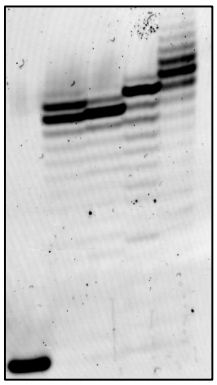
UNIVERSITA' DEGLI STUDI DI PAVIA

Dipartimento di Biologia e Biotecnologie

"Lazzaro Spallanzani"

Study of translesion synthesis dynamics and their role during genome replication



Antonio Maffia

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXXI – A.A. 2015-2018

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Abstract

Genomic DNA can present impediments to the replication machinery. Different factors assist the replication fork permitting DNA duplication even in challenging conditions. Among them, the DNA damage tolerance (DDT) pathway has evolved to allow an efficient bypass of obstacles for the fork, especially after DNA damage. Two important players in this pathway are PCNA and DNA polymerase η. Recently, polη has been involved also in the replication of genomic loci that present secondary structures that are difficult to replicate, or present sparse replication origins (Common Fragile Sites). Considering the expanding role of polη, both in the presence but also in the absence of DNA damage, it becomes crucial to be able to follow polη activity on the genome. Tracking the activity of DNA polymerases has been achieved in the last few years thank to mutants that incorporate ribonucleotides in the synthetized DNA. These can be specifically isolated and used to build DNA libraries for NGS. Sequencing of ribonucleotides would allow to track the activity of the polymerase along the genome. Here we show that DNA polη mutated in F18 (F18A) caused a higher accumulation of ribonucleotides both *in vitro* and *in vivo*. Characterisation of the mutant cell lines has highlighted a partial deficiency in DNA damage bypass, possibly due to the activity of RNase H2. Nevertheless, we present the first human cell system suitable for the tracking of DNA polymerases by ribonucleotide incorporation. DDT relies on a complex network of highly regulated proteins. Post translational modifications of PCNA are central in the pathway. In fact, ubiquitylation and SUMOylation, regulate two branches of the DDT: translesion synthesis and template switch. While the latter is an error free process that uses recombinational intermediates to bypass the damage, translesion synthesis employs mutagenic polymerases to continue replication. Lysine 164 (K164) on PCNA has a pivotal role in regulating DDT. However, its role in humans is still ill defined. To fill this gap in knowledge, we established a PCNA K164R cell line using the CRISPR/Cas9 genome editing approach. The characterisation of an heterozygous clone has already provided intriguing phenotypes, thus stressing the importance of this residue in DNA damage bypass. Post translational modifications of PCNA regulate TLS polymerases recruitment at the fork to bypass DNA lesions. However, it has been recently shown that TLS polymerases may be exploited also during the normal replication of the genome. Non B-form DNA is among the hypothesised

substrates of polη. We have verified the role of TLS in g-quadruplex dynamics by the use of the well characterised G4-stabilising compound pyridostatin. Either presence or absence of the polymerase influenced the activation of the DNA damage response, thus stressing the role of DNA polη in replicating past these structures.

Acknowledgements

At first, I would like to acknowledge my supervisor, Dr. Simone Sabbioneda, for giving me the unique opportunity to work on the projects here presented, but most of all for supporting my scientific development both technically and personally.

I am also grateful to Prof. Antonio Torroni for being my scientific tutor from the University of Pavia and for being supportive and patient with my requests and doubts.

My gratitude goes to our collaborators at the University of Milano, Prof. Marco Muzi Falconi, Dr. Federico Lazzaro and Dr. Alice Meroni; for sharing critical ideas, suggestions, precious materials and fundamental protocols. Moreover, I am grateful to Dr. Catherine Green from University of Oxford for checking the karyotype of our cell lines. I must also acknowledge Dr. Marco Di Antonio from the Imperial College in London, for the kind gift of the pyridostatin compound and for supporting my hypothesis.

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Contents

1. Introduction

1.1 Genome Replication

Inheritance of the genomic DNA is one of the most crucial mechanisms in living organisms. The genome must be correctly replicated to avoid the onset of detrimental mutations. Given the importance of this mechanism cells have evolved a complex network of proteins to ensure the accurate transmission of DNA by daughter cells. This network comprises a variety of enzymes that actively coordinate DNA replication in time and space but also manage eventual damage that would impede a correct progression of the replicative machinery. Timing of genome duplication is tightly regulated and takes place during the S phase of cell cycle (Zink D 2006). A finely tuned control by the different cyclin and cyclin dependent kinases (cyclin/CDKs) restricts DNA replication to a single duplication event in the S phase. This prevents over replication and provides a single copy of the genomic material. Such a delicate task requires the coordinated action of a considerable number of proteins to regulate DNA replication. Proteins that are directly involved in genome replication are part of the so-called replisome. Enzymes and other accessory factors assemble on nascent DNA where leading and lagging strands separate to form the replication fork. This molecular machinery is capable of unwinding, replicate, eventually repair the template DNA and finally control termination of the process. At the same time, replication has to manage chromatin remodelling by first removing chromatin related proteins, in order to gain access to the double helix, and then to re-establish the chromatin complexes. All of these tasks are finely regulated by different levels of control. The first level of replication control relies on the assembly of replicative complexes at origins of replication (Masai H et al. 2010). In fact, at the beginning of S phase the CMG complex, an 11 subunit complex made of the Cdc45, Mcm helicase, and GINS is activated by phosphorylation cascades at the level of origins (Fig. 1).

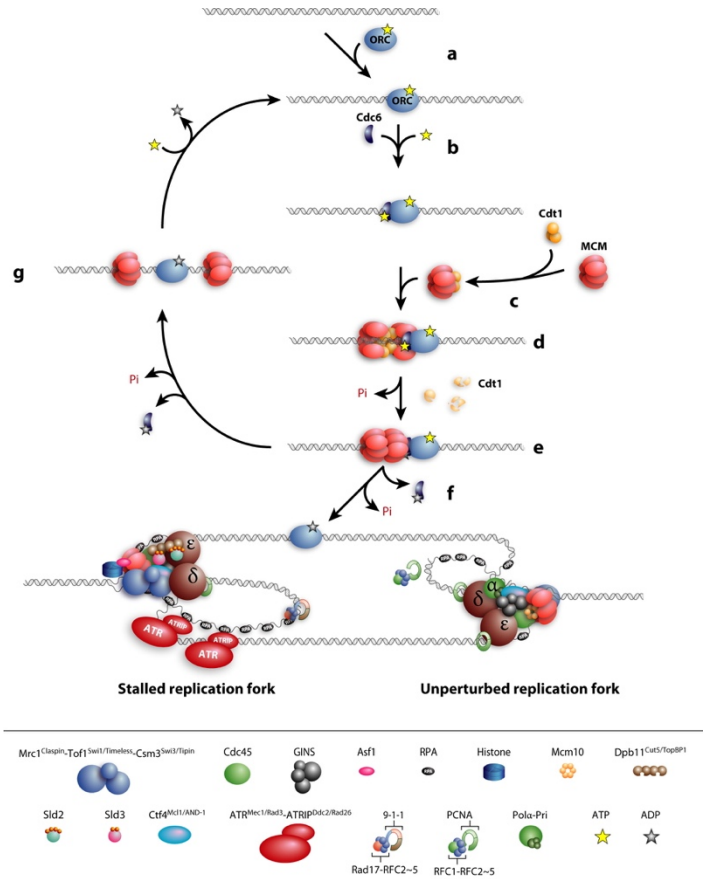


Fig. 1 Replication initiation in eukaryotes

The Origin Recognition Complexe (ORC) binds to replication origins upon its phosphorylation. Following this event phosphorylated Cdc6 associates with the complex. Loading of the Mcm complex with Cdt1 is initiated by hydrolysis of ATP on Cdc6 and its dissociation. These events lead to the assembly of the so-called pre Replication Complex (pre-RC). Binding of numerous other factors such as Cdc45 and GINS guarantees a functional helicase activity. Loading of PCNA by the clamp loader RFC allows engagement of replicative polymerases to start DNA elongation. In the presence of DNA damage forks are protected by ATR -ATRIP complexes.

Adapted from: Masai, Hisao, et al. "Eukaryotic chromosome DNA replication: where, when, and how?." *Annual review of biochemistry* 79 (2010).

The main task of the complex is to begin DNA double helix unwinding. In addition to these proteins, Mcm10 stabilizes the entire complex and stimulates DNA elongation by polymerases thanks to the presence of the Replication Protein A (RPA) bound on ssDNA. Moreover, the RFC complex is required to load the proliferating cell nuclear antigen (PCNA) on DNA. This sliding clamp is composed of a trimer with identical subunits, each containing amino acidic motifs for binding replicative polymerases. The interaction between PCNA and DNA polymerases guarantees their processivity during primer elongation (Choe K and Moldovan G 2017). Many other proteins are required to control Okazaki fragments maturation, recognize and repair mis-pairings and control chromatin reassembly. Protein-protein interactions and post translational modifications coordinate and regulate this very complex network of proteins that constitutes the replisome. These intricate and tightly coordinated interactions manage the entire process of genome replication and guarantee its integrity.

1.2 DNA polymerases

Replicative polymerases are the main enzymes of the replication fork and are all part of the B-family DNA polymerases (Johansson E et al. 2013). Due to their exclusive 5'-3' catalytic activity complementary DNA filaments are replicated either in a continuous (leading strand) or discontinuous (lagging strand) manner. Thus, the replication fork is organized so that the labour is subdivided between the two major replicases (Nick McElhinny S et al. 2008) (Fig. 2). In fact, DNA polε replicates the leading strand, DNA polα primes elongation with RNA primers on the lagging strand and elongates the primers with its DNA polymerase activity. Finally, DNA polδ fills the gaps between primers synthesised by the DNA polα primase activity.

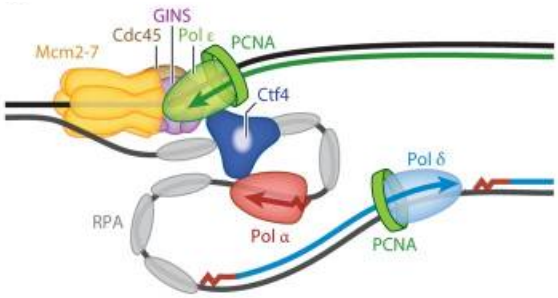


Fig. 2 Replication fork in eukaryotes
Replication fork assembles on the two strands for continous strand replication by DNA polε and discountinous strand replication by DNA polα and DNA polδ. Accessory proteins in the complex, such as the processivity factor PCNA allows an efficient duplication of the genome.
Adapted from: Burgers, Peter MJ, and Thomas A. Kunkel. "Eukaryotic DNA replication fork." *Annual review of biochemistry* 86 (2017): 417-438.

The discontinuous strand synthesis is guided by maturation of Okazaki fragments (Fig. 3). Primers provided by the primase/polymerase activity of DNA polα are elongated by DNA polδ. When reaching the following primer, the polymerase starts a strand displacement process that leaves a 5' flap. This flap is the substrate for a flap endonuclease, FEN1, that removes the structure. Nicks are finally generated by 3' exonuclease activity of DNA polδ that immediately restarts synthesis in an iterative nick translation reaction. To end the process gaps are filled thanks to DNA ligase I generating a continuous filament of DNA. Replicative polymerases share similar general features while having slightly different tasks. The high fidelity of these polymerases is mostly guaranteed by their 3' exonuclease activity that removes erroneously incorporated nucleotides. The proofreading activity is shared by both DNA polε and DNA δ but it is not present in DNA polα. Another important feature is processivity of the replicases, which is the capability of elongating nascent DNA without disengaging from the double helix.

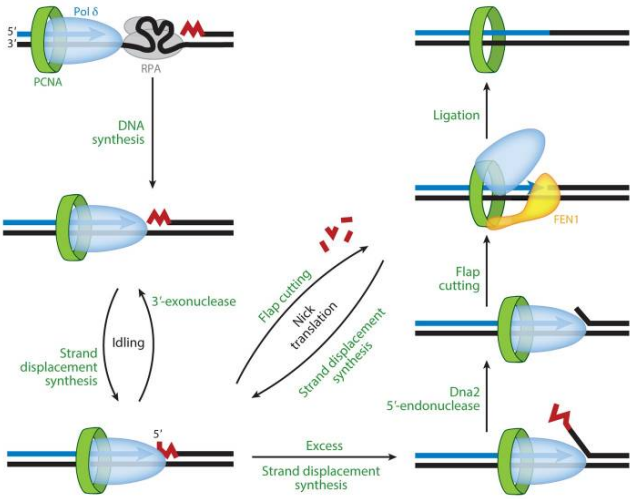


Fig. 3 Okazaki fragments maturation

After primers synthesis by DNA polα, DNA polδ engages the discontinous strand to elongate primers. The strand displacement activity is counteracted by its 3' exonuclease activity. Excessive strand displacement creates a 5' flap intermediate cut by FEN1 exonuclease. The iterative cycle between flap cut and strand displacement generates a continous nick translation reaction that terminates with the ligation of the nicks by DNA Ligase I.

Adapted from: Burgers, Peter MJ, and Thomas A. Kunkel. "Eukaryotic DNA replication fork." *Annual review of biochemistry* 86 (2017): 417-438.

Comparing the different polymerases, DNA polε has the highest processivity thanks to the presence of additional subunits conferring the capability of encircling dsDNA directly. On the other hand, DNA polδ and DNA polα require a direct interaction with PCNA to maintain contact with the nascent dsDNA. In both cases, the sliding clamp acts as a processivity factor and guarantees an efficient polymerisation reaction by DNA polymerases (Chilkova O et al. 2007).

1.3 Sugar discrimination by replicative DNA polymerases

Fidelity of DNA polymerases is not only restricted to the incorporation of the correct nucleotide pair. In this case, both the exonuclease activity and the presence of the mismatch repair system ensure that mutagenesis is maintained at low levels during DNA replication. Another level of complexity for correct nucleotide pairing is given by recognition of the correct sugar during polymerases catalysis. Indeed, cells possess two different pools of nucleotides: deoxyribonucleotides (dNTPs) and ribonucleotides (NTPs) with the latter being from 10 to 100 fold more abundant in eukaryotic cells (Traut W et al. 1994). Given the similarity and the higher abundance, ribonucleotides can be used as a substrate by DNA polymerases and hence be erroneously incorporated during replication (Nick McElhinny S et al. 2010). Despite this tendency, ribonucleotides load on genomic DNA is usually maintained at low levels. Perhaps, misincorporated ribonucleotides are a source of genome instability, mostly because of their higher reactivity and DNA distorting capacity when present in the DNA double helix. Indeed, the presence of ribonucleotides embedded into the genome can cause a transition from B to A forms of DNA. This transition has an immediate impact on the efficiency of DNA replication (Egli M et al. 1992; Ban et al. 1994). Two different strategies have evolved to prevent genomic instability in the case of misincorporated NTPs. The first mechanism resides in the capability of the active site of DNA polymerases to exclude ribonucleotides. Actually, a single residue in the amino acidic chain of the active site is responsible for such efficient exclusion (Brown J et al. 2011). This evolutionary conserved residue has been identified in members of all the DNA polymerases families. For A family it is represented by a glutamine (Glu) residue, while it is an aromatic residue such as tyrosine (Tyr) or phenilalanine (Phe) for B, X, Y and RT family. Such residues are located in the active site in an orientation that causes a clash with the 2' hydroxyl group (2'-OH) of incoming ribonucleotides (Fig. 4).

