Abstract (Versione in italiano)

La sintesi translesione (TLS) è una delle strategie che permette di bypassare il danno al DNA, consentendo alla cellula di continuare il processo di replicazione e completare il ciclo cellulare. La polimerasi η (Polη) è un enzima fondamentale nel processo di TLS. Mutazioni nel gene *POLH*, che codifica per questa polimerasi, possono compromettere la sua funzione e sono associate alla malattia genetica conosciuta come Xeroderma Pigmentoso Variante (XPV). Le varianti di Polη più frequentemente riportate associate a XPV sono quelle chiaramente patogene, spesso rappresentate da mutazioni nonsenso o frameshift, che compromettono significativamente la funzione dell'enzima. Tuttavia, molte varianti missenso identificate nei pazienti rimangono classificate come "varianti di significato incerto" (VUS), a causa dell'assenza di una caratterizzazione funzionale precisa. In particolare, la maggior parte degli studi disponibili si basa su cellule derivate da pazienti, il che introduce variabili difficili da controllare.

In questo lavoro abbiamo analizzato quindici varianti missenso di Polη *in vitro*, otto delle quali anche *in vivo*, utilizzando un sistema sperimentale basato su cellule XP30RO, prive di Polη, in cui abbiamo ri-espresso le varianti di Polη oggetto dello studio.

I risultati mostrano un'ampia eterogeneità di comportamento: le mutazioni sembrano essere coinvolte nella variazione nell'attività enzimatica della proteina, la sua regolazione, la stabilità o le interazioni proteiche. In particolare, è stato osservato che sei varianti (Arg93Pro, Arg111His, Ala117Pro, Thr122Pro, Gly263Val, Cys321Phe) mostrano difetti significativi nella sintesi translesione e sarebbe possibile cambiare la loro classificazione, secondo le linee guida dell'ACMG, da "VUS" a "likely pathogenic" in quanto direttamente responsabili della malattia. Le varianti Lys535Glu e Met595Val, al contrario, presentano un comportamento complesso che necessita di ulteriori studi ed esperimenti, rimanendo perciò classificate come VUS.

Questo lavoro si inserisce in un progetto più ampio volto alla caratterizzazione funzionale delle varianti di Polη, cercando di aiutare a diagnosticare con certezza i casi di Xeroderma Pigmentoso Variante in presenza di nuove mutazioni missenso nei diversi domini funzionali di Polη.



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In vitro and *in vivo* analysis of DNA polymerase η missense variants

Analisi *in vitro* e *in vivo* di varianti missenso della DNA polimerasi η

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1. ABSTRACT

Translesion synthesis (TLS) is one of the strategies that allows to bypass DNA damage, thus allowing the replication process to continue and the completion of the cell cycle. Polymerase η (Polη) is a key enzyme in the TLS process. Mutations in the *POLH* gene, which encodes this polymerase, can impair its function and are associated with the rare genetic disease Xeroderma Pigmentosum Variant (XPV). The variants of Polη most frequently associated with XPV are those clearly pathogenic, often represented by nonsense or frameshift mutations, which significantly impair the function of the enzyme or abolish the expression of the polymerase. However, many missense variants identified in patients remain classified as "variants of uncertain significance" (VUS), due to the lack of precise functional characterization. In particular, most of the available studies are based on patient-derived cells, which introduces variables that are difficult to control.

In this work we analysed fifteen missense variants of Polη *in vitro*, eight of which also *in vivo*, using an experimental system based on XP30RO cells, devoid of Polη, complemented with the Polη variants under study.

The results show a wide heterogeneity of behaviour: the mutations seem to be involved in the variation in the enzymatic activity of the protein, its regulation, stability or protein interactions. In particular, it was observed that six variants (Arg93Pro, Arg111His, Ala117Pro, Thr122Pro, Gly263Val, Cys321Phe) show significant defects and we propose that their classification to be changed, according to the ACMG guidelines, from “VUS” to "likely pathogenic". The variants Lys535Glu and Met595Val, on the contrary, present a complex behaviour, therefore should remain classified as VUS, requiring further studies and experiments.

This work is part of a larger project aimed at the functional characterization of Polη variants, trying to help diagnose Xeroderma Pigmentosum Variant cases with certainty in the presence of new missense mutations in the different functional domains of Polη.

2. INTRODUCTION

2.1. The cell cycle

Deoxyribonucleic acid (DNA) is the nucleic acid that, in all kingdoms of life, stores the genetic information which must be transmitted with high fidelity across generations. DNA replication is therefore a fundamental and highly regulated biological process, essential for maintaining genetic integrity throughout the cell cycle.

The cell cycle consists of a series of events that allow a cell to grow, duplicate its DNA, and divide into two genetically identical daughter cells, which, in the absence of errors, are exact copies of the parental cell. To prevent genetic alterations, this process is tightly regulated, ensuring that DNA is accurately duplicated once per cycle, specifically during the S phase. This precise control is achieved through the coordinated action of various molecules and proteins that govern cell growth in response to mitogenic signals (Khanna and Shiloh, 2009).

The life cycle of a cell includes an interphase and a M phase. The interphase spans almost the entire duration of the cell cycle; it includes G1, S and G2 phases. M phase includes a nuclear division phase, or mitosis, and a cytoplasmic division phase, or cytokinesis. The G1 and G2 phases represent “gaps” in the cell cycle, occurring between two key stages: DNA synthesis and mitosis. During the G1 phase, the cell prepares for DNA replication, the DNA content is 2C. In the S phase, DNA synthesis takes place, resulting in a DNA content ranging from 2C to 4C. The G2 phase is the second gap, during which the cell prepares itself for mitosis (M phase), therefore the DNA content is 4C.

Cells can also exit the cell cycle, either by reaching a terminally differentiated state or by entering a quiescent phase known as G0, in which cells are not actively replicating but have the potential to re-enter the cell cycle. However, the term “G0” has been inaccurately used to describe also terminally differentiated cells (Figure 1) (Shafer, 1998).

The details of the cell cycle vary depending on the organism and can also differ among cells of the same organism depending on their developmental stage. However, the fundamental mechanisms that drive and regulate the cell cycle are unique to all eukaryotic cells. Therefore, these mechanisms can be studied in simple model organisms (for example unicellular eukaryotic cells such as yeasts), to obtain insights that may be validated in more complex systems.

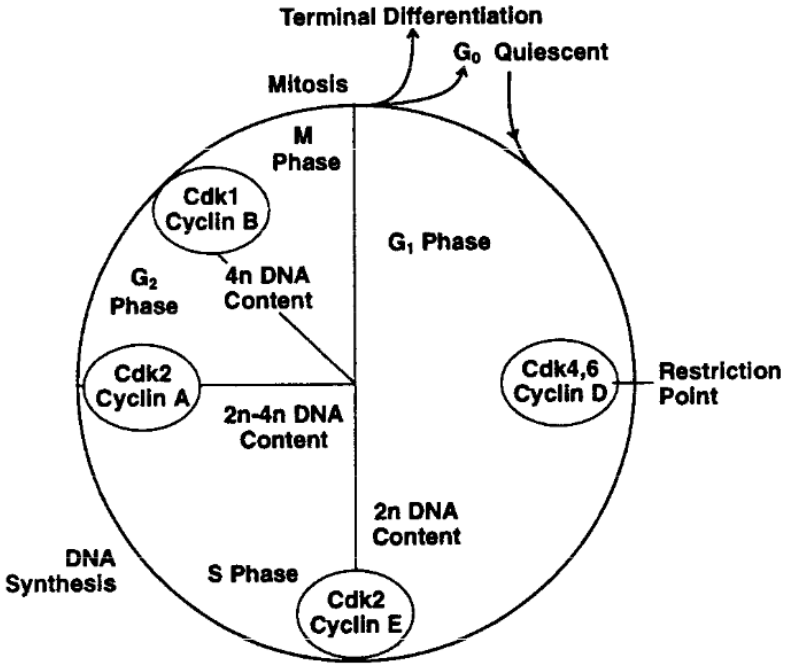

Figure 1
Representative scheme of the cell cycle, including G1, S, G2 and M phases and the complexes of cdks and cyclins that are involved in all phases. Furthermore, we can observe the DNA content in the different phases and the other possible fates when the cell exits the cycle, either terminal differentiation or G0 (quiescent state). From: Schafer KA. The cell cycle: a review. Vet Pathol. 1998 Nov;35(6):461-78. doi: 10.1177/030098589803500601. PMID: 9823588.

The transition from one phase of the cell cycle to the next one is subject to check points to ensure that the cell enters a phase only after completion of the previous one. Such check points are regulated by a related family of protein serine/threonine kinases, which require binding to co-factors, called cyclins, for activation, thus earning the name cyclin-dependent kinases (CDKs) (Pines, 1995).

CDKs phosphorylate specific substrates at distinct points in the cycle, thereby regulating its progression through the different phases. These complexes consist of two subunits: a catalytic subunit with serine/threonine kinase activity, which transfers phosphate groups to serine or threonine residues within specific consensus sequences (S/T P X K/R), and a regulatory subunit, a cyclin, which provides substrate specificity and directs the catalytic subunit to its target (Barnum and O'Connell, 2014).

Cyclins are unstable proteins that are periodically synthesized and degraded during the cell cycle and CDKs are only active when the corresponding regulatory subunit is expressed (Pines, 1995).

Therefore, upstream, CDK activity is primarily controlled by the nuclear availability of cyclins, which are tightly regulated both through transcriptional control mechanisms and ubiquitin-dependent proteolysis (Barnum and O'Connell, 2014).

All cyclins, studied through cloning and sequencing, present a ~100 amino acid region of homology, the cyclin box (Hunt, 1991). Later studies revealed that this protein region plays a role in binding to the CDK family of protein kinases (Kobayashi et al., 1992). Based on sequence homology with the

cyclin box consensus, more than 12 distinct cyclins have been identified in budding yeast, while mammalian cells possess a comparable number, classified from A to H (Pines, 1995).

According to the current model of cell cycle regulation (Wang, 2021), in early G1 phase Cdk4 and/or Cdk6 form complexes with D-type cyclins. The final result of such interaction is the release of transcription factor E2F, which activates the transcription of genes required for the onset of the next phase. Indeed, these genes include E-type and A-type cyclins both forming a complex with Cdk2. However, the Cdk2/cyclin E complex controls G1/S transition, whereas the Cdk2/cyclin A complex phosphorylates substrates involved in DNA replication and controls the progression of S phase and the transition to G2 transition. During late G2 phase cyclin A forms a complex with Cdk1, thus leading to the G2/M transition. Finally, Cdk1 forms a complex with B-type cyclins throughout M phase completion.

2.2. Genome replication

The precise sequence of all developmental events that lead to a multicellular organism starting from a single cell ultimately relies on the genetic information encoded within the DNA molecule. Such information is required for RNA and protein synthesis, both essential for an organism's development, and given its critical role, it is crucial for the cell to ensure an elevated accuracy of replication. As a consequence, cells have evolved a sophisticated molecular machinery dedicated to DNA replication. This process is highly conserved across both prokaryotes and eukaryotes, relying on similar molecular components. Among them, DNA polymerases play a pivotal role, although multiple players are involved in this process.

To fully understand this process, it is essential to start from the very beginning, at the replication origin, where DNA synthesis is first initiated.

The first model that was hypothesised to explain the regulation of DNA replication initiation was the replicon model. It states that replication begins at specific DNA sequences called replicators, which are recognized by initiator proteins, that once bound, help recruit helicases and other replisome components, enabling DNA synthesis to proceed via a bi-directional replication bubble consisting of two forks running in opposite direction (Jacob et al., 1963). The replicon model remains a fundamental concept for understanding prokaryotic DNA replication; however, its applicability to eukaryotic systems has been revised in light of new discoveries. While bacterial replication is controlled by a single, well-defined origin specified by DNA sequences, eukaryotic replication origins are not strictly determined by sequence elements but rather by a combination of chromatin structure and epigenetic factors. Additionally, the original model was based solely on positive

regulation, whereas subsequent research has revealed the presence of negative regulatory mechanisms in eukaryotic replication that prevent unscheduled replication initiation.

Eukaryotic origin function still depends on a conserved initiator protein complex (namely Origin Recognition Complex, ORC) to facilitate the loading of replicative helicases onto DNA during the late M and G1 phases of the cell cycle, a process known as origin licensing (Figure 2). Unlike bacterial systems, eukaryotic replicative helicases are loaded onto origin duplex DNA in an inactive, double-hexameric form. ORC with the assistance of two more proteins, Cdc6 (capable of ATPase activity) and Cdt1, is required to load the helicase hexameric complex (minichromosome maintenance 2-7, Mcm2-7). The loading is of Mcm2-7 is highly cooperative and another helicase ring is recruited forming a head-to-head double hexamer (Remus et al., 2009). However, only a fraction of these helicases (approximately 10–20% in mammalian cells) is activated during each S phase, a process referred to as origin firing.

Indeed, *in vitro* the Mcm2-7 complex is able to slide along the DNA duplex but is not able to unwind the DNA (Remus et al., 2009). For this to happen, two more factors are necessary: Cdc45 and GINS (Pacek et al., 2006). Only after the formation of the CMG (Cdc45-Mcm-GINS) complex the DNA is actually melted and replication can start (Ilves et al., 2010). Therefore, another limitation of the model is its inability to account for the multiple replication origins found in eukaryotic genomes, where more helicases are loaded than activated, allowing for a flexible replication program.

These findings have led to the refinement of the replicon model, which, while still valid for prokaryotes, has been adapted into a more dynamic and context-dependent framework in eukaryotic cells, incorporating both positive and negative regulatory elements to ensure accurate and timely genome duplication (Ekundayo and Bleichert, 2019).

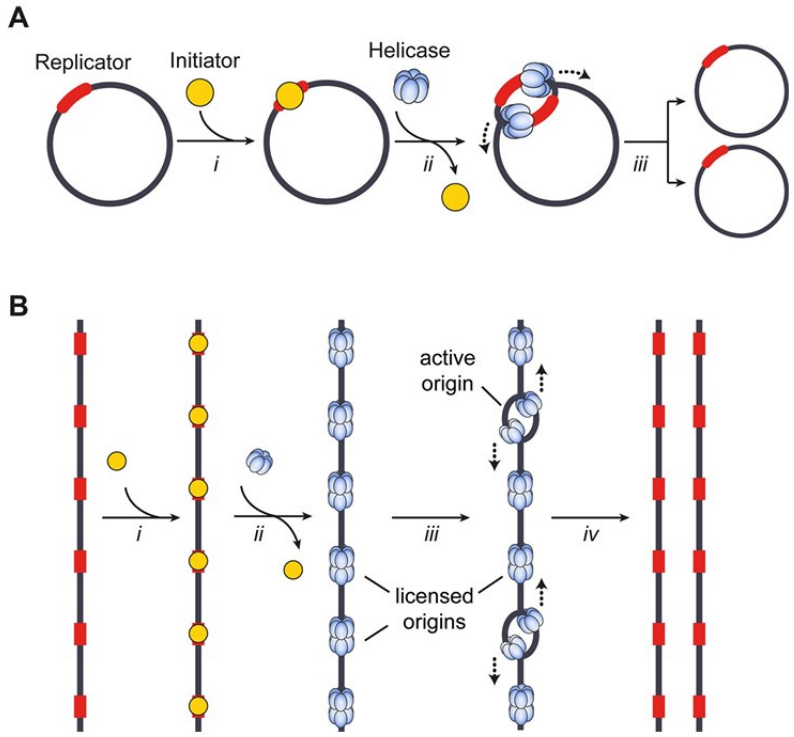

Figure 2
The models of initiation of DNA replication in both bacterial (A) and eukaryotic (B) cells.
(A) **Bacterial DNA replication initiation:** Bacterial circular chromosomes contain a replicator, a specific DNA sequence that marks the site of replication initiation. **(i)** The initiator protein binds to the replicator in a sequence-specific manner, leading to local unwinding of the DNA double helix. **(ii)** This facilitates the loading of helicases onto the single-stranded DNA, which are essential for the progression of the replication machinery. **(iii)** Once the replisome is fully assembled, DNA replication proceeds bidirectionally, generating two identical copies of the bacterial chromosome.
(B) **Eukaryotic DNA replication initiation:** Eukaryotic chromosomes are linear and contain multiple replication origins to ensure complete and efficient genome duplication. **(i)** Initiator proteins bind to replication origins across the genome. **(ii)** This promotes the loading of helicases onto double-stranded DNA, a step known as origin licensing, which marks potential sites for replication initiation. **(iii)** However, only a subset of these licensed origins is activated during a given cell cycle, leading to bidirectional replication from selected origins. **(iv)** Replication continues until adjacent replication forks converge, ensuring complete genome duplication before cell division.
From: Ekundayo, Babatunde, and Franziska Bleichert. "Origins of DNA replication." *PLoS genetics* vol. 15,9 e1008320. 12 Sep. 2019, doi:10.1371/journal.pgen.1008320

Helicases unwind the double-stranded DNA (dsDNA), opening the "replication fork" generating single-stranded DNA (ssDNA), which is quickly bound by Replication Protein A (RPA). This prevents the ssDNA from re-annealing, forming secondary structures, and protects it from degradation by endonucleases (Chen and Wold, 2014).

These regions provide the substrate for priming by the DNA polymerase α (Pol α)–DNA primase complex. This heterotetrameric complex possesses both RNA primase activity, localized in the p48

(Pri1) subunit, and polymerase activity, present in the p180 (Pol1) subunit (Pellegrini, 2012). α (Pol α)–DNA primase synthesizes short RNA primers necessary for the initiation of DNA synthesis.

