1 already attacks the to-be-excised oligonucleotide, and even if it acts after excision this leaves only a 30-nucleotide long window for protein recruitment.

Other critical point to be addressed, are first whether BLM's recruitment is actually dependent on NER activity. Indeed, as discussed in the introduction, UV damage produces other DNA lesions that are not necessarily repaired by NER; for example, oxidative damage. To demonstrate it, we should repeat the induction of local UV damage in NER deficient cells and we'd expect the BLM helicase to not localize anymore. A further question, is whether the localization we are seeing is really due to a novel recruitment of the protein to the ssDNA generated after NER processing and resected by EXO1, and not just the result of DNA double strand breaks generation that we know can happen during COLs processing: in this latter case, it would be likely that BLM would be recruited because of its role in DSB repair. Indeed, it's important to highlight the importance of this protein in Homologous recombination, and to specify that in specific cases this process can occur in G1 cells (such as the ones that were analyzed in our experiments). Furthermore, BLM involvement could also be extended in NHEJ as particular subpathways, like Microhomology-mediated end-joining (MMEJ), require BLM's helicase to inhibit CtIP/MRE11's long resection^{344,345}. Some of the data collected hints that this is not the case: for example, the fact that BLM recruitment preceded the autophosphorylation of DNA-PKcs, however this could simply be an artefact of the experimental conditions (for example the antibody against BLM in the immunofluorescence might be much more sensitive than the one against phosphorylated DNA-PKcs). Using a neutral comet assay in early conditions in which BLM recruitment is already visible could also strengthen this idea: if the helicase is recruited before DSB generation we would expect to observe no comets in these early steps. Another approach, could be through the combined use of apical kinases inhibitors together with UV damage induction: this only works however if ATM (or DNA-PKcs in the case of NHEJ) kinase activity is required for the helicase's recruitment, which is still to be understood in non-S phase cells.

A major concern that will need to be addressed in future experiments on this project regards the method used to quantify the recruitment to sites of UV damage of the protein of interest. Indeed, in the analysis performed so far, we used the signal of EdU present in non-S-phase cells to delimit the area of local UV

damage and quantified the fluorescent signal for other proteins inside that region. However, this only works based on the assumption that the treatments performed do not affect the gap filling step of NER. In the above-mentioned case in which BLM recruitment would need to be assessed in case of NER deficiency, this kind of analysis would be impossible as the bulky lesions would not be excised from DNA and there would be no ssDNA gap to fill. A more ubiquitous analysis would require the use of antibodies targeting directly the DNA lesions; this signal would be used to mark the local UV damage region. As discussed in the introduction, UV damage forms two main kinds of bulky lesions on DNA: CPDs and 6-4PPs. Since the latter are repaired much quicker, in the matter of few hours¹¹, and therefore their repair might invalidate the results obtained at later time points from the induction of damage, targeting the first ones is a more ideal approach.

PROJECT 3: Finally, we were successful in demonstrating the involvement of EXO1, as well as a partial contribution of SNM1b/Apollo, in a NER-independent signaling activation. It is likely that this mechanism is active in all cells, not only NER-deficient ones, and it is only masked by the correct functioning of NER in physiological conditions. Such mechanism, which was already introduced in the Vrouwe et al (2011)³²⁸ work as a NER independent mechanism for ATR activation, was very recently explored in greater details in Gautam et al (2023)³⁴⁶: in this publication, a mechanism of photodimers removal independent from NER but that instead relies on the activity of Base Excision Repair factors like APE1 was shown. The proposed model for this system, is that the UV light used in the experimental conditions (15 J/m² in most of the experiments) would produce both bulky lesions and oxidative damage on DNA. The presence of both kind of lesions would trigger both NER and BER activity independently: however, when both lesions are found in close proximity on the same strand what can happen is that long-patch BER initiated on the oxidated base would remove the bulky lesion as well, without the need of a functional NER apparatus. This indeed would perfectly explain the removal of 6-4PPs in NER deficient fibroblasts that is experimentally shown in the paper. The result is also perfectly in line with the observations made by Vrouwe³²⁸, specifically the APE1 dependent signalling activation after UV, although it does not explain the increased removal of photodimers observed when both XPA and MUTYH (a DNA glycosylase of the BER pathway) are deleted compared to the NER deficiency alone described in Mazouzi et al³³⁰. Additionally, it also does not explain the dependency on EXO1 that is observed in this thesis as neither NER or BER involve the exonuclease in “standard” conditions. On the contrary, if the bulky lesions is still found in close proximity but on the opposite strand, making a sort of reverse COL in which the first event is BER-dependent removal of the oxidated base, the subsequent gap-filling activity may still require PCNA monoubiquitination for lesion bypass and the initial stalling of the canonical DNA polymerase might buy enough time for EXO1 to produce a ssDNA gap large enough to eventually become a DNA double stranded break (Figure 23). To demonstrate this theory, a neutral comet assay in EXO1 silencing conditions in NER deficient cells is imperative.