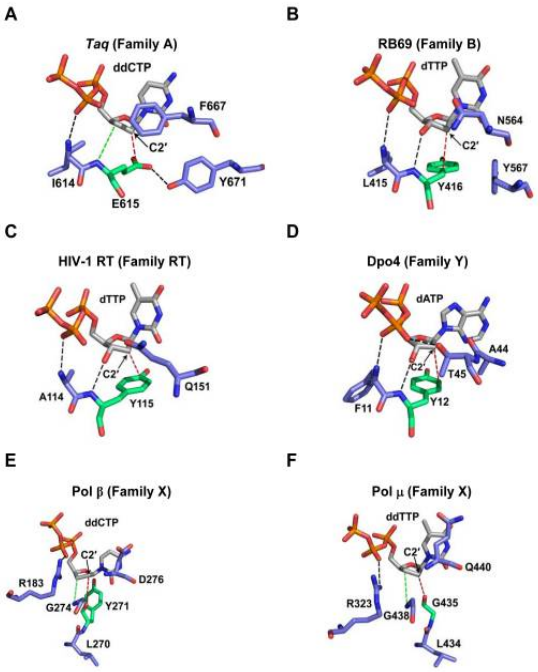


Fig. 4 A conserved steric gate residue prevents ribonucleotides incorporation

DNA polymerases possess a residue in their active site that causes a steric clash with the 2'-OH of incoming ribonucleotides. This is called the "steric gate" residue and efficiently prevents incorporation of ribonucleotides during genome replication. The steric gate has been found evolutionary conserved in both replicative and alternative DNA polymerases. Examples of different families of polymerases are here depicted (from A to F). The steric gate residue is depicted in green, clashing with an incoming ribonucleotide at the level of 2'-OH indicated by the arrows.

Adapted from: Brown, Jessica A., and Zucai Suo. "Unlocking the sugar "steric gate" of DNA polymerases." *Biochemistry* 50.7 (2011): 1135-1142.

Thus, a single amino acidic residue can exclude the wrong nucleotide substrate based on the chemical difference with deoxyribonucleotides. Although this is an efficient strategy, NTPs can escape this first line of control and be erroneously incorporated into genomic DNA (Cerritelli S et al. 2016).

Accordingly, studies in yeast have provided the first evidence of ribonucleotides misincorporation by replicative polymerases. In more detail, in *S. cerevisiae* it was demonstrated that during normal genome replication both DNA polε and DNA polδ mutants were able to misincorporate ribonucleotides *in vivo* (Nick McElhinny S et al. 2010). Surprisingly, precise quantitation of ribonucleotides incorporation revealed that these are the most abundant erroneous base incorporated in DNA during replication. Recently also specialised polymerases, respectively polβ, polγ, and polη have been shown to misincorporate ribonucleotides *in vitro* (Crespan E et al. 2016; Mentegari E et al. 2017). Given the deleterious consequences of genomic embedded NTPs, cells have evolved a dedicated pathway to detect and remove misincorporated ribonucleotides (Sparks J et al. 2012; Williams JS et al. 2016). The pathway is called ribonucleotides excision repair (RER) and takes advantage of the ribonuclease H2 (RNase H2) enzyme (Fig. 5). This is an evolutionary conserved enzyme and in humans is present as an heterotrimeric complex given by the assembly of RNase H2A, RNase H2B and RNase H2C subunits (Reijns M et al. 2014). The RNase H2A subunit has been identified as the catalytic subunit, although it is not able to cleave ribonucleotides per se but requires the correct assembly of the trimer (Chon H et al. 2009). Once correctly assembled, the enzyme can recognize either single or multiple embedded ribonucleotides into genomic DNA and cleave at the 5' the NTP (Nguyen T et al. 2011; Figiel M et al. 2011). The cleaved strand is displaced by replicative polymerases creating a 5' flap that is resolved by Fen1 (Rydberg B et al. 2002). Once gap filling terminates, ligation of the fragments occurs thanks to DNA Ligase I.

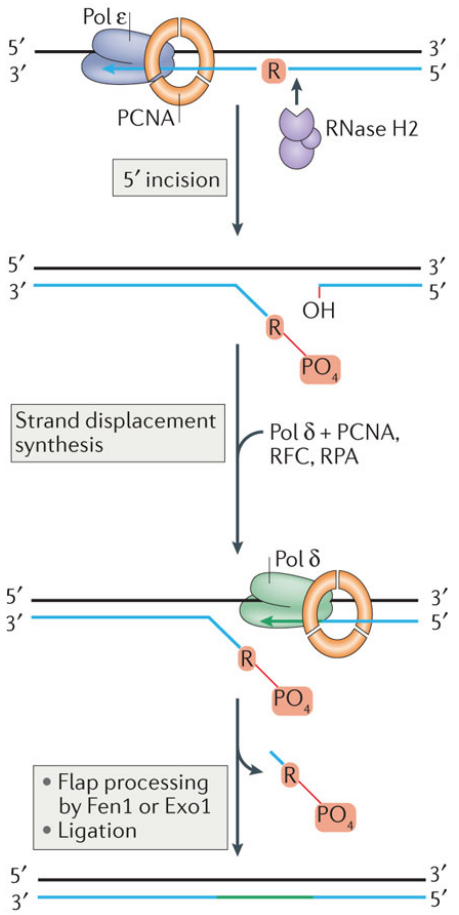


Fig. 5 Ribonucleotides Excision Repair
RNase H2 recognizes genomic embedded ribonucleotides and cleaves at the 5'. This event creates nicks that are filled-in by DNA polδ. The displaced 5' strand is processed by exonucleases and finally gaps are ligated.
Adapted from: Williams, Jessica S., Scott A. Lujan, and Thomas A. Kunkel. "Processing ribonucleotides incorporated during eukaryotic DNA replication." *Nature Reviews Molecular Cell Biology* 17.6 (2016): 350.

Therefore, this process clears DNA from erroneously embedded ribonucleotides and thus prevents genomic instability. However, the exact mechanism by which ribonucleotides accumulation causes genome instability is still under investigation.

Much of the work to characterise RNase H2 activity has been performed in the yeast *S. cerevisiae* model. Indeed, loss of both RNase H1 and H2 in yeast has causes a higher sensitivity to replication stress that can be resolved by post replication repair mechanisms (Lazzaro F et al. 2012). On the contrary, mice that lack RNase H2 show an embryonic lethal phenotype that has been directly linked to the excess of ribonucleotides in genomic DNA (Reijns M et al. 2012). Finally, in humans, mutations of RNase H2, cause an autosomal recessive disorder named Aicardi-Goutieres syndrome (AGS) (Crow Y et al. 2008). This disorder has been classified as a neuroinflammatory autoimmune disease. Indeed, the severe phenotype has been linked to the accumulation RNA:DNA hybrids that can trigger an inflammatory response if left unresolved (Lim Y et al. 2015; Mackenzie K et al. 2016; Mackenzie K et al. 2017). In the past, sugar discrimination by polymerases seemed an obvious property and ribonucleotides accumulation irrelevant. During last years knowledge about ribonucleotides misincorporation has impressively expanded. As a consequence, their influence on human diseases has brought to attention the central role of the RER pathway for genome instability.

1.4 Polymerase's activity tracking

Although being deleterious for the cell, incorporation of ribonucleotides by polymerases has been exploited to track DNA polymerases activity. In fact, one of the strategies to follow DNA polymerases in action along the genome is to follow DNA elongation with nucleotide analogues. If analogues can be isolated, then it will be possible to enrich DNA that has been elongated by the polymerase. Seminal studies in yeast have taken advantage of strand specific mutagenic signatures by catalytic mutants of the three major DNA polymerases (Pavlov Y et al. 2001; Pursell Z et al. 2007; Shinbrot E et al. 2014). Thanks to these studies the roles of DNA polε in replicating the bulk of leading strand and DNA polδ elongation of the lagging strand were directly monitored from yeast genomic mutational patterns (Nick McElhinny S et al. 2008). However, monitoring spontaneous mutations is not an efficient strategy, due to the low probability of misincorporations. In order to improve detection of misincorporations it is necessary to work in a mismatch repair defective background.

Nonetheless, cells carrying this genetic background present an altered viability and have led to different interpretations of the polymerases activity maps (Burgers P et al. 2016; Johnson R et al. 2016). Studies in the field have looked for strategies to force mutagenic nucleotides incorporation by mutant polymerases. While looking for useful catalytic mutants to track replicases mutational signature, variants with a different sugar selectivity were isolated. Mutations in polymerases catalytic site were identified as steric gate mutations that stimulate ribonucleotides incorporation at higher frequency (Nick McElhinny S et al. 2010). In light of these findings, ribonucleotides were designated as useful endogenous nucleotides analogues for polymerases tracking. The use steric gate mutants combined with the loss of RNase H2 in yeast, has led to the development of specific ribonucleotides sequencing methods to map DNA polymerases along the genome. In fact, during the last few years different groups have developed similar but comparably efficient methods to isolate genomic NTPs and precisely map the DNA fragments that contained ribonucleotides (Daigaku Y et al. 2015; Clausen A et al. 2015; Koh K et al. 2015). Briefly, once polymerases have been genetically engineered to allow NTPs incorporation, sequences containing NMPs can be digested either chemically or enzymatically and then the isolated DNA fragments can be used for next generation sequencing (Fig. 6). Similar studies in both budding or fission yeast have provided detailed maps of the asymmetric activity of replicative polymerases along chromosomes. In fact, the division of labor between replicative polymerases has been confirmed by ribonucleotides incorporation (Reijns MA et al. 2015; Clausen A et al. 2015; Daigaku Y et al. 2015). Thanks to these studies, the consequences of ribonucleotides incorporation into genomic DNA have been more precisely assessed. For example, the presence of NTPs and their cleavage by a Topoisomerase I mediated pathway, in the absence of RNase H2, can lead to short deletions or more harmful gross chromosomal rearrangements (Conover H et al. 2015; Williams J et al. 2016). Considering the data obtained in yeast, genetic engineering of polymerases coupled to ribonucleotides sequencing can be exploited as a new and powerful strategy to obtain high resolution maps of each polymerase. Analysis of such maps would help to discern the contribution to genome replication by single polymerases.

At the moment all of such studies have been performed in yeast where whole genome sequencing is advantageous because of the reduced size of the genome when compared to the mammalian genome. Attempts to reproduce such methodology in mammalian cells have to face the obstacle of a larger genome to obtain polymerase sequencing maps.

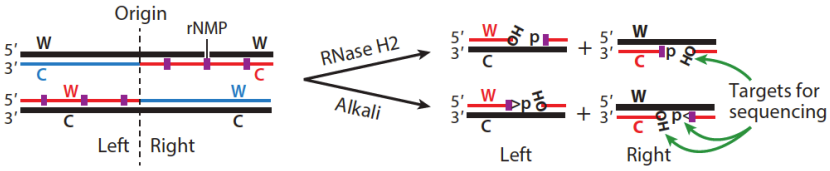


Fig. 6 Isolation of genomic ribonucleotides
Ribonucleotides inserted into genomic DNA on either the leading strand (W) and lagging strand (C), can be cleaved specifically by eithr chemical conditions or enzymatically. Treatment with RNase H2 cleaves at 5‘ generating fragments that can be targeted for sequencing. Alkali instead react with the 2’-OH group producing a 5’-OH group and 2’,3’ phosphate end. Both generated ends can be targeted to build sequencing libraries.
Adapted from: Burgers, Peter MJ. "Polymerase dynamics at the eukaryotic DNA replication fork." *Journal of Biological Chemistry* 284.7 (2009): 4041-4045.

1.5 DNA Damage Response (DDR)

The precise coordination of DNA polymerases in the replication fork guarantees a faithful duplication of the genome. However, numerous sources of DNA damage may cause genomic instability. Exogenous DNA damage can be caused by either chemical or physical treats. Chemicals like alkylating agents or oxidative compounds, can damage DNA bases mainly leading to the formation of abasic sites. Instead, physical damage can be inflicted to DNA either by ionizing radiations causing strand breaks or by UV irradiation causing chemical modifications of DNA bases. In addition to this, not only exogenous damages can offend the double helix and thus compromise genome replication but also the intrinsic nature of the sequence can be an obstacle to the replication fork. In fact, the genome presents regions intrinsically difficult to replicate. Sequence composition, either repetitions or the formation of peculiar secondary structures, can slow the rate of replication with possible deleterious consequences for cell's viability (Zeman M et al. 2014). Any of these circumstances may cause replication stress and activate a pathway called DNA damage response (DDR) (Ciccia A et al. 2010). The main kinases of the DDR pathway are Ataxia Telangectasia Mutated (ATM) and Ataxia Telangectasia and Rad3-related (ATR) (Zhou B and Elledge S 2000). Both are serine/threonine kinases that respond to different genomic stresses. While ATM responds to DSBs together with DNA-PK, ATR is activated by replication fork stresses (Fig. 7). In fact, when encountering an obstacle, replicative polymerases may stall if a lesion is present on the template strand. Even though replication fork progression is impeded, MCM helicase activity continues to unwind DNA, thus uncoupling replication and unwinding processes. These uncoupled reactions produce stretches of single stranded DNA (ssDNA) and this DNA substrate activates the ATR branch of the DDR pathway (Byun T et al. 2005). In brief, the RPA protein binds exposed ssDNA and in turns activates the major kinase of the pathway, ATR (Zou L et al. 2003). The RPA-ssDNA complex recruits the ATR/ATRIP complex at the level of stalled forks and initiates the checkpoint cascade that phosphorylates a plethora of substrates comprising the complex formed by Rad9, Rad1 and Hus1 (9-1-1 complex), TOPBP1, p53, BRCA1, and other players to control both cell cycle progression and damage repair. The cascade finally leads to the activation of the downstream kinase Chk1.