The central role in DNA replication is covered by polymerases, whose function is to actively synthetize DNA. In prokaryotes, DNA replication is carried out by DNA polymerases I, II, and III, whereas in eukaryotic cells, this function is primarily performed by DNA polymerases α, δ, and ε. Despite differences in their amino acid sequences, these enzymes share a remarkably similar structural organization (Steitz, 1999). Every polymerase, except for members of the PrimPol family, can be in fact compared to a right hand, with distinct domains referred to as the "thumb", "palm" and "fingers". The palm domain corresponds to the catalytic domain and it is, in fact, primarily responsible for catalysing the phosphoryl transfer reaction. Based on structural and sequence data, this region appears to be the most highly conserved among all polymerases. The fingers domain plays a crucial role in interacting with the incoming nucleoside triphosphate and, moreover, in binding and orienting the template strand. The thumb is a helical domain that is connected to the others in a flexible manner and it appears to be involved in positioning the duplex DNA and contributing to processivity and translocation (Joyce and Steitz, 1994).

Genome replication is an inherently highly accurate process, with an error rate of less than one per 10^8 bases, which can further decrease to one error per 10^{10} bases when accounting for post-replicative mismatch repair (MMR), thanks to the ability of replicative DNA polymerases to selectively incorporate correctly paired nucleotides and excise misincorporated ones in case of an error. This proofreading function, a hallmark of replicative DNA polymerases, is facilitated by a 3'-5' exonuclease activity, conferred by a dedicated proofreading domain (Loeb and Monnat, 2008; Arana and Kunkel, 2010).

The high intrinsic fidelity of replicative DNA polymerases arises from three key mechanisms: conformational selection, which involves a structural transition from a binary complex (open-finger DNA polymerase bound to the template strand) to a ternary complex (closed-finger polymerase with the template strand and an incoming dNTP correctly paired to the template base) (Doublié et al., 1999); chemical selection, which ensures that a highly precise spatial coordination of three Mg^{2+} ions is required for the formation of the phosphodiester bond (Gao and Yang, 2016); and 3'-5' exonuclease activity cited above, which eliminates misincorporated nucleotides but only becomes operational when a significant distortion of the DNA structure occurs (Fernandez-Leiro et al., 2017).

Regarding the replication fork, the two antiparallel strands of ssDNA are identified as the leading strand and the lagging strand, because they are replicated in different ways. Bearing in mind that DNA synthesis is mono-directional (5'-3'), the leading strand is the one which is replicated in the same direction of the ongoing replication fork, while the lagging strand is elongated in a direction

opposite to that of fork movement. As a consequence, the replication of the leading strand is traditionally considered a continuous process, at least in the absence of obstacles or DNA damage, while the lagging strand replication occurs discontinuously through the synthesis of so called Okazaki fragments. Most evidence supports a model in eukaryotes in which DNA polymerase ε (Pol ε) is the primary enzyme responsible for synthesizing the leading strand in an unobstructed replication fork. On the lagging strand, DNA synthesis initiated by Pol α is limited to approximately 20-30 nucleotides, after which DNA polymerase δ (Pol δ) takes over for the elongation of Okazaki fragments (Burgers and Kunkel, 2017). The processivity and catalytic efficiency of both Pol ε and Pol δ are significantly enhanced by its interaction with proliferating cell nuclear antigen (PCNA), a "sliding clamp" that tethers the enzyme to DNA (Chilkova et al., 2007) (Stodola and Burgers, 2016) (Yeeles et al., 2017). When Pol δ reaches the 5′ end of a previously synthesized Okazaki fragment, it initiates strand displacement synthesis, generating a single-stranded 5′ DNA flap. This flap must be removed to allow ligation of adjacent Okazaki fragments. The primary enzyme responsible for flap removal is flap endonuclease 1 (FEN1), which cleaves the flap through a process known as nick translation. Occasionally, longer flaps resistant to FEN1 cleavage due to secondary structures or RPA binding may form. In such cases, another nuclease, Dna2, which also possesses helicase activity, processes the longer flaps, reducing them in size so that they can be further cleaved by FEN1. Finally, after complete removal of the RNA primer and the filling of any remaining gaps, DNA ligase 1 seals the discontinuities between DNA fragments, completing lagging strand synthesis. (Figure 3) (Burgers and Kunkel, 2017).

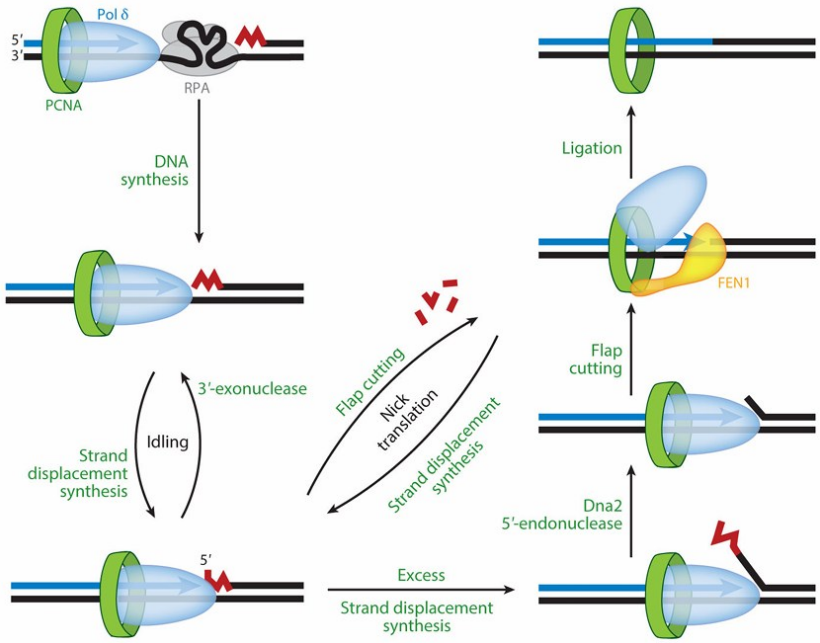

Figure 3
We can observe Okazaki fragment maturation. On the lagging strand, primers are extended by DNA polymerase δ (Pol δ) until the adjacent Okazaki fragment is reached. Pol δ then initiates strand displacement synthesis, which is regulated by its 3′-exonuclease activity (idling) to prevent excessive displacement. In the presence of flap endonuclease 1 (FEN1), the emerging DNA flap is cleaved, allowing strand displacement synthesis to resume. This process is known as nick translation. However, occasional excessive strand displacement results in long 5′-flaps, which are trimmed to shorter fragments by the nuclease activity of Dna2. Once all RNA primers have been degraded, DNA ligase 1 seals the remaining DNA nicks, completing Okazaki fragment maturation.
Abbreviations**:** FEN1, 5′-flap endonuclease 1; PCNA, proliferating cell nuclear antigen; RPA, replication protein A.
From: Burgers, Peter M J, and Thomas A Kunkel. "Eukaryotic DNA Replication Fork." *Annual review of biochemistry* vol. 86 (2017): 417-438. doi:10.1146/annurev-biochem-061516-044709

Given the distinct mechanisms operating on the two strands, it is essential that leading and lagging strand synthesis remain tightly coordinated to ensure smooth replication fork progression and prevent the formation of extensive single-stranded DNA regions. One proposed model to explain this coordination is the "trombone model", in which the lagging strand loops back on itself, allowing both polymerases to function synchronously and in the same overall direction as the replication fork (Sinha, 1980). Recent studies suggest that the yeast protein Ctf4 may play a key role as a replisome coordinator in eukaryotes. Ctf4 forms a homotrimer and can simultaneously interact with Pol α on the lagging strand and with the GINS complex and Pol ε on the leading strand, providing a physical link between the two replication machineries (Simon et al., 2014) (Villa et al., 2016) (Figure 4).
The Ctf4 human homolog, AND-1, has also been shown to interact with Pol δ (Bermudez et al., 2010), however, it remains a highly intricate process about which our current understanding is still limited.

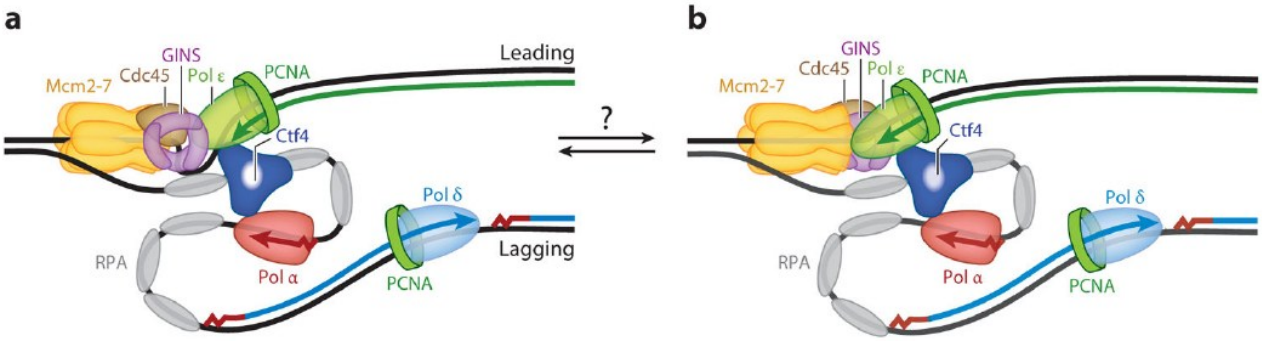

Figure 4
Two proposed models describe the pathway taken by the leading strand before reaching the catalytic site of DNA polymerase ε (Pol ε), suggesting that either (a) ~40 nucleotides or (b) ~20 nucleotides of single-stranded DNA are sequestered. Additionally, it has been proposed that these two configurations may dynamically interconvert. The lagging strand is depicted as forming a loop, enabling both Pol α and Pol ε to advance in the same direction while being stabilized within a complex by Ctf4.
Abbreviations**:** GINS, Sld5-Psf1-Psf2-Psf3 complex; Mcm2-7, helicase complex; PCNA, proliferating cell nuclear antigen; RPA, replication protein A.
From: Burgers, Peter M J, and Thomas A Kunkel. "Eukaryotic DNA Replication Fork." *Annual review of biochemistry* vol. 86 (2017): 417-438. doi:10.1146/annurev-biochem-061516-044709

2.3. DNA damage and DNA repair

The integrity of the genome is constantly challenged by both endogenous and exogenous sources of DNA damage, which, if left unrepaired, can compromise cell viability and lead to genetic instability, a hallmark of aging and many diseases, including cancer (Bouwman and Jonkers, 2012; Ghosal and Chen, 2013; Wolters and Schumacher, 2013). Endogenous DNA damage arises from intrinsic cellular processes such as hydrolysis, alkylation, deamination, oxidative stress caused by reactive oxygen species (ROS), and spontaneous DNA base mismatches during replication (Tropp and Burton, 2012). Additionally, the replication machinery encounters various obstacles that can impede fork progression, including DNA lesions, tightly bound DNA-protein complexes, and atypical DNA secondary structures such as cruciforms, slipped strands, triplexes, G-quadruplexes, Z-DNA, and R-loops. These impediments can induce replication stress, often resulting in replication fork stalling, collapse, or breakage (Zeman et al., 2014).

Exogenous sources of damage include ultraviolet (UV) radiation, ionizing radiation (IR), and various chemical agents such as alkylating compounds and crosslinking agents, which can induce single- and double-strand breaks, bulky adducts, and interstrand crosslinks (Chastain et al., 2006). Among these, UV radiation is particularly notable for causing cyclobutane pyrimidine dimers (CPDs) and [6-4] pyrimidone photoproducts ([6-4]PPs), lesions that, if not repaired before S phase, may block replication forks and lead to double-strand breaks (Lee et al., 1999).

To counteract these potentially harmful events, cells have evolved complex and tightly regulated systems, the DNA Damage Response (DDR), comprising DNA repair mechanisms, damage tolerance pathways, cell cycle checkpoints, and programmed cell death pathways, that collectively mitigate the detrimental effects of DNA damage. The choice of the DNA repair pathway is influenced by several factors, including the type of damage incurred and the specific phase of the cell cycle in which the lesion occurs (Essers et al., 2006) (Figure 5).

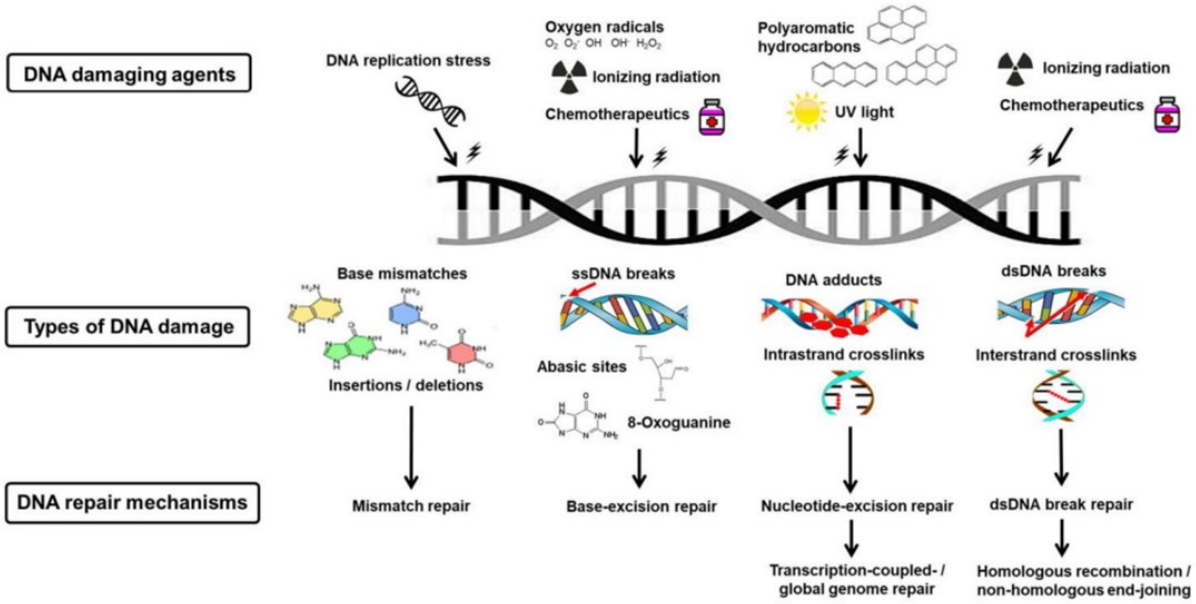

Figure 5
Representation of various types of DNA damaging agents that can cause different types of DNA damage and the related repair mechanisms implemented by the cell.
From: Helena JM, Joubert AM, Grobbelaar S, et al. Deoxyribonucleic Acid Damage and Repair: Capitalizing on Our Understanding of the Mechanisms of Maintaining Genomic Integrity for Therapeutic Purposes. *Int J Mol Sci*. 2018;19(4):1148. Published 2018 Apr 11. doi:10.3390/ijms19041148

DNA damage is initially detected by specialized sensor proteins that activate a cascade of transducers and effectors to coordinate DNA repair and maintain genome stability. The three central kinases, Ataxia Telangiectasia Mutated (ATM), Ataxia Telangiectasia and Rad3-related (ATR) and DNA-PKcs (DNA-dependent protein kinase), play fundamental roles in DDR signalling in mammalian cells (Blackford and Jackson, 2017). Homologous proteins exist in other eukaryotes, such as Tel1 (ATM homologous) and Mec1 (ATR homologous) in *Saccharomyces cerevisiae*, or Rad3 (ATR homologous) in *Schizosaccharomyces pombe*, which share similar functions despite nomenclatural differences (Tannous and Burgers, 2021).

In response to DNA damage, ATM and ATR kinases orchestrate the phosphorylation of hundreds of proteins, primarily at Ser/Thr-Glu motifs, along with additional sites. This extensive signalling cascade plays a central role in coordinating the cellular DNA damage response. In contrast, DNA-

PKcs is thought to target a more limited set of substrates and is mainly involved in the non-homologous end joining (NHEJ) pathway (Matsuoka et al., 2007; Smolka et al., 2007; Bensimon et al., 2010).

Both *in vivo* and *in vitro* studies have shown that ATM and ATR appear to have distinct roles and substrate specificities depending on the type of DNA damage encountered (Figure 6). ATM is predominantly activated by double-strand breaks (DSBs) and involves the recruitment of the MRN (MRE11-RAD50-NBS1) complex, which in turn phosphorylates CHK2 and p53 to halt cell the cycle progression (Aparicio et al., 2014; Bartek and Lukas, 2007). ATR, on the other hand, is able to respond to a broader range of DNA damage, including lesions that interfere with DNA replication. When a replicative DNA polymerase encounters an obstacle, such as a UV-induced DNA adduct, it is unable to proceed further and stalls.

By contrast, the MCM helicase activity is not compromised, so MCM keeps unwinding the DNA to a certain extent, thus generating regions of single-stranded DNA (ssDNA) (Byun et al., 2005). The resulting ssDNA stretches are rapidly coated by the replication protein A (RPA) complex, which plays a crucial role in initiating the DNA damage response by activating the ATR kinase. In fact, ATR by means of its subunit ATRIP (ATR Interacting Protein) is capable of recognizing the RPA-ssDNA complex, thus leading to the recruitment of ATR to the stalled replication fork.

This circumstance triggers a signalling cascade involving a series of phosphorylation events that regulates both DNA repair and cell cycle progression (Zou and Elledge, 2003). Several proteins are involved in this cascade, including TOPBP1, BRCA1, RAD17, HUS1, RAD1, RAD9, and p53. One of the major outcomes of this pathway is the phosphorylation, with consequent activation, of the downstream effector kinase CHK1, which stabilizes the stalled replication fork and prevents premature mitotic entry, thus arresting the cell cycle to allow time for repair. In fact, in the presence of DNA lesions or damage, the progression toward the M phase can be dangerous for genome integrity. Therefore, the ATR axis controls the activation of cyclin/CDKs to avoid deleterious consequences (Liu et al., 2006) (Maya-Mendoza et al., 2007).