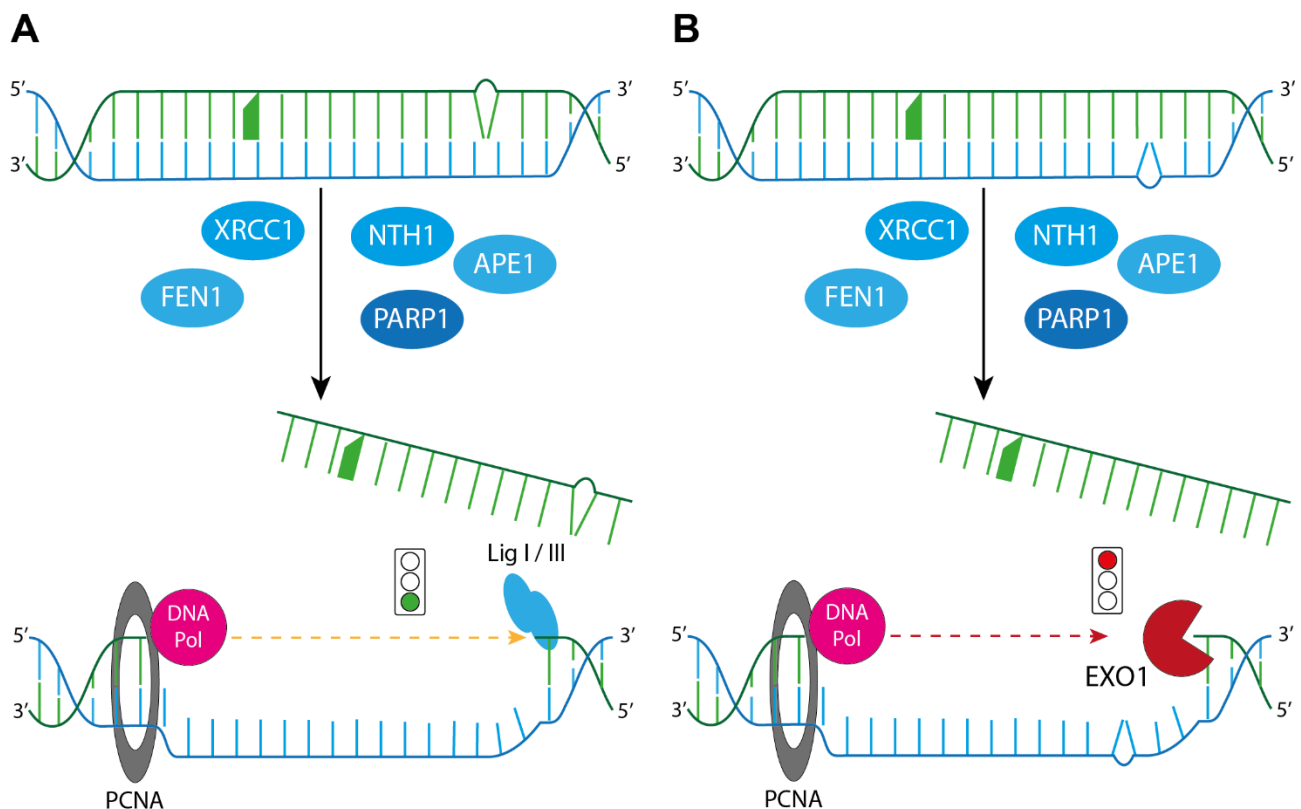


Figure 23: BER-dependent removal of photolesions from the genome and problems associated