In particular, ATR phosphorylates Chk1 on S345 and S317, and while S345 has been demonstrated fundamental to Chk1 activation, the role of S317 is still undefined (Zhao H et al. 2001). The concerted action of ATR and Chk1 permits fork stabilisation, damage repair or fork restart. Most importantly, the phosphorylation cascade controls cell cycle progression. Indeed, entry in mitosis in the presence of stalled forks can lead to more deleterious consequences for genome integrity. To prevent such outcomes, the ATR axis control activation of cyclin/CDKs. In detail, when Chk1 is active, it phosphorylates and thus inactivates CDC25 phosphatases (Liu P et al. 2006). These phosphatases normally remove the inhibitory phosphorylation on CDK1 and CDK2 that if active can form cyclin-CDK complexes and stimulate cell cycle progression. This ATR/Chk1 axis constitutes the intra S-phase checkpoint. This checkpoint mechanism has a major role in preventing incorrect transition from the S phase to mitosis. Moreover the intra S-phase checkpoint also controls origin firing (Maya-Mendoza A et al. 2007; Kumar S et al. 2009), fork stability (Lopes M et al. 2001; De Piccoli G et al. 2012), and DNA repair processes. The control of this considerable number of pathways is achieved thanks to targeted phosphorylation of a plethora of substrates. Overall, the ATR axis guarantees a proficient entry into mitosis alleviating replication stress and thus preventing more deleterious consequences for genome stability.

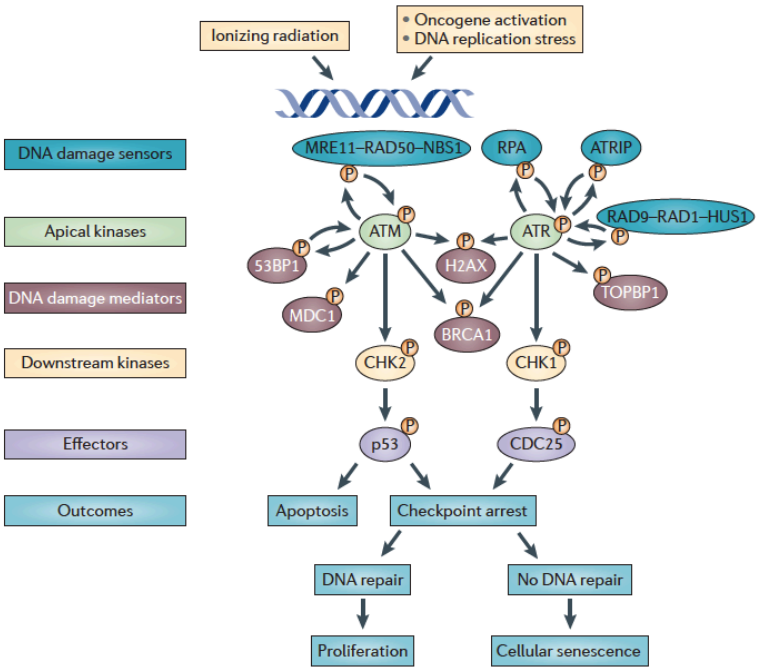


Fig. 7 The ATM/ATR axis

Two different kinds of cellular stresses can activate two branches of the DNA damage response (DDR) distinguished by the two different apical kinases. DSBs are sensed by the MRN complex and stimulate ATM activity. Phosphorylation activates protein mediators, mainly devoted to DNA ends protection, but also the downstream kinase Chk2. The main substrate of Chk2 is p53 which coordinates the final outcome of the pathway. On the other hand replication stress leads to accumulation of RPA coated ssDNA. This is the main target for ATR activation that is recruited to DNA by its partner ATRIP. Here, ATR phosphorylates numerous mediators but including the downstream kinase Chk1. The main substrate of Chk1 is Cdc25 whose activation leads to cell cycle arrest.

Adapted from: Sulli, Gabriele, Raffaella Di Micco, and Fabrizio d'Adda di Fagagna. "Crosstalk between chromatin state and DNA damage response in cellular senescence and cancer." *Nature Reviews Cancer* 12.10 (2012): 709.

1.6 Translesion Synthesis (TLS)

The lesions produced by UV irradiation are between the most characterised obstacles that replication forks may encounter. Both UV-C and UV-B light damages DNA generating bulky adducts with the formation of either covalent bonds between adjacent pyrimidines (cyclobutane pyrimidine dimers or CPDs) or 6-4 pyrimidine-pyrimidone photoproducts (6-4 PPs). The majority of these lesions are repaired before S phase by the nucleotide excision repair system (NER). Thanks to a complex network of proteins, this repair system recognises adducts, excides the DNA tract containing the lesion and then fills the remaining gaps (Marteijn J et al. 2014). The fill-in reaction is performed by replicative polymerases and fragments are ligated by DNA ligase I. Although this is a very efficient pathway, some lesions can be left unrepaired and thus persist at the S phase entry. Replicative polymerases cannot accommodate bulky DNA templates in their active site and as a consequence are blocked on the damaged template. Cells have evolved a dedicated mechanism to cope with the stalling of replicases on damaged DNA and prevent more deleterious consequences. Early works on this phenomenon started from studies in *E. coli*. In this prokaryotic organism, gaps on the newly synthesised DNA strand were noticed after UV irradiation (Rupp W et al. 1968). Initially, it was hypothesised that these gaps may be filled-in by polymerases in post replicative system, that was acting after the bulk of DNA replication was concluded. This replicative system past UV photoproducts was immediately recognised as an error prone system and initially attributed to lower fidelity of replicative polymerases in bypassing adducts (Lemontt J et al. 1971; Kato T et al. 1977). It took years to characterise the Rad30 gene in yeast and to demonstrate that its product was a low fidelity polymerase, albeit having the ability to bypass faithfully thymidine dimers induced by UV (Johnson R et al. 1999). Later on, the polymerase orthologous gene in humans was isolated and correlated to the Xeroderma Pigmentosum Variant (XPV) disease (Masutani C et al. 1999). This is an autosomal recessive syndrome that increases UV sensitivity and causes a higher incidence of skin cancer in patients (DiGiovanna J and Kraemer K 2012). Nowadays the bypass of UV lesions upon replication fork stalling has been characterised as a translesion synthesis mechanism and relies on a dedicated set of DNA polymerases.

DNA polymerase η was subsequently classified as a member of Y-family polymerases (Sale J et al. 2012). The peculiarity of these polymerases lies in their active site. Despite being similar to replicative polymerases in terms of overall structural organisation, the active site in the Y-family is wider and allows accommodation of bulky lesions present on the DNA template. This is a clear advantageous feature for DNA damages bypass, but it becomes detrimental when extending an undamaged template. In fact, the wider catalytic site reduces the capability to discriminate between the correct nucleotide pair with the result of a mutagenic elongation. Increased mutagenesis is also caused by the lack of 3'-5' proofreading activity, which is typical of high fidelity replicative polymerases. Other specialised polymerases have been identified in humans in the last few years and now the Y-family comprises DNA polη, DNA polι, DNA polκ and Rev1. An additional TLS polymerase, DNA polζ had already been identified, although comprised in the B-family of DNA polymerases. To avoid fork stalling, and the eventual fork collapse, the mechanism of translesion synthesis assists the replication fork and employs specialized polymerases to bypass the damage and continue DNA replication. These polymerases share some common features together with structural similarities. In some cases, they are capable of inserting the correct nucleotide in front of the lesion and synthesize short tracks of nucleotides, however they are generally considered error pone when replicating on an undamaged template (Vaisman A and Woodgate R 2017). Another distinctive feature of specialised polymerases is their low processivity. In particular, they tend to synthesize short nucleotide stretches and then immediately dissociate from DNA. This feature defines them as distributive polymerases instead of processive polymerases as the replicative enzymes are. Despite sharing major similarities, each TLS polymerase has its own identity (Vaisman A and Woodgate R 2017) (Fig. 8).

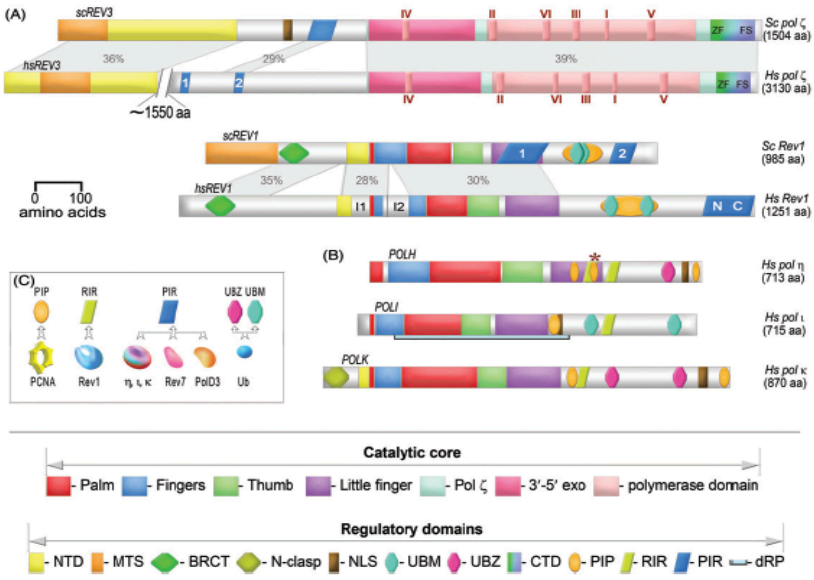


Fig. 8 Comparison of yeast and human Y-family polymerases

A All members share a common right hand configuration similar to replicative polymerases with palm, fingers and thumb domains.

B Y-family polymerases have an additional little finger domain. They lack the 3'-5' exonuclease domain or, as in the case of DNA polζ, it is inactive (A). Mitochondrial signals can be present as in the case Rev1. UBM and UBZ domains allow Ubi-PCNA binding while PIP domains regulate affinity for unmodified PCNA. RIR domains permit Rev1 binding and Rev1 has PIR domains.

Adapted from: Vaisman, Alexandra, and Roger Woodgate. "Translesion DNA polymerases in eukaryotes: what makes them tick?." *Critical reviews in biochemistry and molecular biology* 52.3 (2017): 274-303.

DNA polζ

Polζ has been documented as performing the elongation step after lesion bypass by the other Y-family polymerases. Notably it is part of B-family and thus shares higher similarities with the replicative polymerases. The enzyme is a multisubunit complex and shares accessory subunits with the replicative polδ (Makarova A et al. 2015). In detail, the holoenzyme is a tetramer comprising the Rev3, Rev7, p50 and p66 subunits. Rev3 represents the catalytic subunit and the largest of the complex. Rev7 has been shown to stimulate catalytic activity by up to 30 fold. This subunit also mediates numerous interactions such as with DDK kinase implied in replication initiation, the transcription factor TFII-I and the spindle assembly protein MAD2. In yeast, Pol31 and Pol32 accessory subunits (p50 and p66 respectively in humans) were shown to stimulate catalytic activity and mediate PCNA interactions. Compared to its related replicative polymerases, the holoenzyme has a lower fidelity due to the inactive 3'-5'exonuclease activity. It was shown that about half of the spontaneous mutagenesis in yeast could be related to DNA polζ (Quah S et al. 1980). Interestingly, while being dispensable in yeast, loss of REV3L gene in mice causes embryonic lethality (Wittschieben J et al. 2000; Esposito G et al. 2000). Notably, the mutagenic activity of polζ has been linked to carcinogenesis in humans (Lange S et al. 2011). Much of the informations we have gained on this polymerase are from *in vitro* reconstitution of its activity. Experimental evidence has been shown that polζ has a low efficiency in incorporating nucleotides opposite template lesions, while it has a high efficiency in elongating mismatched primers. Its fidelity on undamaged templates is however higher when compare to the Y-family polymerases. These data led to the present model in which after other specialised polymerases have bypassed the lesion, polζ elongates the primer until replicative polymerases are engaged again on the replication fork (Johnson R et al. 2000).

Polι

Polι has peculiar characteristics in terms of catalysis. In fact, it has been shown to possess both a polymerase and dRP lyase activity, similar to X-family polymerases, that explains its involvement in base excision repair (BER) (Bebenek K et al. 2001). Its processivity is very low, restricted to 2-3 nts and it is the least faithful Y-family polymerase. Catalysis by DNA polι differentiates also in the divalent ions that are coordinated by the active site. Indeed, while the majority of DNA polymerase coordinate Mg^{2+} ions to perform DNA elongation, polι preferentially coordinates Mn^{2+} (Frank E and Woodgate R 2007). The elongation fidelity is highly dependent on sequence context especially while bypassing damaged templates like CPDs (Tissier A et al. 2000; Vaisman A et al. 2003). However, its capability of bypassing such lesions may be exploited, together with DNA polκ, in the case of DNA polη loss such in XPV phenotypes (Jansen J et al. 2014). Despite being faithful opposite a T template, it tends to insert G in front of an A template. Although being a mutagenic activity, this tendency counteracts cytosine deamination and prevents CG to TA transitions (Vaisman A and Woodgate R 2001). While being beneficial to prevent spontaneous mutations, this is detrimental in the case of somatic hypermutation where TLS is required to generate Ig genes diversity. In fact, in this case transitions are at the base of Ig diversity generation and the activity of DNA polι would suppress transitions due to its tendency of inserting G nucleotides in front of A templates. This model explains why DNA polι is usually not involved in the process of somatic hypermutation where other TLS polymerases take part (McDonald J et al. 2003).

Rev1

Rev1 has a restricted catalytic activity and has been demonstrated to insert only C in front G. Its catalytic core presents two amino acidic inserts. The first determines its strict selectivity for both primers and the incoming nucleotides, while the second is a platform for protein protein interactions (Swan M et al. 2009). This insert is fundamental for the non catalytic roles of Rev1 in TLS. In fact, besides having a restricted catalytic activity it is involved in numerous interactions that confers a regulatory role. Interestingly, Rev1 has been demonstrate to play a role in the recruitment of TLS polymerases at stalled replication forks (Gun C et al. 2003; Ohashi E et al.

2004). A C-terminal domain called protein interaction region (PIR) mediates interactions with TLS polymerases (Pustovalova Y et al. 2012). A short motif on DNA polη, polι and polκ called Rev1 interaction region (RIR) is sufficient for their binding to Rev1 (Ohashi E et al. 2009). The role of Rev1 has been elucidated in recent studies. The actual model predicts that Rev1 coordinates the two steps of TLS: damage bypass first and subsequent primer extension. These two steps require the coordination of different TLS polymerases and Rev1 would work as a platform for coordinating polymerases at the fork (Livneh Z et al. 2010; Prakash S and Prakash L 2002). Interactions of Rev1 with the DNA polζ subunit Rev7 would stimulate recruitment of the elongation polymerase displacing the lesion bypass polymerase (Kikuchi S et al.2012; Pustovalova Y et al. 2012). More interestingly, Rev1 may also be able to select the right polymerase to guarantee the most accurate and efficient bypass of the lesion (Ohashi K et al. 2009). Interaction of Rev1 with PCNA mediates PCNA-TLS polymerases binding (Guo C et al. 2006a; 2006b; Wood A et al. 2007). Recent analysis have identified PCNA-Rev1-polη association as a highly dynamic complex that would sample the right polymerase according to the needs (Boehm E et al. 2016). In agreement with these findings Rev1 may have a more prominent role as a scaffolding and regulatory protein in TLS, rather than exploiting its catalytic activity. Its role role is not exclusively nuclear, but it also works in mitochondria (Zhang H et al. 2006). Here it contributes to maintaining mitochondrial DNA stability assisting DNA polγ together with DNA polζ subunits Rev3 and Rev7 (Zhang H et al. 2006).