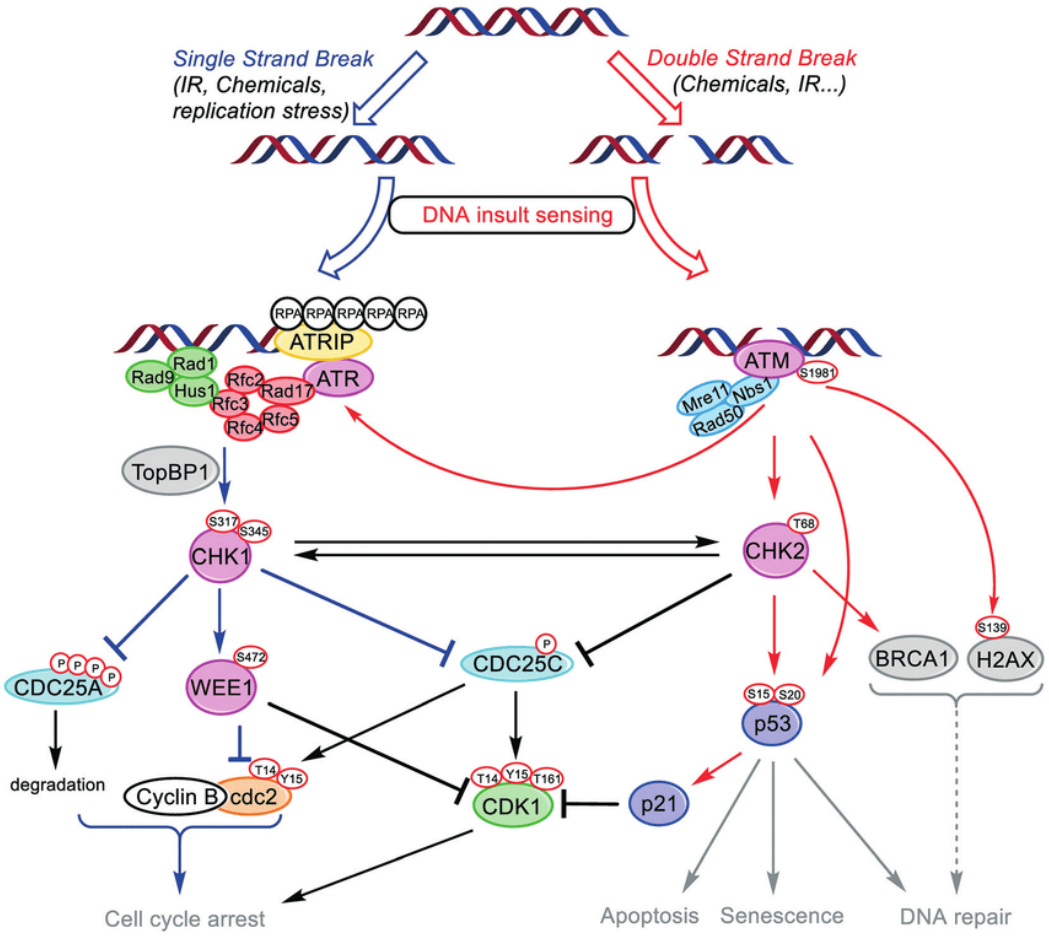

Figure 6
Components of the DNA damage response pathways modulated by ATM, ATR, CHK1 and CHK2 kinases. From: Ronco, C. et al. ATM, ATR, CHK1, CHK2 and WEE1 inhibitors in cancer and cancer stem cells. MedChemComm 2016; DOI: 10.1039/c6md00439c

At least five major DNA repair pathways operate to counteract different types of DNA lesions, including base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end joining (NHEJ) (Jackson and Bartek, 2009).

BER is responsible for dealing with small base lesions caused by oxidative damage, alkylation, and deamination. This pathway begins with the recognition and removal of the damaged base by a damage-specific DNA glycosylase, thus creating an abasic site. This first removal step is then followed by cleavage of the abasic site, gap filling and ligation mediated by endonucleases, DNA polymerases, and ligases respectively (Kubota et al., 1996). BER can occur via short-patch or long-patch mechanisms, with short-patch BER resulting in the replacement of a single nucleotide, and long-patch BER resulting in the initial creation of a longer gap (2-10 nucleotides) to be filled (Frosina et al., 1996).

NER, in contrast, is specialized in repairing bulky DNA lesions, such as UV-induced damage, and involves the coordinated action of the xeroderma pigmentosum (XP) proteins, which remove a 12-25 nucleotide-long fragment containing the lesion, followed by strand resynthesis (Takahashi and Ohnishi, 2005).

MMR ensures the fidelity of DNA replication by recognizing and correcting base mismatches, insertion/deletion loops, and polymerase misincorporation errors. Its molecular mechanism involves a sequential process of mismatch recognition, excision, and resynthesis (Constantin et al., 2005).

For double-strand breaks, cells employ two main repair mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ). HR is an error-free mechanism but requires a homologous sequence as a template. For this reason, it is active in S and G2 phases when a sister chromatid is available (Aparicio et al., 2014; Kass et al., 2013). Conversely, NHEJ is a more rapid but potentially error-prone process that directly ligates broken DNA ends without requiring extensive homology. The lack of such requirement makes it the preferred pathway in G1 phase (Bartek and Lukas, 2007). In addition to the repair pathways described above, interstrand crosslink (ICL) repair is specialized on DNA lesions that covalently link the two strands, obstructing replication and transcription (Bouwman and Jonkers, 2012).

When DNA repair mechanisms fail or become overwhelmed, cells activate programmed cell death pathways to prevent the propagation of genomic instability. This apoptotic response is often mediated by p53, a tumour suppressor that integrates signals from DDR pathways to determine cell fate (Kumar and Huberman, 2009). Importantly, many cancers exhibit misregulation of DDR components, leading to increased mutagenesis and therapeutic resistance (Maya-Mendoza et al., 2007).

Overall, the interplay between DNA damage, repair pathways, and cellular responses determines genomic stability and cell fate.

2.4. DNA damage tolerance and translesion synthesis

During DNA replication, the high-fidelity nature of replicative DNA polymerases becomes a double-edged sword. These polymerases, as we have discussed before, possess extremely stringent active sites that enforce accurate base pairing, however, the downside of their high fidelity is that the replication fork stalls when it encounters a DNA lesion. In fact, the lesion prevents the correct pairing of the incoming dNTP, thereby blocking conformational selection and at the same time, by distorting the DNA template, it also interferes with chemical selection.

Replication fork stalling poses a severe threat to genome integrity. Attempting to excise a lesion in single-stranded DNA at a stalled fork could result in double-strand breaks, leading to fork collapse. To circumvent this, cells employ DNA Damage Tolerance (DDT) strategies that enable replication

to continue past lesions without their immediate removal. DDT mechanisms include two primary pathways: template switching (TS) and translesion synthesis (TLS) (Gao, 2017).

The sliding clamp PCNA plays a key role in DNA Damage Tolerance (DDT) regulation, as its post-translational modifications function as molecular signals that determine which tolerance pathway the cell engages: mono-ubiquitylation of PCNA at lysine 164 (K164) signals TLS engagement, while further K63-linked polyubiquitylation activates TS.

TLS is a conserved mechanism where specialized low-fidelity DNA polymerases replicate across damaged DNA. These TLS polymerases—Polη, Polι, Polκ, Rev1 (Y-family), and Polζ (B-family)—are structurally distinct from replicative polymerases. Their catalytic domains are more open, with shorter "finger" and "thumb" regions and an additional domain known as “little finger” or PAD (Polymerase-Associated Domain) at the C-terminus (Figure 7A). PAD mediates the interaction between the TLS polymerase and the damaged DNA site, determining the recruitment specificity of the most appropriate TLS polymerase based on the type of lesion present (Boudsocq et al., 2004). This configuration allows them to accommodate bulky or mismatched lesions in the template strand. However, their relaxed specificity also makes them intrinsically error-prone, and they lack 3'-5' exonuclease activity for proofreading (Livneh et al., 2010). Although all TLS polymerases are less accurate than replicative polymerases, each TLS polymerase possesses unique features that enable it to accurately replicate specific types of lesions to which it is more suited.

The engagement of TLS polymerases is tightly regulated to minimize mutagenesis. One major control point is the mono-ubiquitylation of PCNA at lysine 164. When the presence of DNA damage arrests the replication fork and RPAs bind the ssDNA, Rad6 and Rad18 are recruited. These two enzymes are respectively E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) ubiquitination enzymes. The process begins with an E1 enzyme (Uba1 in humans) that activates ubiquitin, a small regulatory protein, through an ATP-dependent mechanism. E1 forms a thioester bond, a high energy bond, with ubiquitin, which is then transferred to Rad6 (E2). Rad6 transports activated ubiquitin, preparing it for the attachment to the target, while Rad18 (E3) recognize the target (PCNA) and facilitates the attachment of ubiquitin in cooperation with Rad6 on K164. Monoubiquitinated PCNA appears to have a higher affinity for TLS polymerases that classic replicative polymerases, so TLS are recruited on the damaged site. These polymerases interact with PCNA through specialized domains: PIP (PCNA-interacting peptide), UBZ (ubiquitin-binding zinc finger), or UBM (ubiquitin-binding motif) (Hoege et al., 2002) (Boehm et al., 2016).

The TLS process can occur in two different modalities: in one-step or two-steps bypass.

In the former mechanism, a single TLS polymerase is capable of inserting the nucleotide opposite to the lesion, bypassing the damage, and proceeding with DNA synthesis past the damaged site. A

typical example is the behaviour of Polη with Cyclobutane Pyrimidine Dimers (CPDs) induced by UV. Polη is in fact capable of replicating these types of lesions with a low error frequency (Johnson et al., 1999). However, not all TLS polymerases have this high accuracy: for example, Polι and Polκ, are capable of bypassing different types of damage, inserting nucleotides with a higher probability of errors compared to Polη (Tissier et al., 2000).

One-step TLS is generally more rapid and efficient, but it's limited to damage that can be bypassed with a certain accuracy by a single TLS polymerase (Friedberg et al., 2005).

In numerous cases, instead, it is necessary to rely on the two-step TLS mechanism, in which two polymerases are recruited. Generally, a TLS belonging to the Y family inserts the nucleotide opposite the damaged site, then another polymerase, usually Polζ, extends the DNA strand past the damage. This mechanism is denominated "polymerase switch" (Livneh et al., 2010) (Johnson et al., 2000). Polζ present lower processivity and higher fidelity than the Y-family of TLS polymerases and its role is crucial for TLS, because it allows the completion of DNA synthesis after the first step done by the Y family polymerase, ensuring replication progression (Sharma and Canman, 2012). Replicative polymerases then resume synthesis downstream of the damage (Figure 7B).

Alternative TLS mechanisms can bypass PCNA ubiquitylation entirely, such as the Rev1 scaffold pathway, where Rev1 acts as a bridge to recruit and organize other TLS polymerases (Powers and Washington, 2018).

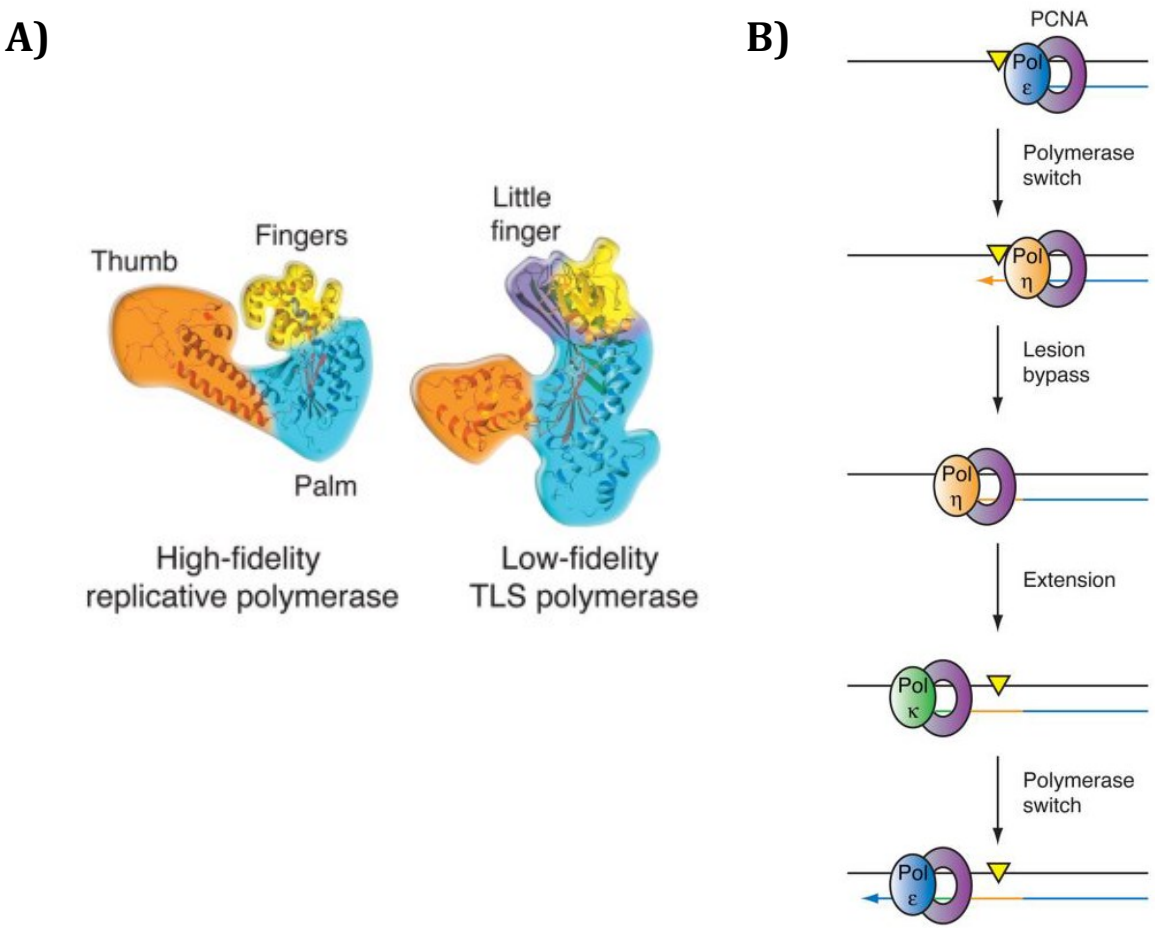

Figure 7
Structural and functional features of TLS polymerases.
(A) Structures of a high-fidelity replicative DNA polymerase and a low-fidelity TLS polymerase. We can observe their similarities and their differences. Both polymerases present "thumb", "fingers" and "palm" domains. The active site of the TLS polymerase (the palm domain, in light blue) is visibly more spacious. In addition, TLS polymerases present a "little finger domain", necessary to mediate the interaction with the damaged DNA site.
(B) Model of "Polymerase Switching" during TLS. Replicative DNA polymerases (blue) stall at DNA template lesions. In the first polymerase switch, a TLS-specialized DNA polymerase (yellow) is recruited to the PCNA sliding clamp (purple) at the stalled fork and proceeds with replication across the lesion (yellow triangle). The "patch" synthesized by the TLS polymerase is then extended either by the same TLS polymerase or by another one (green). The final polymerase switch restores the replicative DNA polymerase to the template, and DNA synthesis resumes.
From: Chang, D. J., & Cimprich, K. A. (2009). DNA damage tolerance: when it's OK to make mistakes. *Nature chemical biology*, *5*(2), 82–90. https://doi.org/10.1038/nchembio.139

When PCNA is polyubiquitinated on lysine 164, it promotes the activation of the error-free template switching (TS) pathway, although the exact details of how this process works remain unclear (Moldovan et al., 2007). In yeast, polyubiquitination on K164 plays a major role in the TS pathway, which is a Rad5 mediated repair, but recent studies seem to underlining that in vertebrate cells monoubiquitylation of PCNA is more relevant even for TS.

In budding yeast, PCNA polyubiquitination on K164 is carried out by Rad5 associated with Ubc13-Mms2, which act respectively as an E3 (ubiquitination enzyme) and an E2 (ubiquitination-conjugating enzyme). Rad5 polyubiquitinates mono-ubiquitinated PCNA, already recruited in the stalled replication fork, by adding K63 poly-ubiquitin linkages. Rad5 is not only involved in polyubiquitination: it was demonstrated that it has also been demonstrated to interact with Rad18 and TLS polymerases (Ulrich and Jentsch, 2000). This seems to suggest that Rad5 could be involved in the direct recruitment of TLS polymerases to stalled replication forks (Gallo et al., 2019). Rad5 contains several conserved domains; among them, the HIRAN domain is involved in the recognition of ssDNA 3'-OH present at stalled replication forks and it is essential for the interaction with PCNA (Gallo and Brown, 2019) (Fan et al., 2018). In human cells, two Rad5 homologues are present, HLTF and SHPRH. Both proteins are capable of carrying out PCNA poly-ubiquitination, but differ in the way they act and in whether they are recruited depending on the type of damage. HLTF is more similar to Rad5, in fact it was demonstrated that it possesses a HIRAN domain and it has the same ability to recognise ssDNA 3'-OH (Kile et al., 2015). Instead, SHPRH lacks a HIRAN domain, but it possesses ubiquitin ligase activity allowing PCNA polyubitination in association with Ubc-Mm2 (Unk et al., 2006). Furthermore, it possesses a PHD domain, that allow SHPRH to bind chromatin and various E2 enzymes permitting to ubiquitinate histones and self-regulate its own activity (Bruhl et al., 2019). Both HLTF and SHPRH can bind Rad18 and the determination of TLS polymerase recruited depends on the Rad18 complex that is formed (Lin at al., 2011). HLTF seems fundamental in UV damage induced responses, preferring the recruitment of Polη, while SHPRH seems fundamental in MMS (Methyl methanesulfonate, an alkylating agent) induced damage responses, primarily promoting Polκ recruitment and Polη in lesser amount (Kondratick et al., 2020)
Current models suggest that TS involves strand invasion, where the newly synthesized strand pairs with the undamaged sister chromatid. This interaction forms a structure known as a sister chromatid junction (SCJ), which serves as a template for filling the gap left by the damage. The SCJ is then resolved, resulting in two intact double-stranded DNA molecules and successful bypass of the lesion. Template switching is considered an error-free DDT pathway. Rather than directly replicating across a lesion, the newly synthesized DNA strand uses the undamaged sister chromatid as a template, copying information through a recombination-like mechanism. This process avoids the introduction of mutations but requires a homologous sequence and more complex coordination.