(A) Mechanism of removal of photolesion from the genome in a NER-independent manner, using the BER machinery instead, as proposed in Gautam et al 2023³⁴⁶. (B) A similar situation but in which the bulky lesion is found on the opposite strand might lead to DNA Polymerase stalling and thus involve EXO1 resection as speculated in the COLs model.

We also observed that this process depends very little on the presence or absence of unscheduled DNA synthesis. In most of the western blot analysis performed we calculated the amount of remaining cycling cells after starvation by EdU incorporation assays and observation at the microscope, resulting in a contamination of cycling cells close to 10% (data not shown). While for these initial experiments we deemed this portion of cells not significant enough to affect the results obtained on the remaining majority of starved cells, it would be better in the future of this project to backup these claims with single cells analysis like immunofluorescences and/or by FACS. Additionally, as working with primary cell lines is particularly challenging and these cells are not easily available, we are currently generating starvable cells (RPE_hTERT) knocked-out for XPA, thus solving both the expensiveness of relying on siRNA transfection for each individual experiment and the intrinsic variability of transient silencing.

The initial data obtained thus far in this project suggest a minor involvement of SNM1b/Apollo in this mechanism; however, data about other human homologs of Pso2, such as SNM1a and SNM1c/Artemis is still to be collected. More importantly, none of the models proposed explain their function in this process. Because of this, this part of this project is still very preliminary and many more experiments need to be performed.

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“If we knew what we were doing, it would not be called research, would it?”

(Albert Einstein)

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11 CONTRIBUTION IN PUBLICATIONS

During my PhD, I contributed to the publication of the following papers:

1. Tau and DNA Damage in Neurodegeneration

(Luca Colnaghi, Diego Rondelli, Marco Muzi-Falconi, Sarah Sertic)

Doi: 10.3390/brainsci10120946

Having collaborated with the first author during my traineeship in neuronal explants preparation and handling, I worked on this review which is focused on the role of the protein Tau in DNA damage in neurons. Tau, encoded by the MAPT (microtubule-associated protein tau) gene, has been associated with many neurodegenerative diseases that go by the common name of tauopathies; while being a cytosolic protein with a plethora of interactors, unexpected new roles in DNA metabolism have recently emerged and are summarized in this work. My contribution to this paper has mainly regarded the review of DNA damage in general considering my background and the work I was doing in the lab, and I also assisted in the writing of the paper and reference check, as well as preparations of the figures.

2. Phosphorylation of H3-Thr3 by Haspin Is Required for Primary Cilia Regulation

(Roberto Quadri, Sarah Sertic, Anna Ghilardi, Diego Rondelli, Guido Roberto Gallo, Luca Del Giacco, Marco Muzi-Falconi)

Doi: 10.3390/ijms22147753

Our laboratory has a long-standing interest in the molecular characterization of Haspin, a nuclear kinase with potential relevance to carcinogenesis. In this work, the involvement of Haspin in the regulation of primary cilia has been presented. The length and resorption of primary cilia is known to be regulated by the histone acetylase HDAC6, whose distribution is instead regulated by the death inducer-obliterators 3 (Dido3). The novel role of Haspin would be to assist the relocalization of Dido3-HDAC6 to the basal body of the cilia. Additionally, it's shown that the kinase is active also in quiescent cells where mitosis is obviously not present, and in these same cells it's responsible for the phosphorylation of its known substrate Histone H3-Thr3 (whose phosphorylation was canonically believed to be exclusively mitotic). The physiological relevance of this mechanism is supported by experiments performed in zebrafish. My contribution to this paper has focused on microscopy analysis of cilia formation under different conditions, preparation of mutated versions of Histone H3 plasmids, and general aid during revision.