DNA polκ

In recent years, DNA polκ has earned major interest by studies in the field. This TLS polymerase has been characterised for its activity in the extension step after DNA lesion bypass. The efficient primer extension capability is mainly provided with an N-terminal extension called N-clasp. This region stabilises the catalytic core and little finger (LF) domain favouring DNA binding (Uljon S et al. 2004). This feature makes the polymerase uniquely specialised for elongating distorted primers (Lone S et al. 2007). The tighter interaction with DNA also guarantees a higher processivity than other TLS polymerases of about 20-30, a sufficient elongation before switching with replicative polymerases. It is also the most accurate between Y-family

enzymes on undamaged DNA with a rate of about $6*10^{-3}$ (Ohashi E et al. 2000). The propensity of elongating misaligned or distorted primers favours introduction of frameshifts mutations by DNA polκ. While being unable to replicate past 6-4 photoproducts (6-4 PPs); it accurately bypasses guanine adducts such as the main cigarette smoke adduct benzo-α-pyrene diol epoxide deoxyguanine (Yamamoto J et al. 2008; Rechkoblit O et al. 2002; Liu Y et al. 2014; Zhang Y et al. 2002). Such findings have led to the hypothesis that this polymerase may have evolved to protect cells from environmental pollutants. The hypothesis is demonstrated by studies in mice where the polymerase was deleted (Ogi T et al. 2002). Moreover, DNA polκ has gained more attention due to its involvement in the NER pathway. Initially the role of polκ in NER was discovered in mice cells (Ogi T and Lehmann A 2006). Loss of polκ in this cells caused a considerable reduction of NER activities and thus demonstrated for the first time the role of this in the main UV damage repair pathway. Subsequent studies tried to elucidate the mechanism by which either NER or polκ were chosen by cells to repair UV lesions. The current model predicts the existence of two modes action in the presence of UV lesions after DNA incision by NER (Ogi T et al. 2010). The first implies unmodified PCNA and replicative DNA polε in copying the DNA template. The second one takes advantage of DNA polδ for strand displacement synthesis at first but then employs PCNA ubiquitylation to recruit DNA polκ. The choice between the two pathways was first addressed to the localisation of the lesions: damages on leading strand may be managed by DNA polε, while damages on the lagging strand required DNA polδ and polκ. However, a more plausible explanation is that either type of repair may depend on the conformation of the lesions and on the chromatin environment. Thus, also in the case of DNA polκ its role is not simply related to the TLS mechanism but has been demonstrated to have a wider implication in maintaining genome stability.

1.7 DNA Damage Tolerance

Given the potential mutagenicity of these polymerases, their recruitment at the level of the replication fork must be tightly controlled. The precise localisation of TLS polymerases at stalled forks is regulated mainly by means of specific protein-protein interactions. The sliding clamp PCNA has a central role in this pathway. Apart from being employed to ensure polymerases processivity, the clamp has been recognised to have additional roles in regulating DNA repair and damage bypass (Choe K et al. 2017). Structural data have revealed the organisation of its domains (Dieckman L et al. 2012). The clamp is an homotrimeric protein with each subunit composed of two globular domains. The domains have an inter domain connecting loop (IDCL) which serves as a platform for binding multiple protein partners (Mailand N et al. 2013) (Fig. 9). On the other hand, PCNA interactors have a conserved aminoacidic sequence that provides affinity for IDCL motifs. This sequence is also called PIP-box (PCNA interacting protein) and is composed of the QXX(I/L/M)XXFF consensus where X can be any aminoacid. Slight modifications of the consensus sequence provide a different affinity to PCNA partners. However, the capability of PCNA to interact with multiple partners is not only given by direct interactions but also mediated by specific post-translational modifications (PTMs) of the trimer. PCNA PTMs are crucial for translesion synthesis, in particular ubiquitylation and SUMOylation (Cipolla L et al. 2016). Upon encountering a damage, replicative polymerases are stopped causing replication fork stalling. This event exposes ssDNA which is a substrate for RPA binding (Davies AA et al. 2008). The presence of such intermediate stimulates the activity of Rad6, the E2 ubiquitin ligase that in turn activates Rad18, the E3 ubiquitin ligase (Hedglin M and Benkovic S 2015). Once Rad18 is stimulated, it ubiquitylates PCNA on a key residue, lysine 164 (K164) localized in the IDCL. This modification provides a landing platform with high affinity for specialised TLS polymerases (Kannouche P et al. 2004) (Fig. 10).

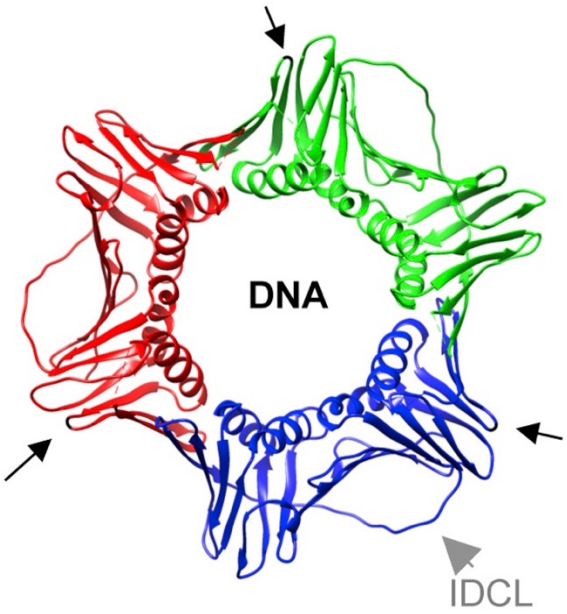


Fig. 9 Structure of the PCNA trimer
The trimer is assembled with identical globular subunits. Domains of each monomer are connected with an interdomain connecting loop (IDCL). Black arrows point at K164 residue, the main regulator of TLS dynamics.
Adapted from: Choe, Katherine N., and George-Lucian Moldovan. "Forging ahead through darkness: PCNA, still the principal conductor at the replication fork." *Molecular cell* 65.3 (2017): 380-392.

Together with a PIP-box in their C-terminal domain, Y-family polymerases also have an UBM or UBZ, ubiquitin binding domains, that confer a higher affinity for the ubiquitylated form of PCNA (Bienko M et al. 2005). Indeed, thanks to PCNA ubiquitylation on K164 the presence of mutagenic polymerases at replication fork is tightly restricted to lesion bypass. Translesion synthesis is not the only mechanism employed to bypass lesions during replication. While TLS uses mutagenic polymerases to overcome DNA lesions, an alternative error free mechanism has evolved.

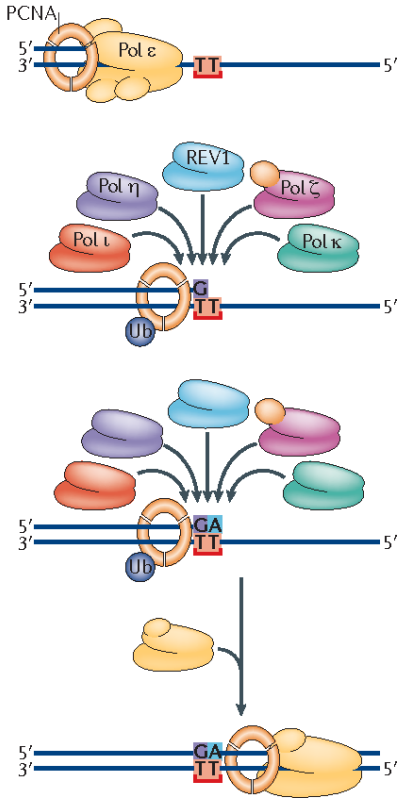


Fig. 10 DNA damage tolerance in human cells.
During genome replication the presence of a lesion stalls the replication fork since replicative polymerases cannot accomodate distorted templates. Stalling of fork activates the DNA damage tolerance pathway (DDT) that leads to PCNA ubiquitylation by the E3 ubiquitin ligase Rad18. TLS polymerases have a high affinity for ubiquitylated PCNA and thus are recruited at the fork to bypass the lesion. After lesion bypass the DNA is further elongated and replicative polymerases can gain access to the fork to restart DNA replication.
Adapted from: Sale, Julian E., Alan R. Lehmann, and Roger Woodgate. "Y-family DNA polymerases and their role in tolerance of cellular DNA damage." *Nature reviews Molecular cell biology* 13.3 (2012): 141.

Modification of K164 on PCNA is the crucial event also in this pathway. This pathway is mediated by K63 linked polyubiquitin chains starting from K164 (Hoege C et al. 2002) (Fig. 10). This modification stimulates a template switch process that uses the newly synthesized sister chromatid to faithfully bypass the lesion. The mechanism resembles homologous recombination by means of strand invasion events. In brief, ssDNA near the lesion is covered by Rad51 and this filament starts homology search with invasion of the homologous region on the sister chromatid. Strand invasion forms a peculiar intermediate called D-loop that must be resolved in a series of events similar to Holliday junctions resolution (Giannattasio M et al. 2014). This template switch pathway has been originally characterized in yeast where the coordinated activity of Ubc13-Mms2 and Rad5, the E2 and E3 ubiquitin ligases respectively, catalyze the formation of the K63 linked polyubiquitin chain on PCNA (Torres-Ramos CA et al. 2002). The search for orthologous of these ubiquitin ligases in mammalian cells has led to the identification of two Rad5 orthologues. The helicase like transcription factor (HLTF) and the SNF2 histone linker PHD RING helicase (SHPRH) (Motegi A et al. 2008; Lin J et al. 2011). However, the role of these enzymes is not well defined yet. In fact, in human cells their loss causes a higher sensitivity to replication fork blocks and PCNA polyubiquitylation is considerably reduced but still detectable (Motegi A et al. 2006, 2008; Unk I et al. 2008, 2010). Instead in mice this sensitivity is lost and polyubiquitylated PCNA is still present (Krijger PH et al. 2011). Thus the players and the mechanistic events occurring during template switch are still to be elucidated. Moreover, how cells decide to shift towards either TLS or template switch still lacks a precise explanation.

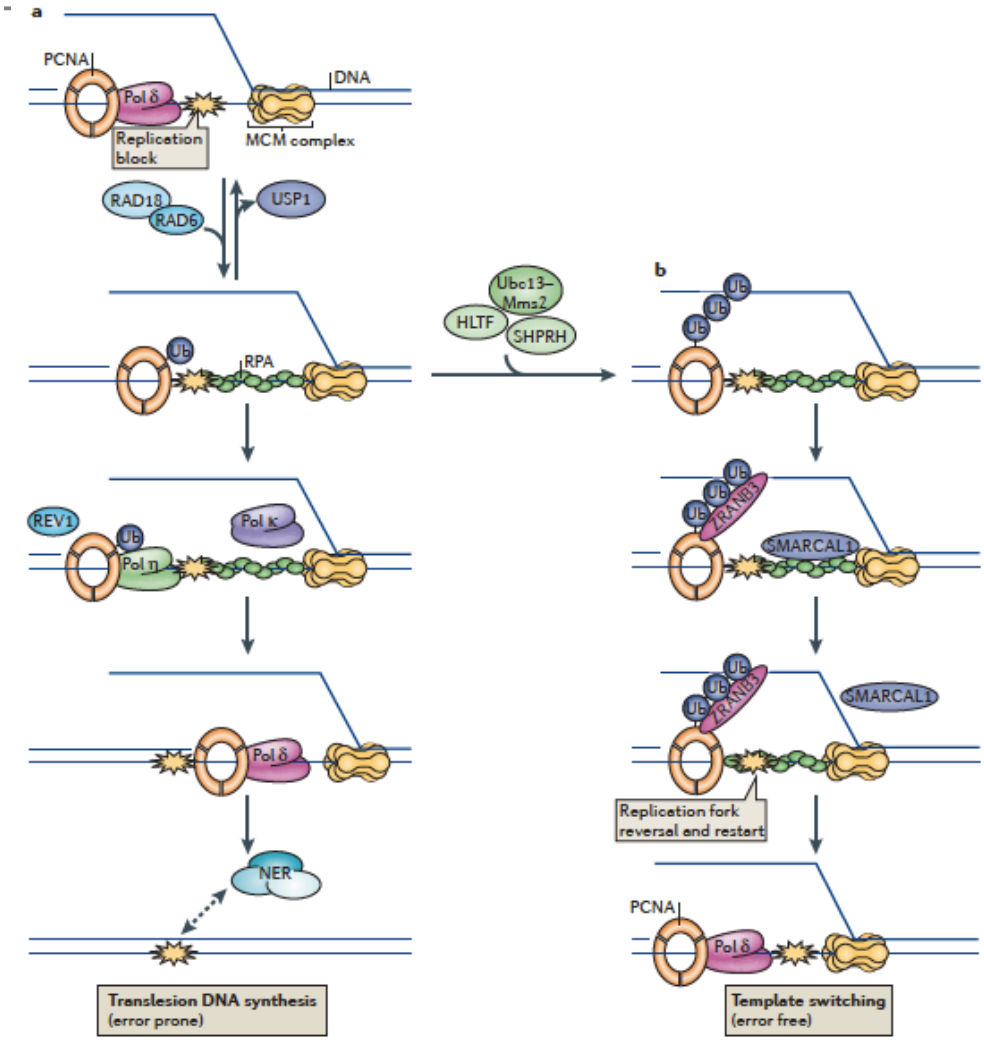


Fig. 11 Error prone and error free damage bypass

A Moboubiquitylation of PCNA upon replication fork stress activates the error prone TLS pathway.

B Poliubiquitylation of K164 with K63 linked ubiquitin chains by the Ubc13-Mm2/HLTF/SHPRH complex stimulates the error free template switch pathway. The translocase ZRANB3 is recruited together with the helicase SMARCAL1. Either fork reversal or template switch facilitate restart of the replication fork.

Adapted from: Mailand, Niels, Ian Gibbs-Seymour, and Simon Bekker-Jensen. "Regulation of PCNA–protein interactions for genome stability." *Nature reviews Molecular cell biology* 14.5 (2013): 269.