Ubiquitination, however, is not the only post-translational modification that occurs on PCNA; in fact, this protein is also SUMOylated. SUMOylation consists of the attachment of one or more SUMO proteins (Small Ubiquitin-like Modifier), small proteins similar to ubiquitin, to the lysine of a target

protein. It has been observed that, in yeast, PCNA is SUMOylated by the combined action of Ubc9 (E2) and Siz2 (E3), predominantly on the amino acid residue K164 and, to a lesser extent, also on K127 (Hoege et al. 2002) during unperturbed S phase. SUMOylated PCNA can interact with the helicase Srs2, which is necessary to inhibit the activation of homologous recombination, one of the main mechanisms for repairing double-strand breaks (DSB) when this kind of repair system is not needed. In humans, the process is similar: the homologs of E2 and E3 SUMOylate the amino acid residues K164 and K254 (Gali et al., 2012), which can then interact with the orthologs of Srs2: PARi (PCNA-Associated Recombination Inhibitor) and FBH1 (F-Box DNA Helicase 1) (Moldovan et al., 2012) (Bacquin et al., 2013).

2.5. DNA polymerase η

The POLH gene, encoding Polη in humans, is located on chromosome 6p21.1-6p12 and consists of 11 exons, one of which is untranslated. The corresponding cDNA encodes a protein composed of 713 amino acids, with an estimated molecular weight of approximately 78.4 kDa (Yuasa et al., 2000). Figure 8 shows the position of the catalytic site subdomains "palm," "finger," "thumb," and "little finger" within the primary structure, as well as the protein interaction sites.

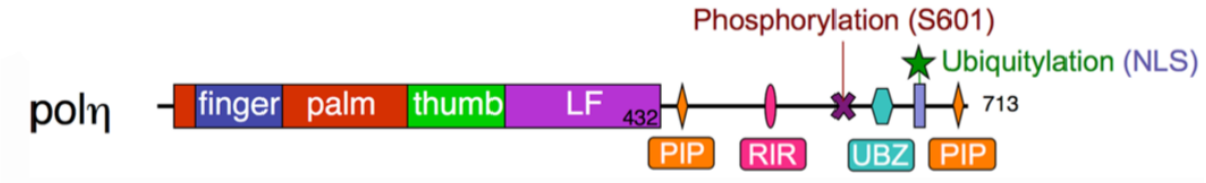

Figure 8
Primary structure of human DNA polymerase η. The different subdomains of the catalytic site "finger," "palm," "thumb," and "little finger" ("LF") are shown, along with the regulatory regions. "PIP" stands for PCNA-interacting peptide, "RIR" for Rev1-interacting region, "NLS" for nuclear localization signal, and "UBZ" for the ubiquitin-binding zinc finger.
From: Yang W. 2014 "An overview of Y-Family DNA polymerases and a case study of human DNA polymerase η." *Biochemistry*. 6;53(17):2793–803

The complete 3D structure of Polη has not yet been fully resolved, primarily due to the intrinsic flexibility and disorder of its C-terminal regions. However, the structure of the catalytic site has been determined and it is associated is also associated with two metal ions, usually Mg^{2+} (Yang et al., 2006) (Figure 9).

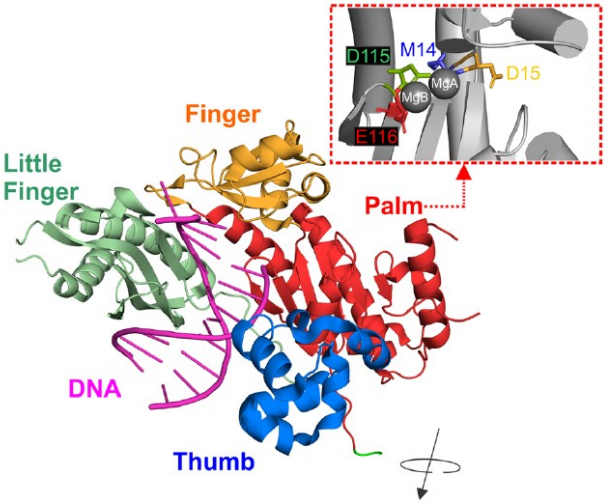

Figure 9
3D structure of the catalytic site of Polη. The subdomains of the catalytic site can be observed: "thumb," "palm," "finger," and "little finger." The inset provides a magnification of the Mg2+ ions and their interactions with the amino acids of the "palm" domain of Polη.
From: Feltes, B. C., & Menck, C. F. M. (2022). Current state of knowledge of human DNA polymerase eta protein structure and disease-causing mutations. Mutation research. Reviews in mutation research, 790, 108436. Advance online publication. https://doi.org/10.1016/j.mrrev.2022.108436

Polη is a TLS polymerase that is particularly efficient in bypassing DNA damage induced by UV irradiation. Rad30 is the homologous protein in *S. cerevisiae* and in the presence of nucleotides, is able to replicate DNA even in the presence of a damaged template (Johnson et al., 1999).

The most common type of UV damage is represented by cyclobutane pyrimidine dimers (CPDs) and Polη is able to bypass them, especially those formed by thymine dimers. In fact, it incorporates A-A with an accuracy comparable to that of an unmodified T-T sequence (Johnson et al., 1999). The efficiency of Polη in CPDs repair is due to the conformation of its active site: crystallographic analyses have shown that the catalytic region (residues 1-432) is particularly spacious and can accommodate two covalently linked adjacent DNA bases, such as a thymine dimer (Biertuempfel et al., 2010) (Zhao et al., 2012). The same activity of translesion synthesis has been seen for other polymerases belonging to Y-family, like pol ι and pol κ (Johnson et al., 2000) (Ohashi et al., 2000). In this group of polymerases with TLS activity, only Polη can replicate this kind of damage in an error free manner.

Polη also bypasses other types of DNA damage, such as 8-oxo-deoxyguanosine (8-oxodG), which can be generated spontaneously as a byproduct of cell metabolism or can be generated by UVA irradiation. In respect to deoxyguanosine (dG), 8-oxoG contains an extra oxygen atom that can lead to transversion mutations of the type G>T. Recent experiments indicate that XP-V cells, which are Polη deficient, had increased G>T mutations, confirming that, *in vitro*, Polη can efficiently and accurately replicate DNA in presence of this type of lesions (McCulloch et al., 2009) (Shibutani et al., 1991).

2.6. DNA polymerase η regulation

Post-translational modifications play a crucial role in the regulation of TLS polymerases.

Several studies have shown that Polη is monoubiquitinated *in vivo* in the absence of damage. The main target of ubiquitination is Lys682, but using recombinant proteins mutated at this amino acid residue, it was shown that Polη ubiquitination still occurred. In fact, there are three other lysine residues (Lys686, Lys694, Lys709) near Lys682, and only the mutation of all four of these residues inhibits Polη ubiquitination (Bienko et al., 2010). This post-translational modification is carried out by PIRH2 (p53-Induced Protein With A RING-H2 Domain) (Jung et al., 2011). The amino acid residues involved in this modification are located at the nuclear localization signal (NLS) at the C-terminal end, adjacent to the PIP box. Polη ubiquitination is believed to lead to a conformational change in the C-terminus, which causes the ubiquitin bound to Polη to interact with the UBZ domain of the polymerase itself. In this way, the PIP domain is masked on one side, while the UBZ domain is made unavailable, resulting in preventing Polη from binding to both PCNA and ubiquitylated PCNA. Therefore, in the absence of damage, Polη cannot be efficiently recruited to the replication fork. However, when DNA damage is detected, Polη must be deubiquitinated to make the domains involved in binding to PCNA available again and thus be recruited to replace the replicative polymerase (Bienko et al., 2010). Subsequent studies have shown that USP7 directly functions as a DUB (deubiquitinating enzyme) specific for Polη (Qian et al., 2014).

After the completion of TLS, to promote its removal from the replication fork and facilitate the switch to a classical replicative polymerase, Polη can be polyubiquitinated by at least two different pathways: the first involving the E3 ligase MDM2, an oncogene acting as an important negative regulator of p53 (Jung et al., 2012); and the second involving the E3 ligase CRL4, (Ma et al., 2017), previously shown to target PIP box-bearing proteins for proteolysis (O'Connell and Harper, 2007).

Another modification that regulates Polη's binding to DNA is SUMOylation. It occurs through the action of PIAS1 (protein inhibitor of activated STAT1) in a RAD18-dependent process at the amino acid residue K163. Mutating the Lys163Arg residue demonstrated that Polη SUMOylation is not required for an efficient response to DNA damage at low UV doses, but at high doses, it is associated with its correct accumulation at damage sites. It is hypothesized that this post-translational modification is primarily involved in maintaining Polη near replication foci under non-stress or mild stress conditions, to be recruited when replicative polymerases encounter difficult-to-replicate sequences. It has also been observed that the SUMOylation of this residue is unrelated to Polη's ubiquitination (Despras et al., 2016). Recent studies have shown the presence of multiple damage-dependent SUMOylation sites, particularly nineteen lysines. Mutating all these SUMO sites abolishes Polη's multi-SUMOylation, but does not block foci formation following DNA damage or its ability

to bypass adducts, supporting the conclusion that SUMOylation represents an important level of modulation for Polη but is not a prerequisite for its activity. However, it seems that SUMOylation is an intrinsic mechanism of TLS-mediated damage bypass to limit the interaction between Polη and PCNA at damaged DNA to avoid harmful mutagenesis (Guerillon et al., 2020).

Finally, Polη can be phosphorylated, and this modification affects both its ability to bypass damage and its inactivation once the molecular event is completed. As observed by Chen and colleagues, Polη is a target of phosphorylation; indeed, both in untreated and UV-treated samples, the incorporation of 33P in immunoprecipitated Polη was observed, although with higher incorporation in the treated samples. Additionally, inhibition of the ATR kinase protein by siRNA led to a reduced accumulation of Polη at damaged sites (Chen et al., 2008). Subsequently, the role of ATR kinase in Polη phosphorylation was confirmed; this modification occurs on serine residue 601 (S601) and is induced by DNA damage. This modification is needed for efficient bypass of the damage (Goehler et al., 2011). It was also shown that the interaction with Rad18 and the presence of Polη's intact UBZ domain are required for the phosphorylation of S601 induced by DNA damage, which occurs only when the polymerase is recruited to chromatin. Indeed, it was observed that the expression of a Rad18 protein lacking the Polη binding domain (Watanabe et al., 2004) led to a strong decrease in phosphorylated Polη, and the same result was obtained by mutating Polη's UBZ domain, preventing its binding to ubiquitin. It was demonstrated that this type of phosphorylation is independent of the mono-ubiquitination of PCNA (Goehler et al., 2011).

S601 is not the only phosphorylation site on Polη; the enzyme can also be phosphorylated by CDK2 (Cyclin-Dependent Kinase 2) at serine 687 (S687). It was observed that inhibition of CDK2 led to a decrease in Polη phosphorylation on S687. Phosphorylation at this residue decreases in a time-dependent manner following UV damage (Bertoletti et al., 2017). Moreover, mutation of residue S687 confers mild UV sensitivity and makes damage bypass less efficient.

Although phosphorylation at S687 is important in the presence of DNA damage, it also plays a fundamental role in the absence of damage. CDK2 kinase, in fact, phosphorylates Polη at S687 in a cell cycle-dependent manner, regulating Polη stability during the cell cycle and allowing its accumulation during late S-phase and G2-phase, when TLS is critical for cell survival. In its absence, polymerase levels decrease, making the cells less efficient in replicating damaged DNA. Therefore, the phosphorylation of S687 by CDK2, along with the phosphorylation of S601 by ATR, makes Polη available and active to be recruited in case TLS activation is needed. Once the lesion is bypassed, the phosphorylation of S687 is removed, reducing stability and leading to a decrease in Polη, thus favoring the resumption of normal replication (Bertoletti et al., 2017).

2.7. Xeroderma Pigmentosum and Xeroderma Pigmentosum Variant

Xeroderma Pigmentosum (XP) is a rare autosomal recessive disorder characterized by sun sensitivity, skin and ocular abnormalities, and multiple malignant skin tumours.

XP is classified into eight complementation groups depending on the gene involved: from XPA to XPG and XPV. The gene products from XPA to XPG are involved in different steps of the NER system (Berneburg and Lehmann, 2001) in repairing lesions caused by UV radiation, such as cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone photoproducts (6-4 PPs) (Clingen et al., 1995).

However, it has been observed that patients affected by XPV (Xeroderma Pigmentosum Variant), while sharing the clinical features of other XP patients, have cells with normal NER activity but show a deficiency in some kind of Post Replication Repair (PRR) (Kannouche and Stary, 2003). In 1999, several research groups independently identified the XPV gene and discovered that its product is DNA polymerase η (Johnson et al., 1999) (Masutani et al., 1999).

XP mainly manifests with an increase in skin lesions, including a high frequency of skin cancers in sun-exposed areas. These lesions include hyperpigmentation with freckles and precancerous lesions such as actinic keratosis, which can develop into tumours. Compared to the general population, XP patients show a 10,000-fold higher incidence of non-melanoma skin cancers and a 2,000-fold higher incidence of melanoma (Kraemer et al., 1994). These symptoms are also present in XPV patients, although they tend to be milder and appear later. Usually, patients with XP-V are not particularly sensitive to sunlight during childhood, but their photosensitivity increases after 15-20 years, without manifesting real sunburn, and subsequently develop more skin tumours. However, the symptoms are less serious and appear later than patients with classic XP.

While some XP patients may develop neurological problems over the course of their lives, this is not observed in XPV patients. These severe neurological symptoms are most likely linked to defective transcription of damaged DNA (Kajitani et al., 2021), due to impairments in transcription-coupled repair, a sub-pathway of NER. Since XPV is associated with TLS deficiencies, this subtype does not present neurological problems.

Since the discovery of Polη gene as the gene responsible for XPV, various mutations associated with this disease were reported. These mutations include single nucleotide substitutions, small insertions or deletions that lead to frameshift or protein truncation (Broughton et al., 2002) (Gratchev et al., 2003) (Itoh et al., 2000) (Johnson et al., 1999) (Masutani et al., 1999). Larger mutations have also been reported in literature, such as break points situated in introns 4 and 9 that result in a deletion involving the intermediate exons, specifically from exon 5 to exon 9 (Guo et al., 2013) and a large deletion of 6kb involving exon 10 (Ben Rekaya and al., 2014).

More than 70 mutations of Polη gene have been documented in literature. Although many mutations causing XPV disease have been found in the catalytic site, specific mutation “hot spots” have not been identified. In fact, variants associated with XPV can be found throughout the entire protein, including the C-terminal regulatory region.

Initially, the most carefully studied Polη variants were clearly pathogenic, often represented by nonsense or frameshift mutations that significantly compromise the enzyme's function, particularly affecting the catalytic domain. However, there is a growing number of missense variants for which a precise functional assessment has not yet been possible. In the absence of solid experimental data, these mutations are classified as variants of uncertain significance (VUS).

Analysis of different variants of DNA-Polη highlights how mutations can distinctly influence enzymatic functionality, the ability to bypass UV-induced damage and, in some cases, be associated with specific tumour types.

The variants Leu11Pro, Arg93Pro and Arg111His, located in the Palm domain, with Arg93Pro and Arg111His involved in DNA binding, are all homozygous and associated with the variant form of xeroderma pigmentosum (XPV). Among these, Arg93Pro severely compromises cell survival after UV damage and caffeine treatment, suggesting the protein’s inability to bypass UV damage. This effect is attributable to the loss of arginine interaction with the phosphate group of the template filament in position -2, as well as to the interruption of the interaction between the finger and little finger domains, essential for maintaining the molecular splint structure that supports the template filament in the B conformation in the presence of UV lesions. Furthermore, the substitution with proline probably introduces a bending in the alpha-helix. Moreover, Arg93Pro variants is reported to be associated with different kind of tumours, including basal cell carcinomas (BCCs), squamous cell carcinomas (SCCs) and melanomas (Santiago et al., 2020) (Ricciardiello et al., 2024) (Opletalova et al., 2014).

Also, Arg111His, although not fully characterized at the functional and tumour level, is positioned close to the catalytic site and is involved in interactions with both the little finger domain and the template filament, suggesting a relevant structural impact: the substitution with histidine explains the appearance of the XPV phenotype (Biertümpfel et al., 2010).

Leu11Pro, on the other hand, although associated with XPV, is not currently linked to known tumours nor has it shown a clear phenotypic characterization.

The Ala117Pro and Thr122Pro variants, also located in the Palm domain, are highly evolutionarily conserved and linked to both XPV and melanoma. However, both are unable to bypass damage in XPV cell lines. In particular, Ala117Pro, located close to the catalytic site, alters the structure of the hydrophobic core, leading to unfavourable interactions (Ling et al., 2001). In particular, A117 is

located adjacent to the catalytic carboxylic acid E116 and its substitution with proline could generate steric hindrances with the carbonyl oxygen of V12 (Biertümpfel et al., 2010). Thr122Pro, a mutation combined with a frameshift, compromises the structural stability of the Palm domain, and is conserved in several species (Broughton et al., 2002).