1.8 The role of PCNA lysine 164 residue

In the last few years, different studies have tried to define the importance of PCNA ubiquitylation on K164. Although this residue is clearly crucial for DNA damage tolerance (DDT) pathways, some contrasting evidence points to a redefinition of the role of this PCNA modification. One of the main questions in the field is whether TLS directly assists the replication fork during DNA synthesis or whether it fills the gaps left by fork stalling by a post replication repair system. In fact, while the polymerase switch requires K164 ubiquitylation *in vitro* (Masuda Y et al. 2010), its necessity *in vivo* is still matter of debate. Notably, PCNA ubiquitylation can still be detected in G2 phase in yeast, even though it has no influence on TLS (Daigaku Y et al. 2010; Karras G and Jentsch S 2010). In brief, both models would require Y-family polymerases to be recruited on chromatin but PCNA ubiquitylation could be dispensable (Waters L et al. 2009). Across the years different model organisms have been employed to study the role of PCNA K164. Despite the efforts, nowadays the role of PCNA ubiquitylation and the precise definition of either a replication-assisted or post replication repair mechanism are yet to be defined. Early studies have provided details of PCNA post translation modification using the yeast model *S. cerevisiae*. The post translational modification axis that ends with the activation of Rad18 and PCNA ubiquitylation has first been characterized in this model (Hoege C et al. 2002). Seminal works on vertebrate models have taken advantage of the chicken DT40 cell line. This cell line was used to establish a PCNA K164R mutant that disrupts the lysine role as ubiquitination acceptor (Edmunds C et al. 2008). In this cellular background, the replicative and post replicative TLS activities can be detected as clearly distinct pathways with different modes of regulation. In the first case, Rev1 may play a major role in maintaining fork progression at the level of damaged DNA. Indeed, this TLS polymerase possesses a non catalytic function required to guarantee replication forks progression and this function does not require PCNA ubiquitylation. On the other hand, the post replicative system is strongly dependent on PCNA K164 ubiquitylation since K164R mutants presented shorter fragments of nascent DNA after UV irradiation, as isolated by alkaline sucrose centrifugation.

Summarising the data obtained by PCNA K164R DT40 cell lines, DNA damage tolerance could be actually envisioned as composed by two different systems. A Rev1 mediated but Ubi-PCNA independent replicative system that directly assists the replication fork and a post-replicative system that would rely on K164 ubiquitylation (Edmunds C et al. 2008). Concomitantly with the works on DT40 cell lines a mouse model was generated carrying either the heterozygous or homozygous K164R mutation on the PCNA locus (Langerak P et al. 2007; 2009). Notably, homozygous mutated mice were viable, demonstrating that the K164R mutation is not embryonic lethal. Although being viable, homozygous mice were infertile due to a complete lack of germ cells. Hypothesis regarding this phenotype point to a role of PCNA poliubiquitylation in the recombination pathway more than to defects caused by loss of monoubiquitylation. In fact, during meiosis recombination is a crucial mechanism to provide allelic diversity. This pathway generates recombination DNA intermediates that if left unresolved can lead to double strand breaks. Hence, PCNA modifications at the level of K164 have been hypothesised to play a role in stimulating resolution of recombination intermediates (Hoege G et al. 2002). Abrogation of K164 not only disrupts the pro-recombinogenic activity of polyUbi-PCNA but also the anti-recombinogenic activity by conjugation of small ubiquitin like modifier (SUMO) on K164. Since recombination is crucial during meiotic divisions, a general unbalance of this pathway could explain germ cells lethality. TLS activity regulated by PCNA ubiquitylation also has a role in somatic hypermutation (SHM) in B cells (Roa S et al. 2008). This pathway starts with introduction of genomic DNA lesions by the AID enzyme in genomic loci coding for the light chains of immunoglobulins (Ig). These lesions are promptly taken over by the TLS mechanism that bypasses the damage employing Y-family polymerases and thus generating variable point mutations (Di Noia J et al. 2007). Patterns of Ig loci genetic variability can be used to trace TLS activity in these specific genomic regions. TLS polymerases are recruited for SHM by means of PCNA monoubiquitylation on K164. A strong reduction of A/T mutagenesis was observed in PCNA K164R homozygous cells, thus stressing the importance of the modification in this pathway. In accordance with this model, homozygous K164R primary mouse fibroblasts showed a great reduction in DNA polη foci formation after UV irradiation (Langerak P et al. 2007).

In light of the data obtained from mutated mice it could be possible to hypothesise that PCNA ubiquitylation is a mandatory event for the recruitment of TLS polymerases at the level of damaged DNA. Attempts to clarify the role of K164 in mammalian cells have led to different lines of evidence that would define a dispensable role of Ubi-PCNA in DNA damage bypass. For instance, mouse embryonic fibroblasts (MEFs) from PCNA K164R/K164R mice were employed to study defects in TLS (Hendel A et al. 2011). Cells showed a higher accumulation of ssDNA as detected by RPA increase with respect to wt cell lines after UV irradiation. Direct analysis of TLS capability by the introduction of exogenous plasmids carrying a TLS substrate lesion confirmed the existence of a defective damage bypass. More interestingly, knock down of each TLS gene in mice, namely Rev3L, POLH or Rev1 in PCNA K164R/K164R cells caused each an increase of UV sensitivity. Such a phenotype unravels the presence of multiple pathways that have a TLS role in an Ubi-PCNA independent background. Further studies on mammalian cell lines seem to overturn this model. In more detail, the use of the proteasomal inhibitor epoxomycin causes a cellular reduction of ubiquitin and thus indirectly prevents PCNA ubiquitylation. In such experimental conditions, DNA polη foci were still detectable in transformed human embryonic fibroblast cell line MRC5 after UV damage (Sabbioneda S et al. 2008). This evidence would favour the hypothesis that K164 ubiquitylation may be dispensable for DNA polη recruitment in discrete foci upon DNA damage. Subsequent studies on mammalian cell lines have investigated the correlation between DNA polη activation and K164 ubiquitylation. After UV irradiation, DNA polη is phosphorylated by ATR on serine 601 (S601) (Gohler T et al. 2011). This modification does not influence the polymerase catalytic activity but instead may mediate its binding to a yet unidentified protein. Interactions with this protein may ensure its localisation into replication factories. Subsequent binding to Ubi-PCNA would retain the polymerase on chromatin. Furthermore, it was shown that loss of Rad18 activity causes a decrease in Ubi-PCNA while it does not affect DNA polη phosphorylation. In fact, despite the reduction of Ubi-PCNA, DNA polη could still be activated upon DNA damage. In contrast to these data, the UBZ domain of the polymerase is necessary to have an efficient S601 phosphorylation.

Altogether this evidence would point to the presence of another ubiquitylated protein on the chromatin for DNA polη binding and activation. All the efforts on studying the role of K164 in DNA damage tolerance have provided different models according to the model organism. At the moment there is no clear and univocal evidence about the role K164 ubiquitylation. For example, it is still not proven whether Ubi-PCNA is the sole molecular platform for DNA polη recruitment or if other proteins can attract the polymerase thus explaining the presence of DNA polη foci during unperturbed S phase (Kannouche P et al. 2001; Sabbioneda S et al. 2008). Moreover, the timing of molecular events that allow lesion bypass and the dependence on Ubi-PCNA is still unsolved. We still lack a clear evidence on whether the bulk of damage bypass occurs during S phase and thus on the ongoing replication fork, or if lesions are left on the genome until a post replication repair mechanism removes them. It is worth to notice that regarding the human model, all studies have taken advantage of ectopic expression of the K164R mutated transgene and we still lack a cell line carrying the K164R in the genomic locus of PCNA.

PCNA deubiquitylation

Any of the predicted models activated by Ubi-PCNA must be tightly regulated to prevent erroneous activity by TLS polymerases. Thus, once ubiquitylated the modification is removed by specialised deubiquitylating enzymes (DUBs). The ubiquitin specific protease 1 (USP1) in association with USP1-associated factor 1 (UAF1) deubiquitylate PCNA (Huang T et al. 2006; Cohn M et al. 2007). The deubiquitylase is regulated to ensure the proper ubiquitylation/deubiquitylation cycle of PCNA. In fact, after UV damage the increase of Ubi-PCNA directly correlates with a reduction of USP1 transcript (Niimi A et al. 2008; Huang T et al. 2006). However, treatments that affect replication fork progression such as MMS and hydroxyurea do not alter USP1 levels. This observation led to the hypothesis that other DUBs could be present to regulate PCNA ubiquitylation. For instance, USP7 has been shown to deubiquitylate PCNA in vitro and to reduce monoubiquitylation in vivo upon DNA damage (Kashiwaba S et al. 2015). Another recently discovered factor, USP10 has a role in remove ubiquitin from PCNA (Park J et al. 2014). Its loss leads to a high accumulation of Ubi-

PCNA following UV damage, however it does not have an influence on the levels of Ubi-PCNA in undamaged cells. This has led to the hypothesis that it may catalyse deubiquitylation in the recovery phase that follows DNA damage. A different mechanism may be mediated by the ubiquitin protease 10 (UBP10), an USP1 ortholog identified in yeast (Gallegos-Sanchez A et al. 2012). UBP10 levels do not change after exposure to UV damage, thus confirming a possible different mechanism of Ubi-PCNA regulation compared to its human orthologous.

Additional PCNA modifications

Ubiquitylation is not the only modification targeting the K164 residue on PCNA. In fact, other PTMs have been identified during recent years. Another modification of K164 is the link to a SUMO (Small Ubiquitin-like Modifier) molecule. Similarly to ubiquitylation, modifications with SUMO proteins require the action of SUMO ligases for conjugation of the modifier protein on target residues. SUMOylation of PCNA was first identified in yeast together with ubiquitylation of K164 (Hoege C et al. 2002) with the additional K127 residue. In yeast, this modification is used to regulate the HR pathway with an inhibitory effect during S phase. In brief, SUMO-PCNA prevents HR during unperturbed S phase by interaction with the helicase Srs2. This helicase acts on the recombination DNA intermediate and displaces Rad51 from the filament thus preventing strand invasion (Pfander P et al. 2005; Papouli E et al., 2005). In human cells, K164 is still the target of SUMO modification together with K254, differently from yeast K127 (Moldovan G et al. 2012). Also in this case, SUMO-PCNA preserves its inhibitory role on HR by interacting with the Srs2 orthologous helicase, PARI (Moldovan G et al. 2012). However, in humans this post translational modification of PCNA seems to have an additional role in DNA damage. In particular, it has been shown that SUMO-PCNA prevents the occurrence of DNA double strand breaks as demonstrated by γH2AX foci reduction (Gali H et al. 2012). The complex pattern of PCNA post translational modifications on K164 is even more intricate as a new PTM was recently discovered. ISG (Interferon Stimulated gene) modification also known as ISGylation was discovered as an ubiquitin-like PTM relying on the interferon I axis for its activation (Haas A et al. 1987; Loeb K and Haas A et al. 1992).

Similarly, to previous ones, this pathway has specific ISG ligases and PCNA was shown to be ISGylated after UV irradiation on residues K164 and K168 by EFP E3 ligase (Park J et. al 2014). Interestingly, mutations that impede ISGylation cause a higher recruitment of DNA polη at the chromatin level at late time points after UV irradiation (Park J et al. 2014). This evidence, together with the active recruitment of the deubiquitylase enzyme USP10 by ISG-PCNA points to a role for this modification in the post replication repair termination.

1.9 DNA polymerase η

Among the many Y-family polymerases identified across the years, DNA polymerase η is the most well-characterised and the main specialised DNA polymerase responsible for UV damage bypass, in particular CPDs. The gene encoding the TLS polymerase (polη) was isolated in human cells after being correlated with the autosomal recessive syndrome Xeroderma Pigmentosum Variant (XPV) (Masutani C et al. 1999). Patients carrying mutations in the alleles for DNA polη have a higher UV sensitivity and as a consequence a higher probability to develop skin cancers. DNA polη, as the other Y-family polymerases, is composed of an N-terminal domain containing the catalytic site and a C-terminal domain with numerous regulatory motifs (Fig. 12). The catalytic site has a typical “right hand” structure. The core of the active site resides in the palm domain where incoming nucleotides are coordinated by divalent ions and the DNA elongation reaction occurs (Biertumpfel C et al. 2010). Compared to other Y-family polymerases, DNA polη possesses the widest catalytic site, able to accommodate highly DNA distorting lesions. Similarly to the other TLS polymerases, a peculiar domain is present: the PAD domain. This is linked to the C-terminus via a flexible region and it is responsible for the distinctive biochemical features of each Y-family polymerase (Wilson R et al. 2013). The tight interactions between the PAD and the catalytic domain constrain the DNA substrate so that frameshift events are efficiently prevented (Biertumpfel C et al. 2010).

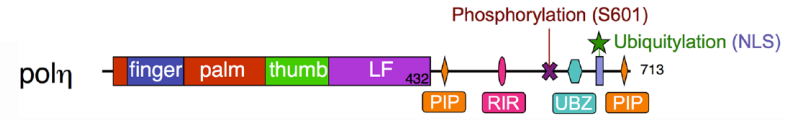


Fig. 12 Structural organisation of DNA polymerase η

As the other Y-family DNA polymerases, DNA polη is encoded as a single polipeptide. The N-terminal domain contains the typical polymerase right hand organisation with the finger, palm and thumb domains. The little finger domain is typical of this family of polymerases and preceeds the C-terminal regulatory region. This region contains numerous regulatory motifs. PCNA interacting peptides mediate PCNA binding. The RIR motif confers capability of binding with Rev1. UBZ domain provides affinity for ubiquitylated targets, including PCNA. A nuclear localisation signal (NLS) is also present for translocations from the cytoplasmic to nuclear compartment. Serine 601 is a crucial phosphorylation acceptor residue for polymerase regulation.

Adapted from: Yang, Wei. "An Overview of Y-Family DNA Polymerases and a Case Study of Human DNA Polymerase η."

Despite conferring the capability to accommodate bulky lesions, the wider catalytic site is not able to exclude incorrect nucleotides incorporation. Thus, DNA polymerase η activity has a dual role in the cell. While being beneficial in the presence of DNA lesions, its activity on undamaged templates can be highly mutagenic. For this reason its presence at the replication fork must be tightly regulated. Control of specialised polymerases can be achieved at different levels. For example, prokaryotes have evolved an efficient transcriptional control of genes after DNA damage, the SOS response (Friedberg E et al. 2006), while eukaryotes rely mostly on post translational modifications (PTMs) of proteins involved in the pathway (Fig. 13). PTMs can either control the spatial localisation of specialised polymerases or regulate directly their catalytic activity. At first, the subnuclear localisation of Y-family polymerases is tightly regulated.

Stalled forks can attract the required specialised polymerases via specific signalling pathways. As already mentioned, the presence of ssDNA stretches is a marker of replication stress and thus leads to the activation of the S phase checkpoint. At the end of the cascade, TLS polymerases gain access to the fork thanks to specific interactions with the sliding clamp, PCNA. The C-terminal regulatory domain of Y-family polymerases contains all amino acidic sequences required for regulation of their sub cellular localisation. As for many other proteins, translesion synthesis polymerases make contact with PCNA thanks to a PIP domain (Moldovan G et al. 2007). This is not the only PCNA interacting motif, two additional ubiquitin binding zinc finger (UBZ) and ubiquitin binding motif (UBM) confer higher affinity for ubiquitylated PCNA (Bienko M et al. 2005). Both the PIP-box and ubiquitin binding domains allow the polymerase switch mechanism with the substitution of replicative polymerases with specialised polymerases (Kannouche P et al. 2004). Post translational modifications on PCNA are not the sole mechanism for Y-family polymerases regulation. Direct modifications of the polymerases can restrict their activity. In the case of DNA polη, ubiquitylation of the polymerase has been extensively characterised (Bienko M et al. 2005, 2010). The ubiquitylation acceptor lysines were mapped in the NLS of DNA polη, partially overlapping with the PCNA interaction region (PIR). Ubiquitylation of the polymerase prevents its unscheduled interaction with PCNA in the absence of DNA damage. When cells are challenged with DNA damaging agents, deubiquitylation of the lysine residues frees the PCNA interaction region and thus permits TLS. Not only direct ubiquitylation/deubiquitylation cycles control DNA polη activity. A further level of complexity to polymerase regulation is given by phosphorilation of the polymerase (Goehler T et al. 2011; Bertoletti F et al. 2017). In this case, ATR was demonstrated to directly phosphorylate DNA polη upon DNA damage when the polymerase is able to interact with Rad18 on the chromatin. The target residue of this phosphorylation is serine 601 (S601) localised in the C-terminal regulatory region. With a mechanism that still needs to be elucidated, phosphorylation of S601 may hypothetically facilitate the exchange of DNA polη from a still undefined protein to ubiquitylated PCNA. This cascade would favour TLS when the ATR signalling cascade is active. Another target residue for phosphorylation is serine 687 (S687).

In this case phosphorylation still influences DNA polη activity, although at a different level. In fact, S687 phosphorylation by CDK2 controls protein stability and tunes DNA polη activity with cell cycle. In particular, it has been hypothesised that this modification, that peaks at the G2/M transition would protect the polymerase from degradation. This intriguing demonstration is in accordance with previous data showing that DNA polη is required during G2 phase of the cell cycle when the bulk of the genome has already been replicated. The idea that DNA polymerase η may have a functional role during unchallenged genome replication is also stressed by its modification by SUMOylation. In fact, it was demonstrated that the polymerase can be modified by addition of SUMO molecules to lysine 163 (K163) in human cell lines (Desprase E et al. 2015). This modification is dispensable for UV damage bypass but required under conditions of replication stress. Rad18 has non-catalytical role in stimulating polη SUMOylation by facilitating the modification by PIAS1 as a SUMO binding enzyme. Thus, it was hypothesised that this modification may be fundamental in the presence of non B-DNA sequences to ensure DNA polη relocation to replication factories. This would facilitate completion of DNA replication in S phase when conditions of replication stress are present. The above mentioned modifications are not the sole post translational modifications in mammalian cells. O-linked β-N-acetylglucosamine modification, namely O-GlcNAc, has been identified as a further PTM widely diffused in the cells (Hart G et al. 2011). Transfer of UDP-GlcNAc substrate relies on an unique O-GlcNAc transferase (OGT) and a single O-GlcNAcase (OGA). DNA polη undergoes cycles of O-GlcNAc conjugation that control its stability. In fact, starting from evidences of the interaction between OGT and DNA polη, it was shown that the alternative polymerase is O-GlcNAcetylated on threonine 457 (T457) (Ma X et al. 2017). The current model identifies this PTM as fundamental for the disassembly of DNA polη from replication forks. In fact mutants in T457 (T457A) showed an impairment in the formation of K48 linked polyubiquitin chains that normally drive the polymerase to degradation. As evident, different levels of mechanisms guarantee polη regulation and ensure that the polymerase is excluded from the replication machinery in the absence of DNA damage.

This preserves the genome from its mutagenic activity. However, even during unperturbed DNA replication, the polymerase is protected by degradation. Recent studies hypothesise that DNA polη may be required to handle replication of genomic regions inherently difficult to replicate. Thus in these cases its wider catalytic site can be exploited for those genomic regions forming non B-DNA structures that would block the replication fork.

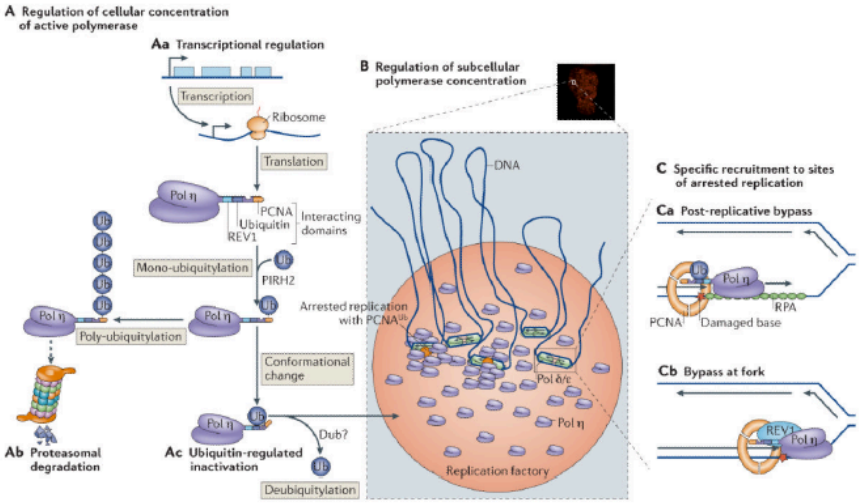


Fig. 13 Regulation of DNA polymerase η

Different levels of regulation control DNA polymerase **η** concentration and activity. Transcriptional control is active in cells, while once translated the polymerase the polymerase can be specifically ubiquitylated by PIRH2 and MDM2 to drive it to proteasomal degradation. Ubiquitylation can be reverted by still unknown DUBs. Another level of control is achieved by regulation of the subcellular localisation of the polymerase. DNA pol**η** has been found associated with replication factories in unperturbed conditions, however the mechanisms for such association are not clear. In the end its recruitment at stalled replicaiton forks is achieved thanks to ubiquitylation of PCNA or by interaction with Rev1.

Adapted from: Sale, Julian E., Alan R. Lehmann, and Roger Woodgate. "Y-family DNA polymerases and their role in tolerance of cellular DNA damage." *Nature reviews Molecular cell biology* 13.3 (2012): 141.

1.10 Replication of difficult to complex DNA regions

The wider catalytic site of DNA polη is exploited by the replication machinery also in processes that are not related with the bypass of exogenous forms of DNA damage. Peculiar regions of the genome can represent a challenge for the replication fork mainly due to their base composition. A clear example of such situation are common fragile sites (CFSs) (Glover T et al. 2017). Metaphase chromosomes were frequently observed to present breaks in such sites. More interestingly, these genomic loci have been proposed to be the cause of chromosomal rearrangements in numerous types of tumours. In fact, if replication is perturbed, these sites are commonly found broken in metaphase chromosome. Nowadays these loci have been precisely mapped after treatment with chemicals that slow the replication fork speed (Le Tallec B et al. 2011). The most used compound with this aim is aphidicolin (Glover T et al. 1984). This drug is capable of inhibiting DNA polymerases but does not block completely DNA replication. A similar compound is hydroxyhurea, a ribonucleotide reductase inhibitor, that has instead a milder effect on fragile sites breakage (Yan Z et al. 1987). Thus, CFSs have been described as genomic loci that are particularly sensitive to replication stress. Their initial genetic characterisation together with recent mechanistic models of CFSs replication have shed light on the possible causes of their instability.

Timing of replication is one of the factors underlying CFSs instability. In fact, many of the identified CFSs are replicated during late S phase and can even remain unreplicated until the G2 phase (Le Beau M et al. 1998). The eventual presence of replication stress would obviously have a consequence on late replicating regions with the possibility of leaving these regions under replicated. Apart from exogenous sources of replication stress, even sequence composition can influence the efficiency of the replication fork. In the last few years many CFSs have been sequenced demonstrating that they are AT rich regions with the presence of AT microsatellites (Boldog F et al. 1997; Ried K et al. 2000; Arlt M et al. 2002). This characteristic correlates with a higher DNA flexibility and a higher propensity to form secondary structures.

As a consequence, these anomalous structures may represent an obstacle for replication fork progression and thus could be the source of replication stress in these regions (Mirkin E et al. 2007). Detailed studies of different CFSs using multiple cell lines have revealed another genetic property of these loci that would further explain replication stress in these regions. In particular, it was demonstrated that CFSs present scarce replication origins. Either directly, by measuring origin firing, or by verifying binding of the pre-replicative complexes, it was shown that these regions have lower density of replication initiation events (Le Tallec B et al. 2011; Letessier A et al. 2011; Miotto B et al. 2016). The scarceness of replication origins forces the replication fork to move for long distances before reaching CFSs further exacerbating the occurrence of replication stress. Finally, transcription of the genes localised in a CFS region can lead to genomic instability. For instance, the majority of CFSs have been mapped in long coding regions. Their length is remarkable and may require an entire cell cycle for transcription with the result of being actively transcribed during S phase. The simultaneous presence of the replication fork and the elongation of the transcript by RNA polII may lead to R-loops formation and finally to instability of the loci (Wilson T et al. 2015; Helmrich A et al. 2011).

1.11 Consequences of CFSs instability

All of these CFSs hallmarks are responsible for their instability (Fig. 14). The main outcome of replication slowing along these sites is their persistence at late stages of S phase as under replicated regions. This in turn causes chromosomal rearrangements that represent the typical CFSs instability phenotype. A number of different mitotic aberrations have been linked to CFSs expression. In particular, persistence of unreplicated DNA until the late G2 or even M phase of the cell cycle may impede its proper condensation into mitotic chromosomes and thus generate breaks and gaps (Minocherhomji S et al. 2015). If replication of these loci is not proficiently completed, then ultra fine anaphase bridges (UFBs) are detectable as non chromatinised DNA linking the two chromatids during M phase. UFBs are responsible for different abnormalities in chromosome separation and thus represent a very dangerous intermediate for the dividing cells (Chan K et al. 2011). Moreover, copy number variations (CNVs) can be the consequences of CFSs instability.

These arise through non canonical breaks repair by template switch micro homology mediated repair (Arlt M et al. 2012; Liu P et al. 2012). Many of the CNVs that arise as a consequence of CFSs instability have been precisely mapped. Interestingly, several of the identified genomic regions upon aphidicoline treatment can also be found mutated in cancer (Wilson T et al. 2015). The majority of the identified lesions are deletions of coding sequences that slightly differ according to the type of tumour. The role of genes containing CFSs in cancer progression is still to be clearly defined. It is clear that the occurrence of deletions in putative tumour suppressor genes may boost cancer progression. The eventual association of these genes with CFSs would make them highly prone to genetic instability and thus increase their probability to be functionally lost. Most of the present work has focused on two CFSs associated genes with putative tumour suppressor functions: the FHIT gene and the WWOX gene (Karras J et al. 2017; Schrock M et al. 2015). While the first has a role in controlling dNTPs balance, the latter is an oxidoreductase whose function in DNA damage is yet to be defined. Loss of either of the two genes in mouse predisposes animals to develop cancer and causes a higher sensitivity to carcinogens (Aqeilan R et al. 2007; Zanesi N et al. 2001). Although other CFSs associated genes have similar functions in cancer development, they are considered to be passenger mutations as the result of a general replication stress phenotype in cancer.

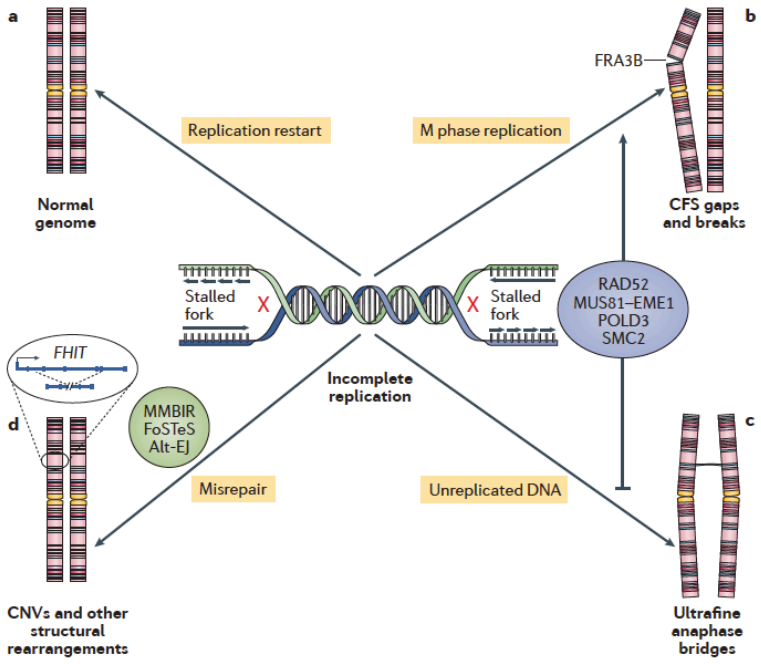


Fig. 14 Consequences of replication stress at CFSs
Properties of CFSs make them highly prone to replication stress. Resolution of replication fork stalling leads to different outcomes for these genomic loci. A) efficient restart of the fork progression may not alter CFSs; B) late stages replication such as during M phase may lead to inappropriate repartition of the chromosomes creating chromosomal breaks; C) presence of unreplicated DNA during mitosis leads to formation of chromosome abnormalities such as ultrafine anaphase bridges; D) attemps to resolve fork stalling with mutagenic pathways may lead to CNVs or other gross chromosomal rearrangements; D) in the end the pre
Adapted from: Glover, Thomas W., Thomas E. Wilson, and Martin F. Arlt. "Fragile sites in cancer: more than meets the eye." *Nature Reviews Cancer* 17.8 (2017): 489.

1.12 CFS replication by TLS mechanisms

Since CFSs are exquisitely sensitive to replication stress, any defect in genes coding for replication associated proteins also causes a higher frequency of CFSs breaks. The first of such genes to be identified was ATR, confirming its role in the maintenance of CFSs stability. In fact, cells lacking the ATR gene have a higher incidence of breaks occurring at CFSs even in the absence of exogenous replication stress (Casper A et al. 2002). Interestingly, also translesion synthesis factors, in particular TLS polymerases have been linked to the maintenance of CFSs stability. Preliminary works showed that upon DNA polη depletion, CFSs breaks increased significatively both in the absence or in the presence of replication stress (Rey L et al. 2009). Afterwards, DNA polη was mapped by chromatin immunoprecipitation (ChIP) at FRA7H.1 , FRA7H.2 and FRA16D (Bergoglio V et al. 2013), showing a greater enrichment when employing a dead-polymerase mutant in which the catalytic site is disrupted. This mutant has a decreased mobility on the chromatin making its presence easier to be appreciated by ChIP (Sabbioneda S et al. 2008). Thus, the model predicts that DNA polη could take advantage of its wider catalytic site to accommodate non-B DNA structures that are frequently found in CFSs. This hypothesis has been confirmed *in vitro* comparing the replication efficiency of DNA polη with that of DNA polδ. The TLS polymerase was able to extend CFSs sequences *in vitro* with a considerable higher efficiency than replicative polymerase even though at the expense of a higher mutagenicity, as demonstrated by the typical TLS DNA lesions bypass signature (Bergoglio V et al. 2013). Deficiency in CFSs replication results in their presence as under replicated regions at late S phase or even M phase (Le Beau M et al. 1998). In accordance to this model and the presumptive role of DNA polη in replicating these regions, loss of the polη gene in mammalian cells caused a higher presence of unreplicated DNA at the level of G2-M phase. Overall, this model suggests that DNA polη catalysis is required when the fork encounters CFSs regions also in the absence of exogenous replication stress. Even if the study cleverly demonstrates the engagement of TLS for replicating past difficult regions, the use of polymerase dead mutants has an impact on cell viability.