Trp174Cys represents a special case: although it is a mutation in the catalytic Palm domain, it allows cells to bypass UV damage. However, the substitution from a hydrophobic to a hydrophilic amino acid could negatively affect the protein conformation. Lys220Glu, present in compound heterozygosity with a frameshift, also retains UV bypass ability and has been associated with melanoma, BCC and SCC (Inui et al., 2008).

In the Thumb domain, the variants Gly263Val, Val266Asp and Gly295Arg are present. The first two are homozygous mutations and do not allow the damage to be overcome, probably due to alterations in primer binding or the stability of the DNA-protein interface. In particular, Gly263Val causes steric hindrance with the carbonyl oxygens of L258, interfering with the binding to the primer by the GGK motif (aa 259–261) (Biertümpfel et al., 2010). Val266Asp, similarly, does not show TLS activity in XPV cell extracts nor does it allow survival after exposure to UV and caffeine (Ricciardiello et al., 2024). Gly295Arg mutants are present in compound heterozygosity with a frameshift and are associated with BCC, SCC and melanoma.

In the Little Finger domain, we find the mutations Cys321Phe and Arg361Ser, both homozygous mutations associated with XPV but still lacking a detailed functional characterization. In particular, Arg361Ser could compromise the stability of the structure for template binding, relaxing the DNA-protein interface and breaking the molecular splint (Broughton et al., 2002) (Biertümpfel et al., 2010).

The Lys535Glu and Lys589Thr variants, located in regulatory regions involved in binding to REV1 and RAD18 respectively, while maintaining a catalytic activity similar to the wild-type *in vitro*, fail to effectively support damage bypass *in vivo*. This underlines the importance of regulatory interactions in the TLS process. (Itoh et al., 2000) (Tanioka et al., 2007).

Finally, the Met595Val variant, located in the C-terminal region and present in heterozygosity, is not associated with XPV, but has been identified in patients affected by melanoma and associated skin carcinomas. Although direct functional data are lacking, it is hypothesized that this substitution, being distant from the catalytic site and relatively conservative, may have a less detrimental impact on enzymatic activity than other XPV-associated mutations (Di Lucca et al., 2009) (Ricciardiello et al., 2024).

Although some information about these variants is present in the literature, many of them require further investigation and functional characterization to understand their impact on the DNA damage bypass process, the cell cycle, and the Polη polymerase itself. Full characterization of these mutations

is not only essential for scientific purposes but also has relevant implications in clinical practice. It is important to remember that every mutation we have cited has been reported in human patients; therefore, a better understanding of the dynamics of these variants can be crucial for improving the diagnosis, prognosis, and treatment of associated human pathologies, such as xeroderma pigmentosum variant, in our case.

3. AIM OF THE WORK

In recent years, a growing number of missense variants of polymerase η (Polη) have emerged, the function of which has not yet been clearly defined. Many of these variants are now classified as variants of uncertain significance (VUS), as there is a lack of sufficient experimental evidence for their correct interpretation according to the ACMG guidelines. Although some of them have already been reported in the literature, their characterization has often been performed on patient-derived cells. This type of approach, although useful, exposes to significant variability due to the cellular context of origin, making it difficult to attribute with certainty the observed effects to the single mutation. For this reason, the pathogenicity of the variants could not be clearly assigned as per ACMG guidelines.

The aim of this work is therefore to functionally and independently characterize fifteen missense variants Polη *in vitro* and *in vivo*, by taking advantage of the experimental framework that we used previously to analyse the Thr191Pro mutation in our laboratory (Ricciardiello et al., 2024).

This approach will hopefully allow to precisely isolate the functional impact of each mutation and represents a robust strategy to contribute to the correct classification of variants according to rigorous and reproducible criteria, helping patients and clinicians in correctly diagnosing Xeroderrma Pigmentosus Variant.

4. MATERIALS AND METHODS

4.1. Cell Lines and Culture Conditions

All experiments were conducted using XP30RO human fibroblast cell line, immortalized with SV40. The XP30RO line is derived from a patient with Xeroderma Pigmentosum Variant (XPV) and carries a homozygous deletion in the POLH gene, resulting in a truncated Polη protein consisting of only 42 amino acids, rendering the line completely deficient for this polymerase.

Isogenic XP30RO eGFPpolη, overexpressing eGFP-tagged wild-type or mutated Polη, were also used.

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (EuroClone) supplemented with 10% fetal bovine serum (FBS) (Gibco), 200 mM L-glutamine (Lonza), and 10 mg/mL gentamicin (Lonza). XP30RO cells were grown in 75 cm^2 flasks at 37°C in a 5% CO_2 atmosphere. The XP30RO expressing wild type or mutated eGFPpolη cell lines were cultured under the same conditions with the addition of 0.5 mg/mL neomycin (G418) (EuroClone) to maintain selection and ensure stable selection of eGFPpolη.

4.2. Plasmid and cloning

Stable XP30RO cell lines expressing Polη variants were established using the N-terminal eGFP-tagged full-length cDNA of Polη, which was cloned into the peGFP-C3 vector (Kannouche et al., 2001). The pCDNA3.1-Polη construct was previously described in the same study.

Plasmids were mutagenized using the QuickChange site directed mutagenesis kit (Stratagene) according to the protocol provided.

Chemically competent TOP10 *E. coli* cells were transformed with the eGFP-tagged constructs. Transformation was carried out by heat shocking at 42°C followed immediately by cooling on ice. Transformed cells were plated on LB-agar with 50 µg/ml kanamycin (LB-KAN). Individual colonies were expanded and cultured in liquid LB-KAN medium. Plasmid DNA was extracted using the Macherey Nagel NucleoBond Xtra MIDI or MINI kit as described by the manufacturer. Finally, the entire coding sequence of Polη was sequenced to check for the introduction of the intended mutations.

4.3. Plasmid transfection

Cells were seeded in 3.5 cm dishes. After 24h, the culture medium was aspirated, and 2 mL of Opti-MEM medium (Gibco, ThermoFisher) was added. A transfection mix was prepared with the following quantities for 3.5 cm dish: 96 µL Opti-MEM, 1 µg of plasmid, and 3 µL of ViaFect

transfection reagent (Promega). The mix was accurately vortexed and incubated at room temperature for 15 minutes, then added drop by drop to each dish.
The plates were then incubated for 4 hours, after which the medium was replaced with 2 mL of complete medium, as previously described.

4.4. Stable Cell Line Generation

Stable XP30RO eGFPpolη cell lines were obtained by integrating different eGFPpolη alleles. Briefly, 3×10^5 cells were transfected with the plasmid of interest using Viafect (Promega), following the manufacturer's instructions. After 24 hours, cells were diluted into four 10 cm dishes in DMEM supplemented with 10% FBS. After an additional 24 hours, 1 mg/mL G418 was added to the medium. Colonies that survived after 15 days were selected using cloning disks (Sigma Aldrich), expanded, and screened for eGFPpolη expression by western blot and flow cytometry.
Cells expressing eGFPpolη were further sorted using a Bio-Rad S3 Cell Sorter (FACS) to monitor Polη expression levels.

4.5. UV radiation

Cells are seeded in 3.5 cm plates. At the time of irradiation, in our experiments 24h after the seeding, the UV lamp was measured using a UV meter to determine the required exposure time for delivering the correct UV dose to the cells. The plates were retrieved from the incubator, the medium was aspirated, and the cells were washed with 1X PBS. After removing the PBS, the cells were dry irradiated with a UV-C lamp at the required dose for the experiment. In the crystal violet assay, the samples were irradiated, or mock treated, with 2 J/m² and 4 J/m² and analysed after 72h, while in the Propidium Iodide assay, cells were exposed to 10 J/m² and processed after 24h.
Following UV irradiation, fresh medium was added in the dishes and cells were placed again in the incubator.
All cells included in the experiments undergo the same treatment, including the negative control cells, with the exception of UV irradiation. The plates are then placed back in the incubator at 37°C for further processing.

4.6. Total Protein Extraction

The culture medium was aspirated from the plates, followed by a quick 1X PBS wash, which was aspirated immediately. Protein extraction was performed by adding an appropriate volume of 1X Laemmli Buffer to the sample, typically 30-90 µl for a 3.5 cm dish. The Laemmli quantity is related

to every sample's cell density, to ideally have the same final concentration of cells in all samples. This is useful to have balanced and better interpretable results.
The sample was collected by scraping and subsequently sonicated for three cycles of 30 seconds. The lysates were then boiled at 95°C for 10 minutes to denature the extracted proteins. The protein extracts can either be stored at -20°C or directly loaded onto an SDS-PAGE gel.

1X Laemmli Buffer Composition
Tris-HCl pH 6.8 – 50 mM
Glycerol – 5%
SDS – 1%
Dithiothreitol (DTT) – 100 mM
Bromophenol Blue – 0.05%
H_2O – to volume

4.7. SDS-PAGE

Protein extracts are loaded onto a denaturing SDS-PAGE gel containing either 8% or 10% polyacrylamide. The gel consists of two layers: the stacking gel, which contains the loading wells and ensures uniform alignment of the samples, and the running gel, located below, which separates proteins based on their molecular weight.
The gel is prepared by pouring the liquid running gel mixture between two glass plates with a 1 mm spacer; once solidified, the stacking gel is added on top along with the appropriate comb to create the loading wells. The electrophoresis apparatus is filled with 1X Running Buffer to the appropriate level. Four microliters of Sharp Mass VI protein marker (EuroClone) and the appropriate volume of samples are loaded into the wells. Initially, a voltage of 90 V is applied to allow the samples to align. After the samples pass through the stacking gel, electrophoresis is performed at a constant voltage of 180 V for approximately one hour.

Running Gel 8%	ml	**Running Gel 10%**	ml	**Stacking gel**	ml
H2O	2.3	H2O	1.9	H2O	1.4
30% Acrylamide	1.3	30% Acrylamide	1.7	30% Acrylamide	0.33
1.5M Tris HCl pH 8.8	1.3	1.5M Tris HCl pH 8.8	1.3	1.5M Tris HCl pH 6.8	0.25
SDS 10%	0.05	SDS 10%	0.05	SDS 10%	0.02
APS 10%	0.05	APS 10%	0.05	APS 10%	0.02
TEMED	0.003	TEMED	0.002	TEMED	0.002
Total Volume	5	Total Volume	5		2

SDS-PAGE Running Buffer 1X, pH 8.3, 1 L	Quantities
Glycine 192 mM	1440 g
Tris base 25 mM	303 g
SDS 0.1%	100 g
H2O	to 1L

4.8. Western blot

Proteins from the SDS-PAGE gel were transferred onto Trans-Blot Turbo nitrocellulose membranes (Bio-Rad) using Trans-Blot Turbo transfer cassettes (Bio-Rad), applying an electric field of 2.5 mA for 10 minutes. The efficiency of the gel run and protein transfer was verified using Ponceau S staining.

To prevent nonspecific antigen–antibody interactions, membranes were blocked in 5% milk in 1× PBS with 0.1% Tween-20 for 1 hour.

Three 10-minute washes were then performed using 1× PBS with 0.1% Tween-20, after which the membranes were incubated overnight at 4°C under constant agitation with primary antibodies. The following primary antibodies were used in our experiments:

- α Polη (1:1000 Cell Signaling 13848)
- α Vimentin (1:20000 Sigma V6389)

All primary antibodies were diluted in a solution of 5% milk in 1× PBS with 0.1% Tween-20 and 0.01% NaN_3.

Three additional 10-minute washes were performed using 1× PBS with 0.1% Tween-20, after which the membranes were incubated with secondary antibodies conjugated to peroxidase, selected based on the species of the primary antibody:

- Goat anti-rabbit 1:5000 (Jackson ImmunoResearch)

- Goat anti-mouse 1:5000 (Jackson ImmunoResearch)

All secondary antibodies were diluted in 1× PBS with 0.1% Tween-20.

After a 2-minute incubation with either Westar nova 2.0 (Cyanagen) or Westar Antares (Cyanagen) substrates, proteins were visualized using the ImageQuant LAS 500 system (GE). Since the signal intensity is directly proportional to the amount of protein, quantification was performed using ImageQuant TL software.

4.9. *In vitro* coupled transcription and translation (TnT)

The TnT T7 Quick Coupled Transcription/Translation System (Promega) was used according to the manufacturer's instructions. The TnT reaction was performed by incubating rabbit reticulocyte lysates with methionine and pCDNA3.1 plasmids, previously obtained through E. coli cultures and encoding for either wild-type or mutated Polη, for 90 minutes at 30°C in the thermomixer.

An aliquot of 1 µl was taken from each sample to verify the presence of Polη through western blot analysis. The remaining sample was frozen in liquid nitrogen and stored at -80°C.

4.10. Primer extension assay

Primer extension assays were performed using equimolar quantities of Polη (0.1 pmol). Samples derived from the TnT reaction were divided into two aliquots: one aliquot was used to perform the primer extension assay either with a 30-mer template containing a thymidine dimer (TT) at position +13 annealed with a 5' FAM-labelled 16-mer or with a 51-mer damaged template containing a thymidine dimer (TT) at position +20, annealed with a 5' FAM-labelled 31-mer; the second aliquot was used to perform the primer extension assay with the undamaged control template.

5′ – CACTGACTGTATGATG – 3′

3′ – GTGACTGACATACTACTTCTACGACTGCTC – 5′

5′ – GTCCGGTAGTTCTGTTGTGGGTGGAGGTGAC – 3′

3′ – CAGGCCATCAAGACAACACCCACCTCCACTGTTCTCACACTATCTCACACT – 5′

The primer extension assay was carried out in a total volume of 10 µl using a replication buffer:

Replication buffer
40 mM Tris-Cl pH 8
5 mM $MgCl_2$
10 mM DTT
0,25 mg/ml acetylated BSA
60 mM KCl
2,5% glycerol

dNTPs were added to a final concentration of 100 µM. The reaction mixture was incubated at 37°C for 15 minutes and then stopped by adding a loading buffer (98% deionized formamide, 10 mM EDTA pH 8, 0.025% xylene cyanol, and 0.025% bromophenol blue).
Samples were denatured at 95°C for 5 minutes and immediately chilled on ice. Reaction products were separated on a 15% acrylamide gel containing 7 M urea and subsequently visualized using a Typhoon imager (GE HealthCare).

4.11. Cristal violet assay

For every cell line, nine 3.5mm dishes were seeded with 80000 cells and placed in the incubator. A dish containing only 10% FBS MEM was also prepared; this will serve as a "blank" for the experiment. After 24h cells were UV irradiated at 2 or 4 J/ m^2 as previously descripted and 10% FBS MEM medium containing 75µg/ml caffein was added. After 72h, dishes were washed in PBS and incubated with crystal violet solution for 20' at RT. Dishes were then washed with tap water and were left to dry for at least 30 minutes. Dishes were incubated for 20' with 1ml of lysis buffer. Afterwards a multiwell was prepared and 100µl of every sample was added each in a well. Since the dye is proportional to the number of cells that survived the treatment, absorbance at 560nm is evaluated and data are analysed.

Crystal violet solution
1g crystal violet powder
160ml H2O
40ml Methanol

Lysis buffer
5,88g sodium citrate
HCl to adjust the pH to 4,2
H2O to obtain 100ml of total volume
100ml 50% EtOH

4.12. Flow cytometry

150000 cells were seeded in 3.5mm dishes and placed in the incubator. After 24h cells were UV irradiated at 10J/ m^2, as previously descripted, and re-incubated.

After another 24h dishes were quickly washed with PBS and trypsin, then incubated 5' at 37°. 500µl of PBS 1x was added to each sample. Cells were resuspended and 450µl of each sample were added drop by drop in the related tube containing cold EtOH while vortexed.

When tubes needed to be analysed were retrieved from 4°C and centrifugated at 2000rpm for 5'. Supernatant is then removed and a mix containing 500µl PBS 1x + 0.1% Tween, 25µl RNAseA (10mg/ml) and 25µl Propidium Iodide (1mg/ml) is added to each sample. After the incubation at 37°C for 15 min, each sample was filtered and DNA content was evaluated with Bio-Rad S3 Fluorescence Automated Cell Sorter (FACS).

5. RESULTS

5.1. *In silico* analysis of Polη missense mutations

The role of DNA polymerase η in replicating damaged DNA was first discovered by investigating the mutations causing the disease xeroderma pigmentosum variant (XPV). The patients with XPV, as previously mentioned, are extremely sensitive to sunlight and are subject to skin cancer with high frequency due to a nonfunctional Polη.

Understandably, a number of pathogenic variants of Polη were the first to be characterized and turned out to be either loss of function truncations or frameshift mutations, mainly affecting the catalytic domain. Nonetheless, there are many missense variants that are not reported as pathogenic or likely pathogenic due to the lack of functional validation. These variants are referred to as variants of uncertain significance (VUS).

As we can see in Figure 10, using the Alphafold model, it is possible to predict the impact of amino acid substitutions across the primary sequence. Although the model is generally accurate, it is important to underline that these predictions necessitate experimental validation to be considered clinically and prognostically meaningful.