In fact, catalytic mutants showed a chronic activation of the checkpoint as demonstrated by phosphorylation of Chk1. This chronic activation of the checkpoint finally leads to apoptosis at higher frequency when compared to polη wt expressing cells. Thus, a different and more sensible approach is required to avoid the use of such mutants but still be able to detect the activity of DNA polη on the genome. In a more recent work the possible mechanism of CFSs replication by TLS was reconstituted *in vitro* (Barnes R et al. 2017). In reconstituted reactions past CFS sequences, after loading of DNA polδ and PCNA on DNA, only the addition of either DNA polη or DNA polκ resulted in the efficient elongation of the primers. Moreover, specific mutagenic signature with T to C transitions was present along the CFS region thus confirming that the replicative polymerase exchanged with the TLS polymerase to elongate the CFS template. More surprisingly, the exchange between replicative and specialised polymerases did not require Ubi-PCNA, once again proposing the presence of a different mechanism for polymerases switch at least *in vitro*. Altogether evidences of the last years suggest a major role for TLS polymerases during replication stress occurring at the level of CFS. Nonetheless, previous studies still lack a precise identification of the mechanisms occurring *in vivo* and the identification of specialised polymerases activity in those regions of the genome.

1.13 Replication of structured DNA

The human genome presents other difficult to replicate regions. For instance, guanine rich sequences can form non B-DNA structures called G-quadruplexes (Sen D and Gilbert W 1988). When ssDNA arises during dynamic processes such as replication and transcription, four adjacent guanines can arrange in a quaternary structure stabilised by hydrogen bonds. Stacking of G-quartets forms a G4 structure extruding the adjacent filaments as ssDNA loops (Davis J 2004) (Fig. 15). Monovalent cations stabilise the structures in the central cavities neutralising the repulsion between guanines (Williamson J et al. 1989).

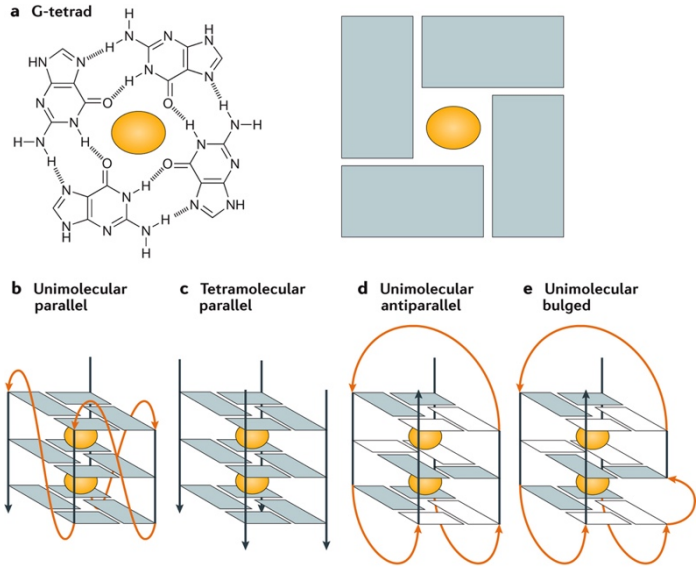


Fig. 15 G-quadruplex structure
G-quadruplex can form from adjacent guanines with the coordination of an ion in the central cavity (a).
More complex structures arise from interactions between the planar tetrads and are classified according to their composition (either a single strand or multiple strands) and their orientation (b-e).
Adapted from: Hänsel-Hertsch, Robert, Marco Di Antonio, and Shankar Balasubramanian. "DNA G-quadruplexes in the human genome: detection, functions and therapeutic potential." *Nature Reviews Molecular Cell Biology* 18.5 (2017): 279.

Despite being deeply characterised *in vitro* in the last few years, G4s have been first computationally predicted (Todd A et al. 2005; Huppert J and Balasubramanian S et al. 2005) and only recently identified and mapped *in vivo* in mammalian cells (Biffi G et al. 2013; Henderson A et al. 2014). Sequencing of G4s has revealed they are not randomly distributed and they are conserved among organisms from yeast to mammalian cells. These properties may suggest a functional role for these structures (Capra J et al. 2010).

A high prevalence of G4 structures has been identified at the level of telomeric DNA given by the high GC content and the presence ssDNA (Henderson E et al. 1987). Additional G4 sequences have been identified at satellites, near promoters, at ribosomal DNA and mitotic or meiotic DSBs (Capra J et al. 2010; Hershman S et al. 2008; Huppert J et al. 2005; Rawal P et al. 2006; Nakken S et al. 2009). G4s at telomeric regions have been extensively characterised and have served for modelling G4s resolution. Telomeric DNA protects linear chromosomes from shortening and prevents chromosomal aberrancies during cell division (Zakian V 2012). Intriguingly, telomeric regions consist of G rich tracts prevalently running 5' to 3' from centromeres to telomeres. These stretches are longer than their complementary strand thus forming a G-rich tail. Hence, telomeric tails have been demonstrated to form G4 structures *in vitro* (Henderson E et al. 1987; Sundquist WI et al. 1989). Most of the studies on G4s telomeric DNA employed the ciliate Stylonychia lemnae as a model organism, and relied on the development of antibodies against its G4 telomeric DNA. These studies led to identification of G4 telomeric DNA *in vivo* and its mechanism of assembly and unfolding (Paeschke K et al. 2005; Schaffitzel C et al. 2001; Paeschke K et al. 2008). The relationship between DNA replication and folding/unfolding of these structures was first demonstrated in this model organism. In particular, it was shown that these structures are actively resolved during DNA replication to prevent fork stalling (Schaffitzel C et al. 2001). TEBPβ is phosphorylated during genome replication and as a consequence disassembled from G4 structures. Its disassembly favours G4 resolution. Interestingly also telomerase and helicases of the RecQ family are necessary for G4 unfolding at late stages of the S phase (Paeschke K et al. 2005; Paeschke K et al. 2008; Juranek SA et al. 2012; Postberg J et al. 2012). The role of telomerase in G4 unwinding is still to be defined. Evolutionary conservation of TEBP proteins suggests that similar mechanisms may be conserved in vertebrates. Lack of specific G4 binding molecules against higher organisms G4 structures has left their resolution mechanisms undetectable for years. Only recently two specific antibodies have been developed, namely BG4 and 1H6 (Biffi G et al. 2013; Henderson A et al. 2014). Immunofluorescence analysis with these antibodies in mammalian cells revealed a cell cycle dynamic for G4 formation with a peak during S phase.

Moreover, staining of metaphase chromosomes revealed a telomeric localisation even though this was not exclusive with diffused staining along the chromosomes (Biffi G et al. 2013). Telomerase and G4 binding antibodies co-stained in a relevant number of chromosomes thus confirming the data obtained in the ciliate specie (Moye A et al. 2015). Chemical synthesis of G4 trapping compounds has expanded the possibilities of their detection and dynamic analysis (Rodriguez R et al. 2012; Huang WC et al. 2015; Shivalingam A et al. 2015). G4 stabilising compounds and in particular pyridostatin (PDS), have been employed in a newly established sequencing protocol, G4-seq. Stalling of the polymerase during sequencing reaction at the level of stabilised G4 structures has allowed their precise mapping (Chambers V et al. 2015). Such sequencing maps have revealed a higher number of G4 than expected in the human genome, arriving at about 700,000. Interestingly, these structures matched with promoters and 5' UTRs and correlate well with genes overexpressed in numerous cancers (Chambers V et al. 2015). The use of G4 stabilising compounds combined with the use of specific anti-G4 antibodies are a promising strategy for the precise capture and sequencing of G4 structures and analysis of their dynamic formation.

1.14 Consequences of G4s on genome replication

The high prevalence of G4 outside of telomeres poses the question of how transcription of replication can resolve their structure without blocking any of the two processes. As already mentioned G4s are present at the level of many promoters. More interestingly, they were found in promoters of well known oncogenes such as myc and kras (Siddiqui-Janin A et al. 2002; Cogoi S et al. 2006). By using G4 ligands, the expression of these genes was altered, thus suggesting that G4 structure may have a role in transcription of these loci. In addition to this, binding of G4 antibodies is enriched at transcriptionally active chromatin regions in human cells (Haensel-Hertsch R et al. 2016). Across the years different helicases have been identified as G4 resolving enzymes. These comprise the Werner syndrome protein (WRN) and the Bloom syndrome protein (BLM) and mutations in these helicases predispose to cancer (Monnat et al. 2010). In addition, their alteration influences transcription of genes that are predicted to have G4 motifs (Johnson J et al. 2010; Nguyen GH et al. 2014; Tang W et al. 2016).

Recent findings speculate that the role of G4s in transcription is highly dynamic and can be thought as an additional layer of transcription regulation. However, the presence of structured DNA constitutes an obstacle to the replication fork progression (Fig. 16). As many other lesions encountered during genome replication cells need to resolve such non B-DNA structures to avoid replication stalling. Apart from being present as regulatory elements, G4 motifs may also form transiently and spontaneously during DNA replication. In particular, during lagging strand synthesis that transiently exposes ssDNA or upon fork slowing. In any of the previous cases G4s must be resolved and different helicases have been hypothesised to unfold them. A plethora of DNA helicases have been studied for their G4 unwinding activity over recent years, however WRN, BLM, FANCJ and Pif1 are the best characterised. The most characterised in human cells is FANCJ. Mutations of this helicase cause a form of Fanconi anaemia and patients carry genomic deletions in regions that are predicted to have G4 structures (London TB et al. 2008). In addition, the use of telomestatin, a G4 stabilising compound, provokes a higher level of DNA damage in the FANCJ mutated cells and finally leads to apoptosis (Wu Y et al. 2008). Pif1 helicases instead was initially studied in the *S. cerevisiae* yeast model (Paeschke K et al. 2011). Genomic mapping of Pif1 binding revealed that it binds around 25% of the total G4 predicted sequences in this organism. In human cells PIF1 has been hyphotesised to have a similar role. Treating human cell lines with pyridostatin as a G4 stabiliser, caused DNA damage detectable as γ–H2AX foci. These foci co-localised with fluorescently labelled PIF1, indicating a possible role of this helicase at the level of G4 motifs (Rodriguez R et al. 2012). The occurrence of DNA damage was already documented in yeast. In fact Pif1 deficient cells had a high mutation rate at the level of G4 involving a possible error prone process during their replication (Paeschke K et al. 2011).

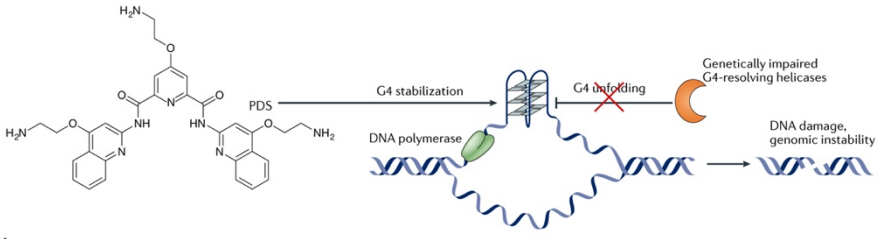


Fig. 16 Consequences of G4 stabilisation
G4s can cause replication fork stalling. Presence of either G4 stabilising compounds (such as Pyridostatin) or mutations that impair helicases activity prevent G4s resolution. Unresolved G4 structures may trigger double strand breaks (DSBs). Stabilisation of G4s to cause DSBs may be a promising strategy to treat cancer in a DSBs repair defective background.
Adapted from: Hänsel-Hertsch, Robert, Marco Di Antonio, and Shankar Balasubramanian. "DNA G-quadruplexes in the human genome: detection, functions and therapeutic potential." *Nature Reviews Molecular Cell Biology* 18.5 (2017): 279.

1.15 Replication of structured DNA by TLS mechanisms

A possible involvement of TLS and their specialised polymerases in replicating past G4 sequences was documented. In detail, Rev1 has been demonstrated to take part in replication across G4 motifs in DT40 chicken cells (Sarkies P et al. 2010). Moreover it was shown to be able to bind and disrupt G-quadruplex DNA *in vitro* displacing and stabilising the template (Eddy S et al. 2014). To have more insights into the role of TLS in bypassing G4 structures, the role of the main TLS polymerases DNA polη, DNA polκ and DNA polι was investigated (Betous R et al. 2009). Analysis were performed in cell lines where each of the three polymerases were independently and stably silenced via shRNA. Use of telomestatin, a G4 ligand, did not influence the survival of DNA polι depleted cells while showing a great decrease in survival rate for both DNA polη and DNA polκ depletion.

This would suggest that while DNA polι may not have a role in G4 structures resolution, the other two TLS polymerases may be directly implied in the resolution of structured DNA. Loss of the specialised polymerases increased the presence of DSBs events, events that have been linked to replication fork stalling when G4 motifs cannot be resolved. Supporting a role of TLS in G4 resolution, DNA polη was shown to be able to bind G4 motifs and replicate across them *in vitro* (Eddy S et al. 2015). During last few years there is mounting evidence on the role of specialised polymerases in assisting replication past structured DNA. However, how replication fork progresses through these sites and the coordination between the S phase checkpoint and Y-family polymerases recruitment is yet to be defined. Understanding the mechanisms of G4 resolution and the response of the replication fork to these structures will be fundamental. The presence of G4 motifs in many oncogenic sequences has raised the interest to the analysis of the molecular events that permit their resolution, since any factor involved would be a promising druggable target.