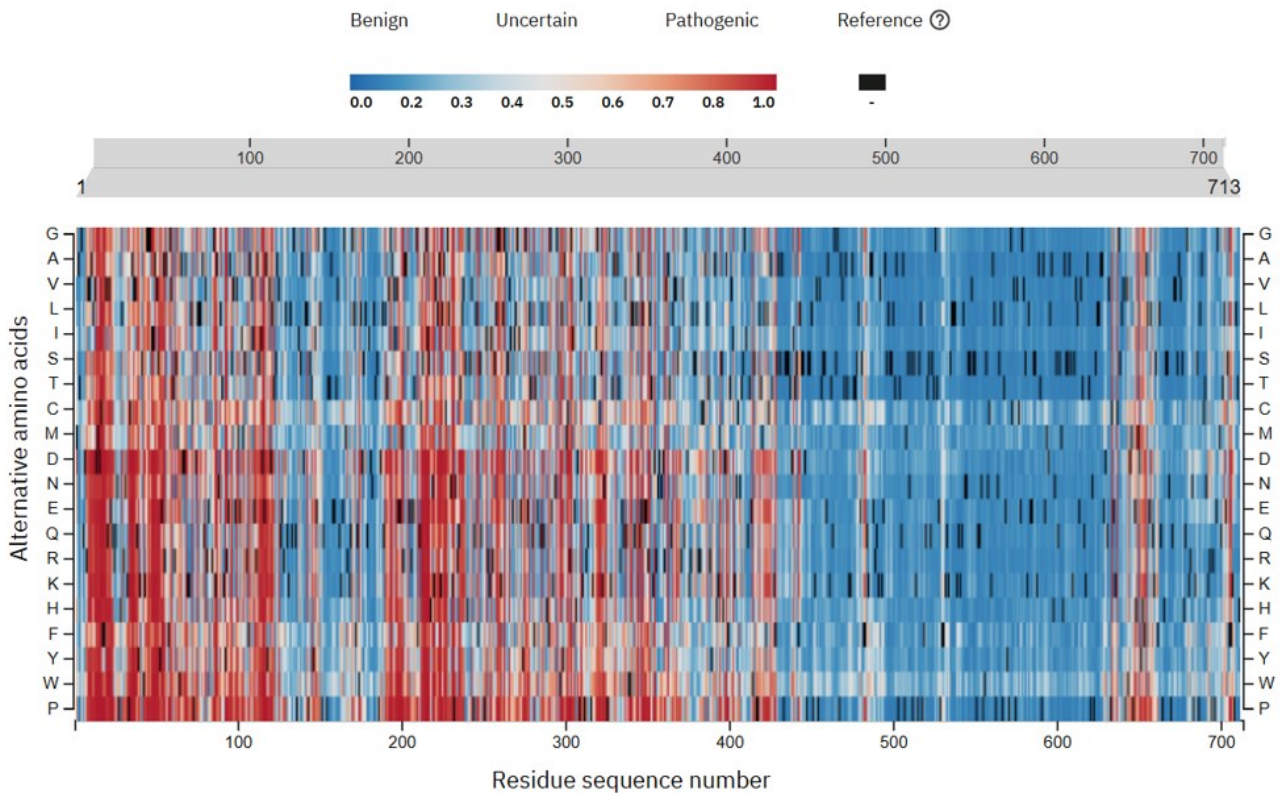

Figure 10
Representation of AlphaMissense Pathogenicity Heatmap of DNA-Polη. It can be observed the prediction of the impact of amino acids substitutions throughout the primary sequence of DNA-Polη.
From: https://alphafold.ebi.ac.uk/entry/Q9Y253

In our laboratory *in vivo* and *in vitro* approaches have allowed us to conduct the first functional molecular characterization of Polη missense variant, upgrading Polη Thr191Pro from "VUS" to "likely pathogenic mutant" (Ricciardiello et al., 2024).

Polη variant	**Zygosity**	**Domain**	**XPV**	**Tumor type**	**Damage Bypass in XPV cell lines**	**Alphamissense Score/Effect**
Leu11Pro	Homozygous	Palm	Yes	None	Not characterized	0.9633/ Likely Pathogenic
Arg93Pro	Homozygous	Palm (DNA-binding)	Yes	BCC, SCC, melanoma	No	0.9921/ Likely Pathogenic
Arg111His	Homozygous	Palm (DNA-binding)	Yes	Not characterized	Not characterized	0.4529/ Uncertain
Ala117Pro	Homozygous	Palm	Yes	melanoma	No	0.9917/ Likely Pathogenic
Thr122Pro	Compound heterozygous with frameshift	Palm	Yes	Not characterized	Not characterized	0.7548/ Likely Pathogenic
Trp174Cys	Homozygous	Palm	Yes	melanoma	Yes	0.9287/ Likely Pathogenic
Lys220Glu	Compound heterozygous with frameshift	Palm	Yes	BCC, SCC, melanoma	Yes	0.9617/ Likely Pathogenic
Gly263Val	Homozygous	Thumb	Yes	Not characterized	No	0.9202/ Likely Pathogenic

Val266Asp	Homozygous	Thumb	Yes	Not characterized	No	0.9857/ Likely Pathogenic
Gly295Arg	Compound heterozygous with frameshift	Thumb	Yes	BCC, SCC, melanoma	Not characterized	0.9857/ Likely Pathogenic
Cys321Phe	Homozygous	Little finger	Yes	None	Not characterized	0.798/ Likely Pathogenic
Arg361Ser	Homozygous	Little finger	Yes	Not characterized	Not characterized	0.9693/ Likely Pathogenic
Lys535Glu	Compound heterozygous with Lys589Thr	C-terminal	Yes	None	No	0.0985/ Likely Benign
Met595Val	Heterozygous	C-terminal	No	melanoma	Not characterized	0.0757/ Likely Benign

Table 1
Overview of the main naturally occurring missense variants of Polη that lack functional characterization. Adapted and modified from: Ricciardiello, Roberto et al. "Homozygous substitution of threonine 191 by proline in polymerase η causes Xeroderma pigmentosum variant." *Scientific reports* vol. 14,1 1117. 11 Jan. 2024, doi:10.1038/s41598-023-51120-1

In Table 1 it is possible to observe a summary of the main naturally occurring Polη missense variants that are associated with XPV and are currently lacking functional validation.

5.2. Testing catalytic activity and CPD damage bypass of Polη variants

The first step of the analysis involved the evaluation of the enzymatic activity of the mutant variants of Polη. For this purpose, each mutation was inserted into a plasmid vector used in an *in vitro* transcription and translation (TnT) system based on reticulocyte extract.
The quantity of the synthesized proteins was then measured by western blot (Figure 11), using 0.5 ng and 5 ng of purified Polη as a quantitative reference (lanes 9 and 11).

The results showed that most of the variants are expressed in similar amounts, with the exception of the Gly295Arg mutation, whose expression is significantly reduced (lane 15). For this reason, this mutant was not carried out in further experiments.

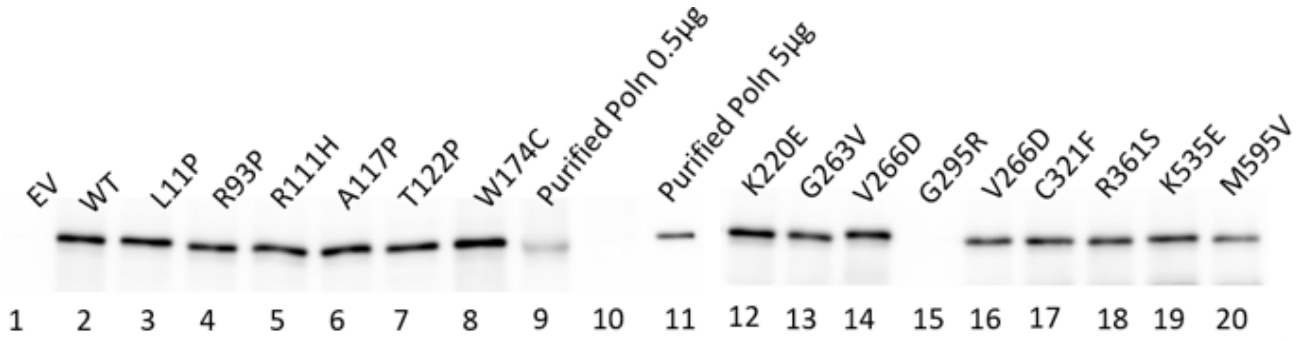

Figure 11
Western Blot representing the *in vitro* transcription and translation reaction of mutated Polη. As we can observe, the western blot confirms the presence of Polη in all samples, except for the G295R variant. The samples, probed with an anti-Polη antibody, were compared to purified Polη used as a reference control.

Once established that Polη is present, a primer extension assay can be performed to test the catalytic activity of Polη mutants and their capability of bypassing CPD damage.

The primer extension assay is a technique used to analyse the activity of DNA polymerases, with particular reference to their ability to extend a primer using a DNA template strand, even in the presence of lesions. This type of assay is particularly useful in the study of polymerases involved in translesion synthesis (TLS), such as Polη, as it allows to directly evaluate their efficiency in bypassing specific DNA damages, such as those induced by UV-C rays.

The principle underlying the assay consists in the use of a substrate consisting of an oligonucleotide (primer), labelled at one end with a fluorochrome (in our assay, FAM), hybridized to a DNA template strand that contains a defined lesion. For this assay we used a 16-mer 5' FAM-labelled primer annealed with two different 30-nucleotide templates: one without lesions and one containing a CPD (thymidine dimer) in position +1 with respect to the 3' end of the primer (Figures 12A and 12B).

This system is incubated with the polymerase under examination, for our assay every Polη variant, in the presence of the four dNTPs, under controlled conditions. If the enzyme is active and able to overcome the lesion, it will extend the primer along the template synthesizing a product of greater length. Conversely, if the enzyme is defective or sensitive to the presence of damage, extension will stop before or at the lesion.

At the end of the reaction, the synthesized products are separated by denaturing gel electrophoresis (15% acrylamide gel containing 7 M urea), which allows to distinguish the fragments based on their length. The analysis of the resulting pattern allows to evaluate the ability of the polymerase to carry

out synthesis beyond the damage: the presence of short bands indicates an early arrest, while the presence of longer bands testifies to an effective extension and therefore a functional TLS activity. Therefore, this experimental approach allows to determine to what extent specific mutations influence the ability of the enzyme to overcome DNA damage and complete replication *in vitro*. During the analysis, three parameters were taken into account: the extension of the primer to a full length product (red box), the bypass efficiency just after the lesion (orange box) and the degree of consumption of the initial primer (blue box) (Figure 12C).

In presence of undamaged and damaged template, wild type Polη showed a highly processive behaviour, managing to elongate the primer to its full length. Even the reticulocytes containing the empty vector produced a modest extension in presence of the undamaged template, suggesting a residual polymerase activity of the extract. However, this residual competence fails to bypass the CPD damage completely.

The variants tested showed a diverse range of behaviours.

Mutations located in the C-terminal regulatory region, Lys535Glu, Lys589Thr and Met595Val, maintained a similar performance to that of the wild-type enzyme, managing to carry out a processive extension even in the presence of CPD.

In contrast, the Leu11Pro variant, which affects the palm domain, showed a strongly reduced catalytic activity: in the presence of undamaged DNA, it produces only a minimal extension, comparable to the EV, while in the presence of CPD it is completely unable to extend the 16 nucleotides of the primer.

The Arg93Pro and Arg111His mutations are both located in the palm domain and situated in the site involved in DNA binding. Arg93Pro shows an activity similar to the empty vector when in presence of undamaged template, indicating a very weak catalytic activity; while, when in presence of damaged template, it is able to partially overcome CPD damage, adding three nucleotides before stopping. Arg111His, on the other hand, can extend the primer to the complete product on an intact template, although with lower efficiency than the WT. With CPD template, the activity is reduced, showing a more distributive behaviour, but it still manages to overcome the lesion substantially, reaching in part full length product. Position 111 in Polη is close to the DNA and the incoming nucleotide: these results suggest that the substitution from arginine to histidine, although slightly modifying the properties, does not completely compromise the function of the enzyme probably due to the maintenance of polarity of the two amino acids.

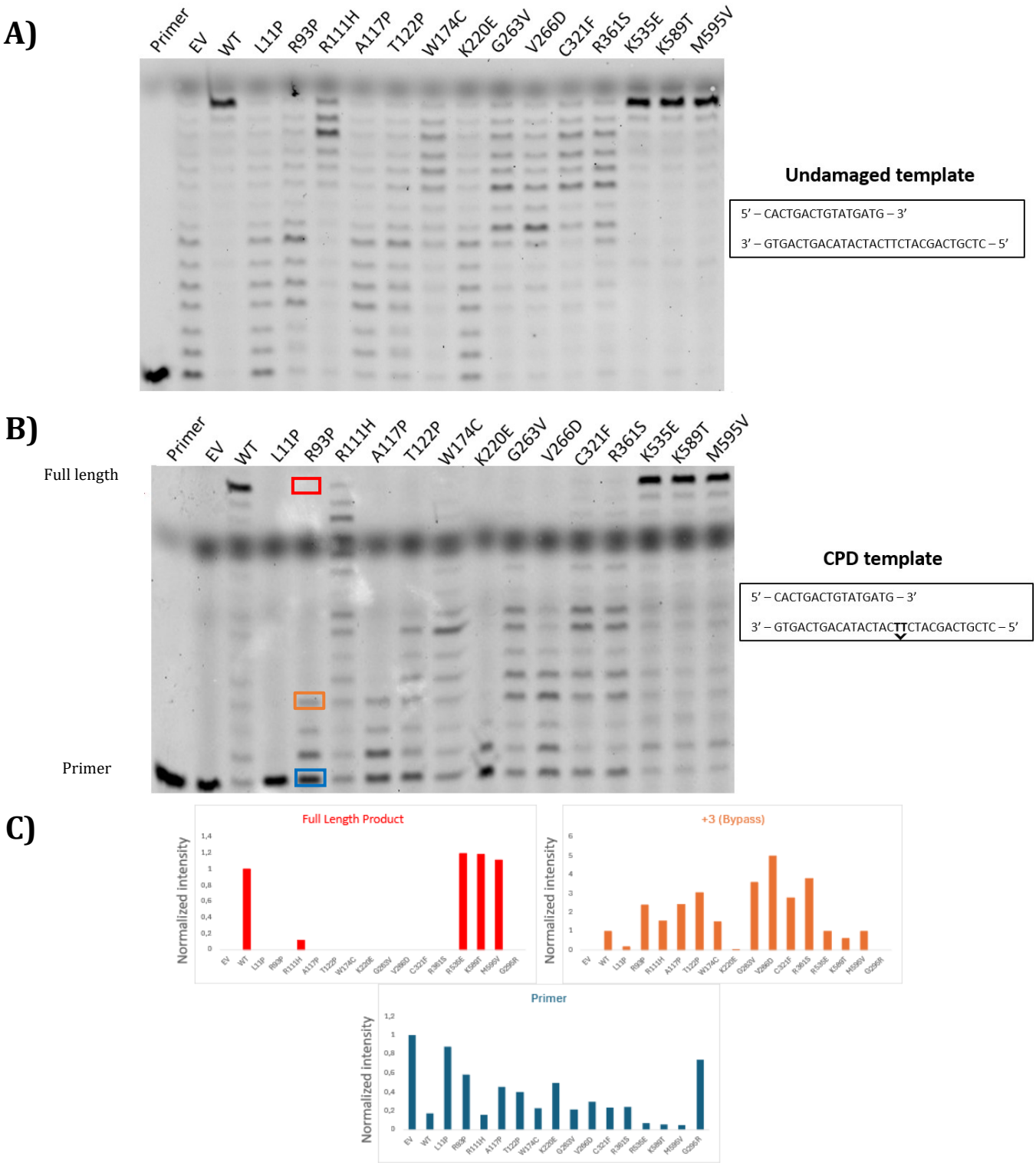

Figure 12

In vitro primer extension assay in which TnT DNA Polη, either wild-type (WT) or carrying specific mutations, was tested for its ability to extend a primer on two types of DNA templates: one undamaged and one containing a cyclobutane pyrimidine dimer (CPD). The EV (Empty Vector) control represents the reticulocyte extract incubated without Polη.

A. Primer extension assay using the undamaged template, with relative template sequence.

B. Primer extension assay using the template containing a CPD at position +1 with relative template sequence.

C. Quantitative analysis of the mutant variants' ability to: generate the full-length product (red box), bypass the CPD by adding three nucleotides (orange box), and consume the primer (blue box). The quantification was normalized to WT Polη for the red and orange metrics, and to the EV control for primer consumption.

The variants Ala117Pro, Thr122Pro and Lys220Glu show a behaviour similar to the empty vector when polymerizing the undamaged DNA, whereas in presence of CPD they perform differently. In fact, Ala117Pro is able to bypass the damage but it blocks after the third nucleotide insertion, Thr122Pro is the most processive although it not able to reach full length product, and Lys220Glu is able to consume only a minimum part of the primer, arriving at the second nucleotide insertion with difficulty.

The variant Trp174Cys, situated as the previous ones in the palm domain, is also able to overcome the lesion and continue the synthesis, although not reaching the full length, similarly to the Thr122Pro variant. Trp174Cys, located on the same α helix as the Thr191Pro mutation (already characterized in our laboratory), maintains a greater catalytic activity *in vitro* (Ricciardiello et al., 2024).

The variants Gly263Val and Val266Asp, located in the thumb domain, and Cys321Phe and Arg361Ser, located in the "little finger" domain, show a distributive activity both with undamaged DNA and with CPD. In the presence of the lesion, they show an even lower polymerization efficiency, generating mainly short fragments.

We can conclude that the only variants capable of generating full-length products are found outside the catalytic site. Leu11Pro is confirmed to be completely ineffective in bypassing the damage, while the other mutations, although showing a compromised functionality, retain at least part of the catalytic capacity.

In order to confirm the results and exclude sequence specific behaviours we decided to use, the same experimental approach with a different substrate. Another TnT reaction was performed, using the same method to evaluate the effective presence of Polη (Figure 13), controls containing 10 µg of purified Polη were used. In addition, as can be seen in Figure 13, we were able to produce the G295R allele that was missing from the first analysis.

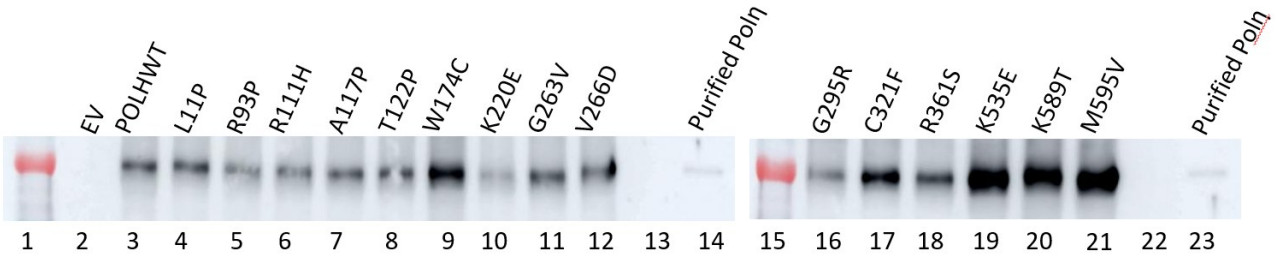

Figure 13
Western Blot representing the *in vitro* transcription and translation reaction of mutated Polη. As we can observe, the western blot confirms the presence of Polη in all samples. The samples, probed with an anti-Polη antibody, were compared to purified Polη used as a reference control.

Polη mutants were then used to perform the primer extension assay using a different template: a 51-mer damaged template containing a thymidine dimer (TT) at position +20, annealed with a 5' FAM-labelled 31-mer to evaluate the *in vitro* activity of Polη in different conditions. This approach allowed to analyse the efficiency of DNA synthesis in the presence of the new substrate/template, comparing the performances of the different protein variants in relation to their ability to carry out translesion synthesis (TLS) (Figure 14).

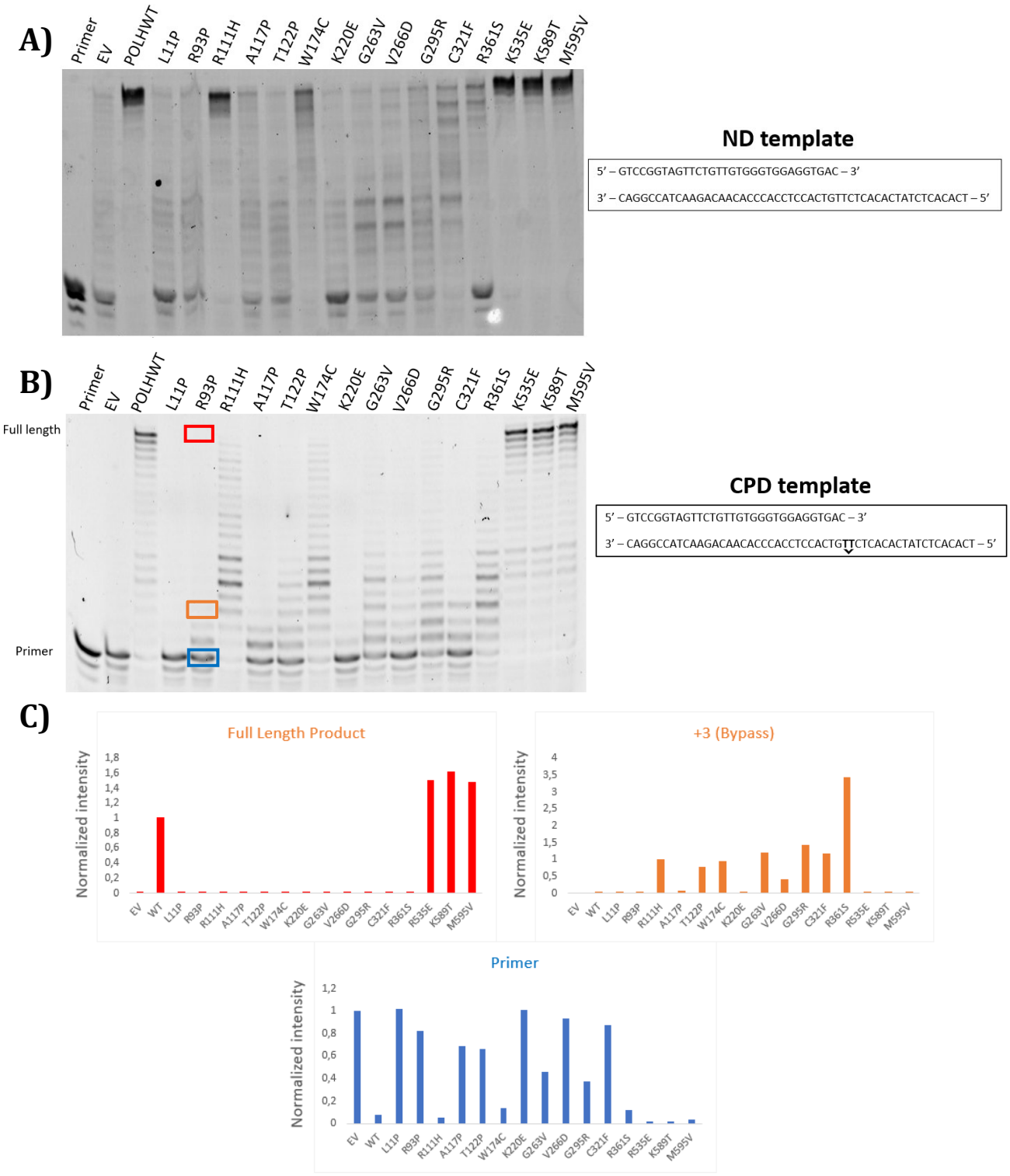

Figure 14

In vitro primer extension assay in which TnT DNA Polη, either wild-type (WT) or carrying specific mutations, was tested for its ability to extend a primer on two types of DNA templates: one undamaged and one containing a cyclobutane pyrimidine dimer (CPD). The EV (Empty Vector) control represents the reticulocyte extract incubated without Polη.

A. Primer extension assay using the undamaged template, with relative template sequence.

B. Primer extension assay using the template containing a CPD at position +1 with relative template sequence.

C. Quantitative analysis of the mutant variants' ability to: generate the full-length product (red box), bypass the CPD by adding three nucleotides (orange box), and consume the primer (blue box). The quantification was normalized to WT Polη for the red and orange metrics, and to the EV control for primer consumption.

5.3. Cell cycle analysis of Polη variants exposed to UV irradiation

Primer extension analysis provided an initial overview of the catalytic activity of the different Polη variants. However, to fully understand their impact, it is also necessary to evaluate the cellular phenotype *in vivo*. To this end, we stably complemented XP30RO cells, that are Polη deficient, with each of the mutant Polη variants, fused with the eGFP tag, in order to analyse the damage bypass capacity by monitoring the S phase progression after exposure to UV irradiation damage. While we were able to establish most of the variants, of Polη, some mutants failed to express *in vivo* probably due to instability of some mutated proteins.

All the stable cell lines were seeded after cell sorting for eGFP positive cells expressing similar levels of Polη and then irradiated with 10 J/m² of UV light and, after 24 hours, they were fixed in ethanol (see material and Methods). Subsequently, samples were stained with propidium iodide (PI) and cell cycle distribution was analysed by flow cytometry (FACS).

Propidium iodide is an intercalating fluorochrome that binds stoichiometrically to double-stranded DNA. Since its fluorescence is proportional to the amount of DNA present in the cell, it is possible to distinguish cells in the G1 phase (diploid content, 2C), in the S phase (with an intermediate DNA content between 2C and 4C) and in the G2/M phase (tetraploid content, 4C).

The results of FACS are displayed as a histogram: on the X-axis is the PI fluorescence intensity (so, the DNA content) and on the Y-axis is the number of cells. Typically, two peaks are observed: one in G1 (on the left) and one in G2/M (further to the right), with the space in between that represents the S phase. Therefore, this type of approach allows us to analyse the DNA content and to correlate it to the different phases of the cell cycle (G1, S, G2/M).

Observing the mutant cell lines DNA content allows us to evaluate effects of the mutated polymerase on proliferation or cell cycle progression.

This analysis, therefore, is particularly useful for monitoring how a given experimental condition, such as the absence or presence of a mutated variant of Polη, can influence the cell cycle progression. By applying this method to our cell lines, it was possible to compare the DNA distribution profiles in basal conditions and following UV damage, to evaluate the actual ability of the different variants of the polymerase to support the progression of the cycle.

In untreated samples (-UV in figures 15-18), the cell cycle profile is similar for all cell lines, with a predominant peak in the G1 phase, characterized by a DNA content of 2C.

Following UV irradiation, XP30RO cells complemented with wild-type Polη (fused to eGFP) were able to overcome the damage and advance toward the G2 phase as inferable by a second peak corresponding to a DNA content of 4C. In contrast, uncomplemented XP30RO cells, lacking functional Polη, enter but fail to progress through the S phase, as suggested by a peak slightly shifted

on the right if compared to the 2C peak. The tested variants show different cell cycle progression profiles after UV irradiation; However, none were able to completely bypass the damage and reach the G2 phase, although some were less compromised than uncomplemented XP30RO cells (+UV in figures 15-18).

Firstly, we analysed mutations located in the palm domain, particularly in the catalytic site: Arg93Pro and Arg111His variants (Figure 15).

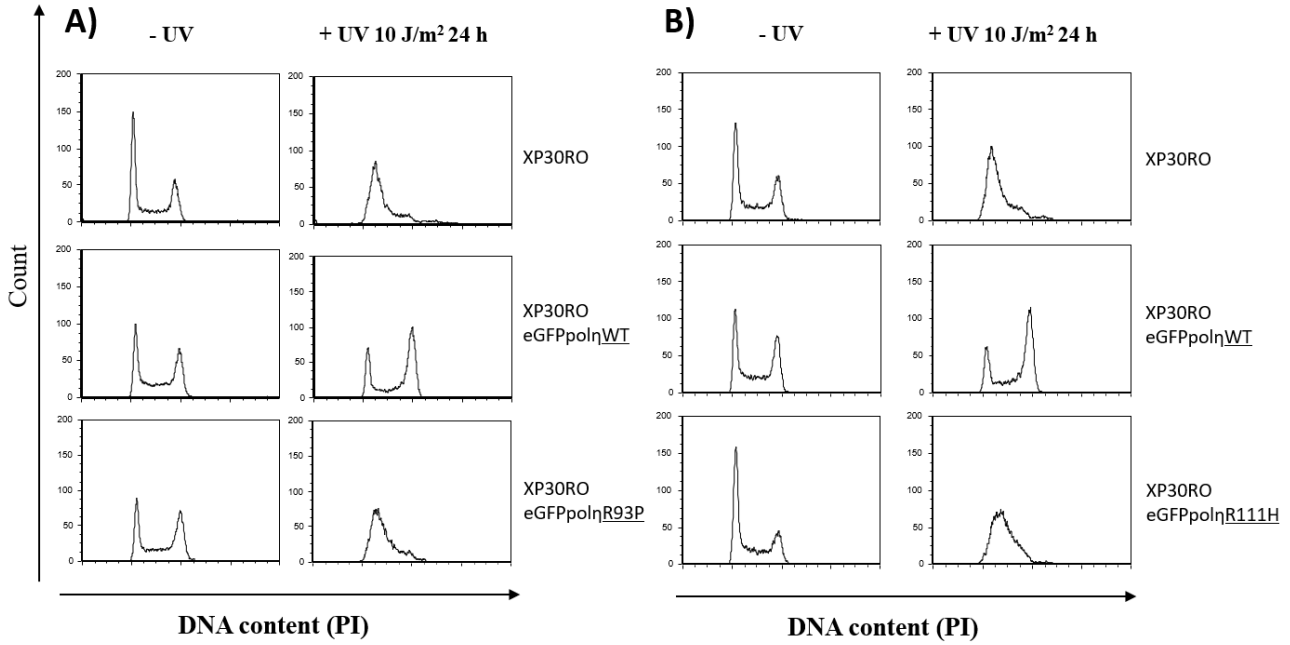

Figure 15
Analysis of the cell cycle progression of the R93P (A) and R111H (B) Polη variants located in the palm domain, more specifically in the catalytic domain.

As we have observed in the primer extension assay, Arg93Pro mutated cell line shows, after UV irradiation, a block in the G1-early S phase, very similar to the XP30RO behaviour. This profile indicates that these cells are unable to bypass the UV-induced damage and complete the cell cycle.
Arg111His mutated variant, which *in vitro* resulted one of the most promising in the bypass and elongation of the primer, presents a peculiar cellular phenotype. In fact, even in the absence of UV treatment, the cell cycle distribution appears altered if compared to both cells complemented with Polη WT and Polη deficient cells (XP30RO). Being the G1 peak relatively higher and the G2 peak relatively lower, the ratio G1 to G2 is shifted toward the former. In presence of UV damage, G1 and G2 peaks do not show up, substituted by a single broad peak shifted to the right if compared to that of XP30RO cells. This finding suggests, that cells expressing this variant are able to progress further in S phase compared to XP30RO, a behaviour that reflects what was observed in *in vitro* assays.

Then, we analysed other variants located in the palm domain, Ala117Pro and Thr122Pro, that seem to influence cell cycle progression in a similar way to the ones described above (Figure 16).

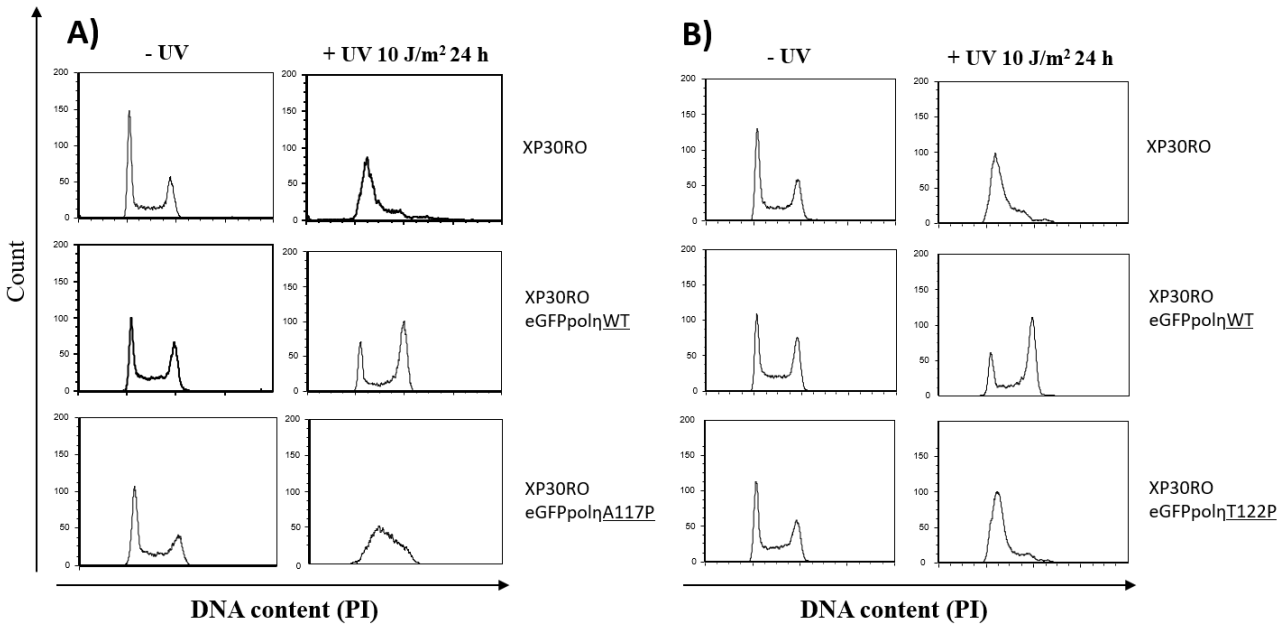

Figure 16
Analysis of the cell cycle progression of the A117P (A) and T122P (B) Polη variants located in the palm domain.

Cells complemented with Ala117Pro mutated Polη fail to go past the S phase after UV damage. This behaviour also reflects the *in vitro* data, which describe this variant as capable of bypassing the DNA damage, however it stops soon after it.

We have also observed that in the primer extension assay, the Thr122Pro variant, is able to bypass CPD damage. *In vivo*, however, this variant is not able to complement the lack of Polη activity and the cells fail to complete the cell cycle and remains blocked at early S phase similarly to uncomplemented XP30RO cells

We managed to obtain stable cell lines with mutations situated in the thumb and in the little finger domains, respectively Gly263Val and Cys321Phe (Figure 17).

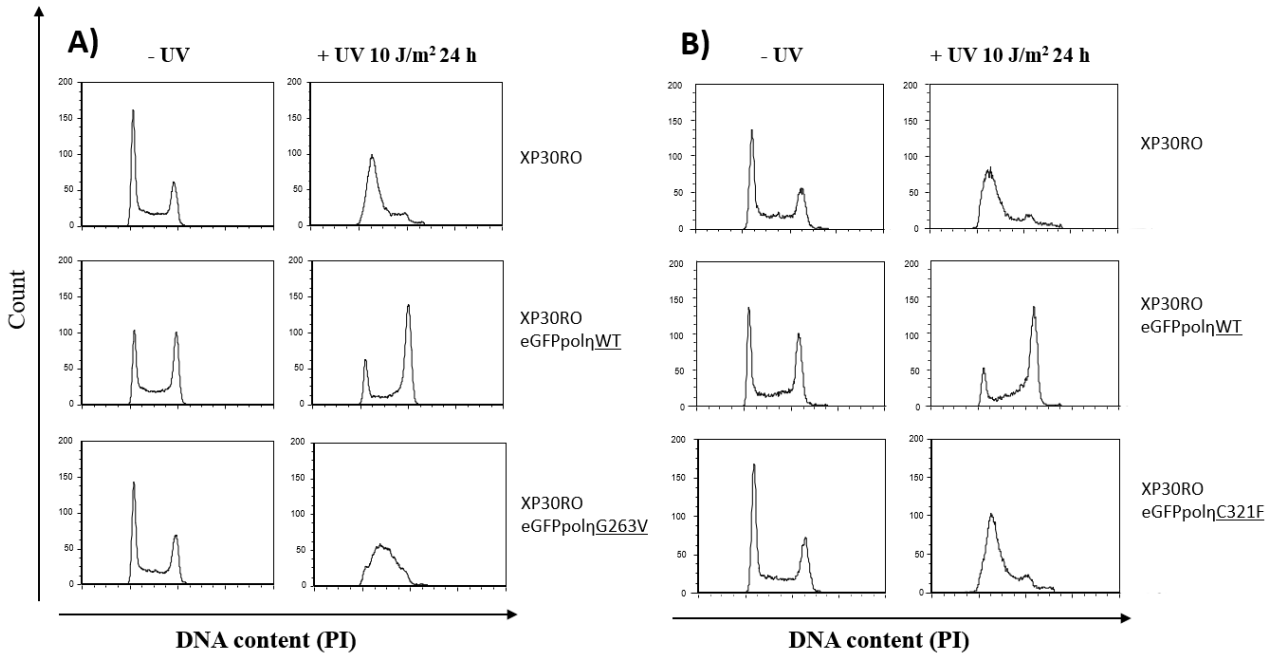

Figure 17
Analysis of the cell cycle progression of the G263V and C321F Polη variants located respectively in the thumb and in the little finger domain

From the *in vitro* analysis, we know that these two variants show similar results in bypassing CPD and elongate the primers. However, the cell cycle progression profiles are quite different. Stable cell lines complemented with Gly263Val mutated Polη, after UV damage, result able to bypass the damage and enter S phase, but they are unable to go past the S phase. In fact, in respect to the wild-type Polη cell line, this mutant does not present any peak in 2C nor 4C DNA content, but it shows an intermediate phenotype.

Gly263Val mutants present a profile similar to Ala117Pro mutants (Figure 16A) in terms of shape, but the broad peak is shifted to the right. This observation suggests that even though also these cells, after UV damage, are unable to go past the middle stages of S phase. This finding is in accordance with the primer elongation data, which showed a higher bypass capacity of the variant Gly263Val when compared to Ala117Pro.

Cys321Phe Polη variant cell line behaviour, instead, after UV irradiation, present a profile comparable with the XP30RO cells profile, with a high peak in 2C DNA content and a lower quantity of cells in an intermediate DNA content, suggesting that Cells are able to reach S phase, bypassing the damage, however they are incapable of reaching the G2 phase (Figure 17).

Lastly, we established and analysed the cell cycle progression of the cell lines that contain missense mutations situated in the C-terminal regulatory domain of Polη: Lys535Glu and Met595Val (Figure 18).

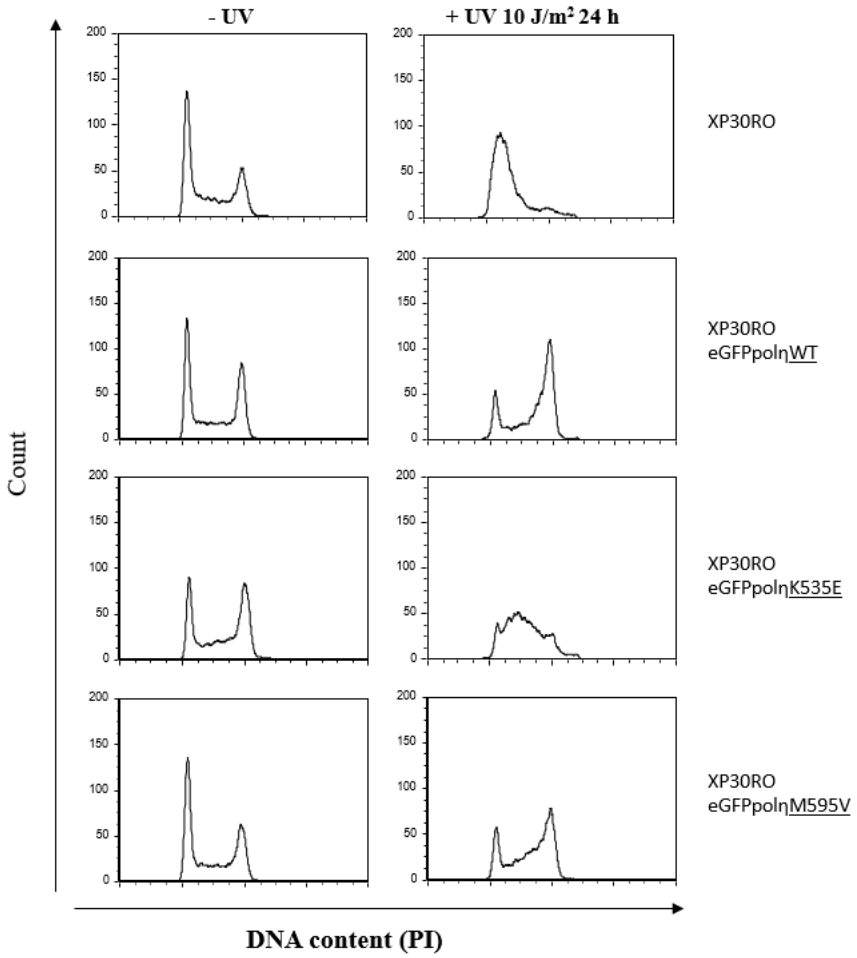

Figure 18
Analysis of the cell cycle progression of the K535E and M595V Polη variants located in the regulatory domain.

Lys535Glu and Met595Val mutations are among those that, *in vitro,* showed an enzymatic activity comparable to that of the wild-type protein. Cell cycle analysis revealed that, after treatment with UV light, these two mutants behave in very distinct ways. In fact, cells complemented with Lys535Glu Polη variant present an intermediate profile between WT Polη cell line and the cell line lacking Polη, suggesting a partial functionality, despite the ability of the mutant protein to overcome CPD damage. Furthermore, the Lys535Glu variant appears to alter the cell cycle even in the absence of damage, indicating that this mutation could interfere not only with the damage response mechanism, but also with normal replication dynamics.

This data highlights the crucial role of Polη regulation in the context of DNA damage bypass.

On the other hand, cells complemented with Met595Val Polη, show a profile comparable to the WT Polη cell line, in presence and in absence of DNA damage. Met595Val variants are able to bypass the damage and complete the cell cycle, arriving in G2 phase.

5.4. Testing *in vivo* Polη variants survival exposed to different doses of UV irradiation

To try to understand how different Polη variants can influence cell survival after UV damage, we used a crystal violet assay. It is a fairly simple but very useful method that allows us to get an idea of how many cells actually manage to survive after irradiation. The principle is that the crystal violet dye binds to the DNA of living and adherent cells: therefore, the intensity of the colour is directly proportional to the quantity of cells that have survived the UV irradiation. After cell sorting the cell lines are seeded and after 24 hours irradiated with different doses of UV-C light (0, 2 and 4 J/m²). Cells are then incubated in medium for 72 hours. During this time, the cells that were unable to respond to the damage stopped proliferating or underwent cell death, while those able to tolerate the damage remained viable. Finally, we stained the plates with crystal violet and measured its absorbance by spectrophotometer (see Materials and Methods). The absorbance values obtained reflect proportionally the number of surviving cells.

Each experimental condition was analysed in triplicate, and, for each cell line, multiple independent experiments were performed ($n \geq 2$). The collected data were averaged to obtain a reliable estimate of cell survival at each UV dose. The results were then plotted, comparing the different Polη variants with each other and with the wild-type protein (Figure 19).

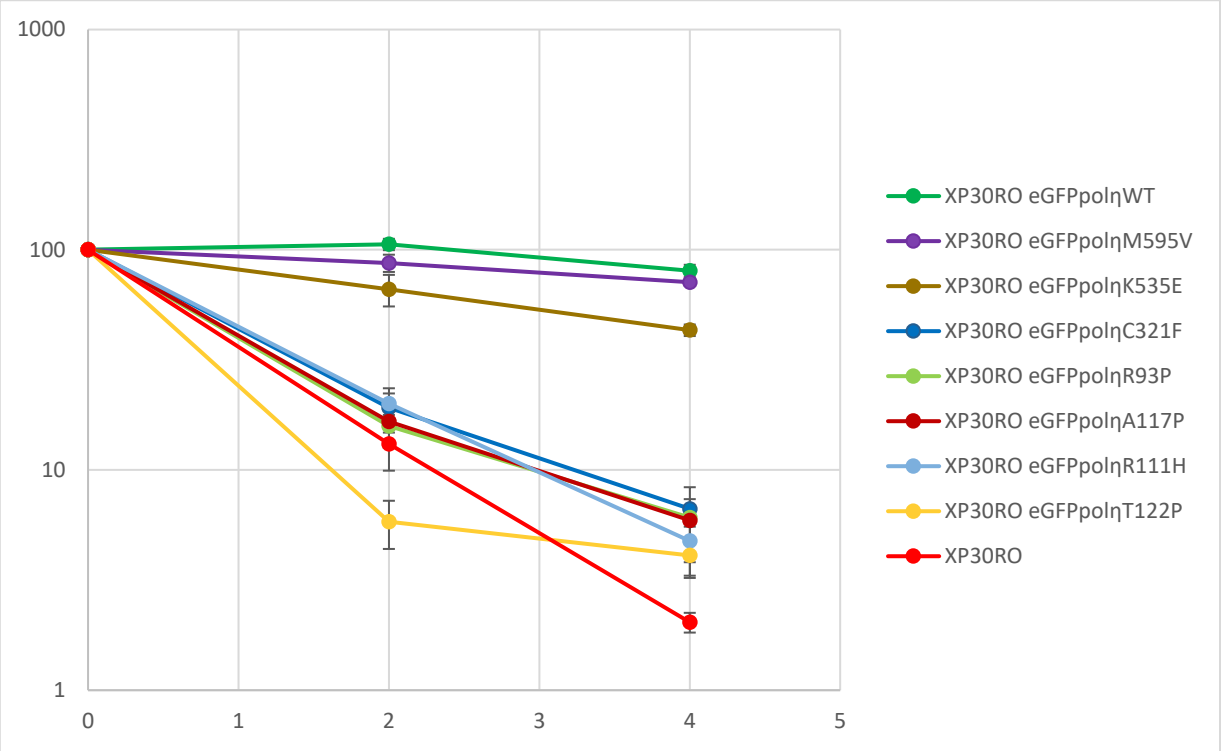

Figure 19
Quantification of cell survival in crystal violet staining assays performed in presence of caffeine (75 µg/ml). Error bars represent the standard error of the mean.

Cell survival analyses conducted by crystal violet assay confirmed what was observed in previous assays, highlighting marked differences in the ability of the different Polη variants to protect cells from UV damage. As expected, XP30RO cells, lacking the functional polymerase, are sensitive to irradiation, showing a drastic reduction in viability, arriving at 2% survival rate in samples irradiated with 4J/m^2.

In contrast, cells expressing wild-type Polη are well tolerant to UV treatment, showing high survival rates in all samples (approximately 80% in samples irradiated with 4J/m^2). This result confirms the ability of the native protein to efficiently bypass UV damage and maintain cell proliferation.

As we expected, mutations situated in the C-terminal of Polη, Lys535Glu and Met595Val, retain the major viability. Over 70% of Met595Val mutants can survive UV irradiation, resulting the most similar to the wild-type. Lys535Glu mutants, however, present a significantly reduced survival rate of 43%.

The tested variants showed approximately homogenous behaviours. In fact, many of them behave similarly to the non-complemented XP30RO line, suggesting a marked inability to restore an effective response to damage *in vivo*. In fact, mutants Arg93Pro, Arg111His, Ala117Pro, Gly263Val, Cys321Phe retain a very low survival score, in a range from 4 to 10%.

An interesting response is observed in the Thr122Pro variant. All the other cell lines are very consistent with drops of survival rate associated to an increasing of UV irradiation; on the other hand, Thr122Pro mutant cell line present a substantial drop in the $2J/m^2$ UV treated sample, presenting for the latter a survival rate lower than the XP30RO, but they seem to not suffer that much the increase of UV irradiation.

In conclusion, this type of approach allowed us to directly observe the functional effect of individual Polη mutations, going beyond molecular analyses and evaluating whether and to what extent each variant can actually protect cells from genotoxic damage. Together with the other assays, the crystal violet assay provided important confirmation of the role of these mutations in regulating survival and response to UV damage, helping to outline a more complete picture of the cellular consequences of a deficit in the TLS system.

6. DISCUSSION

Translesion synthesis (TLS) is one of the essential mechanisms for DNA damage tolerance. In this study, through functional characterization of Polη missense variants, we evaluated the impact of specific mutations on the ability of the enzyme to perform and its implication on cell cycle and cell viability. Polη is a key element in the bypass of DNA damage, in particular caused by UV (cyclobutane pyrimidine dimers and [6-4] pyrimidone photoproducts), contributing to genomic stability and prevention of skin tumorigenesis. In fact, mutations regarding Polη itself or its regulation, as observed in patients affected by xeroderma pigmentosum variant (XPV), can predispose to the development of different tumours, specifically basal cell carcinomas (BCCs), squamous cell carcinomas (SCCs) and melanomas, distinctive traits of various Polη variants. In this perspective, it is essential to understand how specific mutations can alter the activity of the enzyme and what functional consequences derive from it.

Mutations located in the catalytic site in the palm domain have been shown, in general, to be highly deleterious for the enzymatic activity. In particular, the Arg93Pro and Ala117Pro variants showed almost no primer extension *in vitro*, indicative of a significant loss of polymerase activity. This severe impairment is consistent with the localization of the mutated residues in regions critical for interaction with DNA or dNTPs. *In vivo* data confirm these results: the lines expressing these variants are unable to complete the S phase after UV irradiation, suggesting an inability of the TLS system to effectively bypass the damage. Even the Arg111His mutation, despite affecting a key catalytic residue, maintained partial activity *in vitro*, presumably thanks to the preservation of the positive charge and the ability of histidine to mimic some interactions of arginine with DNA. However, the anomalous cellular behaviour observed, with an altered cell cycle distribution already in basal conditions, suggests that this mutation may interfere with regulatory aspects of the enzyme, perhaps by influencing its stability or interaction with other protein partners.

The Thr122Pro variant presents itself as a particularly interesting case. Despite being located in the catalytic region, it shows an *in vitro* enzymatic activity superior to other nearby mutations, partially bypassing the damage. However, *in vivo* data indicate a reduced ability to sustain the progression of the cycle after UV and a compromised cell survival. This discrepancy between *in vitro* and *in vivo* data reflects the fact that this residue is important for the structural integrity of the palm domain (Broughton et al., 2002), therefore it reflects a structural instability of the mutated protein whose implication *in vitro* is limited but it is crucial when inserted in a cellular context. Moreover, this amino acid alteration could cause a reduced affinity for co-factors necessary for the correct execution of TLS in the cellular context.

Mutations located in the "thumb" and "little finger" domains, such as Gly263Val and Cys321Phe, also show a negative impact on Polη function. These regions, although not directly involved the catalytic site, are functional to the correct positioning of the DNA and the processivity of the enzyme. The two variants show a reduced ability to extend the primer beyond the damage and a distributive behaviour *in vitro*, consistent with a poor and discontinuous action. Cellular data support this observation: although cells expressing them are able to enter S phase, they are unable to complete it, probably due to the accumulation of non-bypassed damage and the activation of replicative checkpoints.

The results obtained for mutations in the regulatory C-terminal region, Lys535Glu and Met595Val are particularly relevant. Both show an enzymatic activity *in vitro* comparable to the wild-type protein, suggesting that they do not directly interfere with the active site. However, *in vivo*, the observed phenotypes are different. The Lys535Glu variant, in particular, induces an alteration of the cell cycle even in the absence of damage and a reduced survival after exposure to UV rays. These data suggest that the mutation may perturb regulatory functions of the enzyme, which is essential for an effective response to DNA damage. On the contrary, the Met595Val variant behaves in a completely analogous way to the wild-type in all the conditions tested, both *in vitro* and *in vivo*. This data appears in apparent contrast with its association with cases of melanoma and associated skin carcinoma reported in the literature. A possible explanation is that Met595Val is not in itself sufficiently deleterious to compromise TLS in standard conditions, but that it represents a predisposing variant, whose effect emerges only in the presence of additional predisposing genetic or environmental factors, such as excessive exposure to UV radiation or concomitant mutations in other DNA repair genes.

Overall, the results obtained outline a complex and multifaceted picture, in which mutations in different regions of Polη can distinctly compromise the enzyme function: through the loss of catalytic activity, the reduction of processivity, or the alteration of regulatory mechanisms and interactions with other components of the damage tolerance system.

7. CONCLUSIONS

There is a growing number of Polη missense variants that lack a precise functional assessment, which are classified as variants of uncertain significance (VUS). Many of these variants have been characterized in several studies and reported in the literature, however, the prevalent use of patient-derived cells does not allow obtaining the level of evidence necessary to classify these variants as pathogenic, according to the PS3 criteria of the American College of Medical Genetics and Genomics (ACMG) guidelines. In our laboratory, we analysed fifteen mutations *in vitro*, eight of which were also studied *in vivo*, by complementing XP30RO cells, lacking Polη cells, with mutated Polη. This approach allowed us to study Polη variants in a controlled and independent context, obtaining results that depend exclusively on the mutation, without the influence of unknown variables related to the cellular context of patient cells.

Through the analysis of these eight missense mutations of polymerase η it was possible to observe how each one has different effects on DNA damage tolerance. The results obtained show a heterogeneous picture, which highlights how the positioning and interactions of even just one residue can influence the stability of the protein, its activity or regulation and the interaction with other co-factors, leading to deleterious conditions for the cell and more generally, for the organism.

Given the results of the experiments carried out, we propose that the mutations Arg93Pro, Arg111His, Ala117Pro, Thr122Pro, Gly263Val, Cys321Phe can be classified as likely pathogenic mutants according to ACMG guidelines. On the contrary, given the complexity of results relating to Lys535Glu and Met595Val mutants, it is not currently possible to change their classification, which remains that of variant of uncertain significance, requiring further investigations and characterization.

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