2. Aims of the research

DNA translesion synthesis has been proven to be a critical damage tolerance system essential for the survival of the cells after genotoxic stress. New evidence suggests a more widespread role for TLS also in normal genome replication. The role of polymerase η and PCNA ubiquitylation in this process is still ill defined and our aim was to establish new tools and strategies to ascertain their function in mammalian cell lines. One of the goals of this project was to establish a reliable cell system for tracking TLS polymerase activity in human cells. We propose to alter the sugar discrimination of polymerase η, as a proof of concept, and study how this tool could help unravelling how TLS works both in the absence and in the presence of DNA damage. The final aim of the study will be to construct DNA libraries from ribonucleotides containing genomic DNA for NGS sequencing. Activation of the DNA damage tolerance pathway relies on PCNA and in particular on post translational modifications of the K164. Previous works, in a number of model systems, have not unequivocally defined the role of this residue, especially in human cells. To this end, we propose to establish a human cell lines mutated to K164R by the CRISPR/Cas9 technique in MRC5 and XP30RO backgrounds that differ for the presence or the absence of DNA polη respectively. Overall the establishment of such human cell lines would help in clarifying the role of modified PCNA K164 in translesion synthesis. Finally, DNA polη has also been involved in the replication of structured DNA sequences such as the ones formed by G-quadruplexes. Preliminary results suggest that polη could replicate past G4 DNA *in vitro*, but its role *in vivo* is only speculative. We plan to verify its role after treatment with a G4 stabilising compound, especially by comparing the activation of the DNA damage response in polη deficient and polη proficient cells. Ultimately, this will help shedding some light on the role of the polymerase in G4 quadruplexes methabolism, a new and exciting target for the development of novel cancer therapies.

3. Materials and methods

Table 1. List of primers

1	5’ CTCGTGGACATGGACTGTTTTGCCGTTCAAGTGGAGCAGCG 3’
2	5’ CGCTGCTCCACTTGAACGGCAAAACAGTCCATGTCCACGAG 3’
3	5’ ACCACAGTCCATGCCATCAC 3’
4	5’ TCCACCACCCTGTTGCTGTA 3’
5	5' TAATGCGTCCCCTTGTGACT 3'
6	5' GGCAGATTTTAGTCCCTCAGC
7	5' TGAGCCATTCTGTCACCAAG 3'
8	5' AACCTTCCTACTGCCTGCTG 3'
9	5' TCCTGTGGAAGGGATATTTA 3'
10	5' CCCCTCATATTCTGCTTCTA 3'
11	5' TGTTGGAATGTTAACTCTATCCCAT 3'
12	5' CATATCTCATCAAGACCGCTGC 3'
13	5’ATATGCCGAGATCTCAGCCATATTGGAGATGCTGTTGTAATTTCTTGTGCAAGGGAC GGAGTGAAGTTTTCTGCAAGTGGAGAACTTGGAAATGGAAACA 3'
14	5'TGTTTCCATTTCCAAGTTCTCCACTTGCAGAAAACTTCACTCCGTCCCTTGCACAAGAAA TTACAACAGCATCTCCAATATGGCTGAGATCTCGGCATAT 3'
15	5' GATCGGAATTCTTTGTTCTTAATAGGAACAG 3'
16	5' GATATGGTACCTCCTAACAATGTTATGAGG 3'
17	5' GTCATCCTAGGAAGAGCTTAGCATATGCTGTG 3'
18	5' GTCTAGTCGACTATCTTACCAAGGGGTACATC 3'
19	5' GTAATTTCCTGTGCAAGGGACGGAGTGAAATTTTCTGCAAGTGG 3'
20	5' CCACTTGCAGAAAATTTCACTCCGTCCCTTGCACAGGAAATTAC 3'
21	5' TCTGAGTTGTTTAGGTGTTGCC 3'
22	5' TCTGAGTTGTTTAGGTGTTGCC 3'
23	5' AGGAGGCCTTCCATCTGTTGC 3’

Cell culture and treatments

SV-40 transformed human primary fibroblasts, MRC5V1 and XP30RO human fibroblasts from XP-V patients have been cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. In the case of XP30RO cells complemented with transgenes carrying neomycin (G418) resistance medium was supplemented with 0.5 mg/mL G418. Cells were sorted with Biorad S3 Fluorescence Automated Cell Sorter (FACS) for eGFP to monitor transgenes expression. XP30RO complemented stable cell lines were obtained by integration of the polη wt or polη F18A transgenes. In brief, $3*10^5$ cells were seeded and transfected using Viafect (Promega) transfection protocol according to manufacturer's instructions. The day after cells were diluted into 10 cm dishes in DMEM 10% FBS supplemented with 1 μg/ml of G418. Surviving colonies were picked after 15 days using cloning disks (Sigma Aldrich). Single colonies were expanded and screened for the presence of eGFP DNA polη by western blot analysis. Growth rate of each line was calculated by seeding the same number of cells and counting at different time points. All values were normalised on the initial seeding numbers.

Transfections were performed using Viafect (Promega) according to manufacturer's instructions. Briefly, plasmids were dissolved into Opti-MEM medium (Gibco) and 3 μL of Viafect for each μg of plasmid were added to the mix. Transfection mixes were vortexed thoroughly and incubated at RT for 15 min. After that, the mix was added dropwise to the cells. Cells were incubated overnight and, unless otherwise stated, further analysis were performed after 24 hours.

UV irradiation was performed at indicated UV doses after removing media from the cells. Recovery was performed in DMEM 10% FBS at indicated timepoints. Unless otherwise stated cells were fixed with 4% paraformaldehyde for immunofluorescence analysis or lysed for SDS PAGE as indicated afterwards.

Hydroxyurea (HU) treatment was done by dissolving HU 1 M into DMEM 10% FBS. Dilutions of the concentrated mix were used to supplement media at indicated concentrations. Cells were grown in the HU supplemented media for 24 hours and then either fixed for FACS analysis or lysed for SDS page as indicated afterwards.

Pyridostatin (PDS) treatment was performed by diluting a 5 mM solution into DMEM 10% FBS at the indicated concentration. Cells were incubated in the PDS supplemented media for 24 hours and then lysed to obtain total protein extracts or cell's fractions for SDS PAGE as indicated afterwards.

Plasmids and cloning

To obtain the eGFP-polη constructs for *in vivo* analysis, we used the N-terminal eGFP tagged full length cDNA of DNA polη wt in a peGFP-C3 vector (Clontech) (Kannouche et al. 2001). The plasmid was mutagenised to obtain F18A mutant by Quickchange site directed mutagenesis (Strategene) with the indicated primers (Table 1, primers 1 and 2). For protein expression and purification, we mutagenized C-term His tagged full length DNA polη cDNA contained in a pET-21 vector by site directed mutagenesis as previously indicated. Either TOP10 or BL21-λde3 Codon+ *E. coli* chemically competent cells (NEB) were transformed with eGFP tagged or His tagged constructs respectively. Competent cells were heath shocked by rapid incubation at 42 °C followed by incubation on ice and plating on LB-agar plates to isolate single colonies. MIDI preparations from single bacterial colonies using Macherey Nagel Nucleobon Xtra MIDI kit according to manufacturer's instruction. Presence of the desired mutation was verified by sequencing plasmids. For generating a plasmid for homologous recombination following CRISPR/Cas9 genome targeting, we used the pDonor-D09 plasmid (Genecopoeia). Homology arms were subcloned from genomic DNA amplification of the PCNA locus spanning 1130 bp before and after the site targeted by the gRNA. Target regions were amplified by PCR using primers to insert restriction sites (Table 1, primers from 15 to 18). EcoRI and KpnI restriction sites were added for cloning the left homology region while SalI and AvrII restriction sites were added for right homology region. Both plasmid and amplification products were digested using the

indicated restriction enzymes according to manufacturer's instructions (NEB). Vector and inserts were purified and ligated overnight using T4 DNA ligase (NEB). Ligated products were transformed by heat shock into *E. coli* TOP10 chemically competent cells (NEB). Isolated colonies were expanded and plasmid DNA was purified by MIDI preparation (Macherey Nagel). To obtain K164R plasmid as homology donor template we mutagenised the constructs by Quickchange site directed mutagenesis (Stratagene) according to manufacturer's instructions with the indicated primers (Table 1, primers 19 and 20). Mutated products were transformed and plasmids isolated from single bacterial colonies were sequenced to check for the presence of the K164R mutation.

Total RNA extraction and retrotranscription

Total RNA was extracted using Nucleospin RNA kit (Macherey Nagel) according to manufacturer's instructions. Samples were quantified by spectrophotometric analysis and 1 μg of total RNA was used for retrotranscription. cDNA was obtained by retrotranscription with M-Mulv Reverse Transcriptase kit (Roche). Relative quantity of cDNA in each sample was assessed by amplification of GAPDH locus by PCR using the indicated primers (Table 1, primers 3 and 4).

Immunofluorescence

For detection of epifluorescence, cells were fixed with 4% paraformaldehyde (PFA) and coverslips directly mounted using Aqua-Poly/Mount (Polyscience). For immunofluorescence staining using the S9.6 antibody, cells were washed and then fixed with 4% PFA and then permeabilised with 0.5% Triton X-100. After that, slides were treated with 0.5% SDS followed by blocking with 3% BSA - Tween 20 0.1% - 4x SSC. Incubation with S9.6 antibody was performed at 1 ng/μL concentration in 3% BSA - Tween 20 0.1% - 4x SSC and incubated overnight at 4 °C. Fluorescent secondary antibody, Alexa Fluor 555 was used at 1:1000 dilution in 3% BSA - Tween 20 0.1% - 4x SSC and incubated for 1 hour at room temperature. Nuclei were stained with DAPI and coversliprs mounted as previously indicated. RNase HII treatment was performed prior to fixation. In brief, cells were permeabilised with 1% Tween 20 in PBS 1x for 10 min on ice.

After that, 10 U of E. coli recombinant RNase HII (NEB) were dissolved in ThermoPolBuffer 1x and the solution was used to treat slides at 37° C for 25 min in a humid chamber. Afterwards, cells were fixed with 4% PFA and stained with S9.6 antibody as previously indicated.

Clonogenic assay

Cell lines were seeded and UV irradiated the following day at increasing UV doses. After 15 days cells were fixed with 70% ethanol and stained with methylene blue (0.1% Page-Blue, 50% methanol, 7.5% acetic acid). Plating efficiency was calculated and used to normalise values.

Cell cycle analysis

Cells were seeded and the day after treated with either UV or HU to monitor cell cycle progression. After 24 hours cell were resuspended in PBS and fixed in cold ethanol to a final concentration of 70%. Fixed samples were left at 4 °C overnight. Once fixed, cells were pelleted by centrifugation at 1500 rpm for 5 min. Pellets were resuspended in 50 μg/mL propidium iodide, 5 μg/mL of RNase A in PBS 1x, 0.1% Tween 20. Solutions were incubated at 37 °C for 15 min and then analysed at FACS.

Cell's viability assay

Cell's viability was assessed by staining with Trypan Blue. In brief, cells were detached with trypsin-EDTA solution and diluted in a 0.4% Trypan Blue solution at 1:1 volume ratio. Percentage of viable cells was obtained by counting unstained vs stained cells.

SDS PAGE and western blot

Whole cell extracts were obtained by resuspension of cells into Laemmli buffer 1x (0.1% beta-mercaptoethanol, 10% glycerol, 2% SDS, 63 mM Tris-HCl pH 6.8). Proteins were separated by SDS PAGE and transferred onto 0.22 μm nitrocellulose membrane. 5% skim milk in PBS 1x, 0.1% Tween 20 was used as a blocking agent. Primary antibodies were all incubated overnight at 4 °C. After that, membranes were incubated with HRP-conjugated secondary antibodies and signal revealed by chemiluminescent substrates (Millipore). A LAS500 Imager was used for signal detection coupled with ImageQuant software for immunoblots analysis (GE-Healthcare).

Table 2. List of primary antibodies

anti-Check1	mouse monoclonal	(Cell Signal)
anti-DNA polη	rabbit polyclonal	(Abcam)
anti-histone H3	rabbit polyclonal	(Abcam)
anti-PCNA (pc10)	mouse monoclonal	(custom made)
anti-phosphoCheck1	rabbit monoclonal	(Cell Signal)
anti-Rad18	mouse monoclonal	(Abcam)
anti-RNase H2A	rabbit	(Abcam)
anti-RPA	rabbit polyclonal	(Abcam)
anti-tubulin	mouse monoclonal	(Sigma Aldrich)
anti-UbiPCNA	rabbit monoclonal	(Cell Signal)
anti-vimentin	mouse monoclonal	(Sigma Aldrich)

Cell fractionation

Cells were lysed in a hypotonic buffer (10 mM Tris-HCl pH 8, 2.5 mM MgCl2, 0.5% Igepal, 1 mM dithiothreitol, protease and phosphatase inhibitors) and incubated on ice for 1 min. Soluble fraction was obtained by centrifugation at 6000 rpm at 4 °C for 1 min, an aliquot of supernatant was used for protein quantitation by Bradford assay and samples were resuspended in Laemmli buffer for SDS PAGE. Pellets were washed in hypotonic buffer and centrifuged at 6000 rpm at 4 °C for 1 min.

After that, pellets were washed in isotonic buffer (10 mM Tris-HCl pH 8, 150 mM NaCl with protease and phosphatase inhibitors) and centrifuged again as previously indicated. To release chromatin-bound proteins, DNA was digested with benzonase (Merck) according to manufacturer's instructions, for 30 min at 4 °C. Chromatinic fractions were isolated as by centrifugation at 13200 rpm at 4 °C for 1 min. Samples were diluted with Laemmli buffer 1x for SDS PAGE as previously indicated.

Transcription and translation reactions

In vitro transcription and translation reactions (TNT T7 Quick Coupled Transcription/Translation System, Promega) were performed according to manufacturer's instructions. In brief, 1 μg of plasmids coding for His-tagged polη were incubated with the TNT master mix for 90 min at 30 °C. Aliquotes were immediately frozen in liquid nitrogen and stored. An aliquot was taken to check for DNA polη by western blot analysis.

Purification of 6His-DNA polη F18A

BL21-λde3 Codon+ *E. coli* competent cells were heat-shock transformed with a codon optimized plasmid carrying the C-terminal His tagged DNA polη F18A full length cDNA. Glycerolate stocks were used to inoculate 300 mL of LB medium supplemented with 50 μg/mL of ampicillin and 25 μg/mL of chloramphenicol. Culture was grown overnight at 30 °C, shaking at 200 rpm. Growth rate was calculated by calculating spectrophotometric absorbance at 600 nm (OD600). Culture was diluted to an OD600 of about 0.1 into 3 liters of culture medium supplemented with ampicillin and chloramphenicol at previously indicated concentrations. The 3 L culture was incubated at 37 °C until it reached an OD600 of 0.6. At this point the culture was immediately chilled on ice for 20 minutes and induction was performed adding IPTG to a final concentration of 0.5 mM. Following induction, the culture was grown at 12 °C for 40 hours. After 40 hours the entire culture was centrifuged at 5000 rpm for 10 min at 4 °C. Supernatant was discarded and pellets were resuspended in 15 mL of resuspension buffer 10 mM KPO4, 300 mM NaCl, 10% glycerol, 0.2 mM PMSF supplemented with protease

inhibitors (Roche). 30 mg of lyophilised lysozyme (Sigma Aldrich) were added directly to the 15 mL bacterial suspension to lyse bacterial cells. Bacterial suspension with lysozyme was incubated at 4 °C for 10 min rocking. To get rid of bacterial debris lysates were sonicated in a Bioruptor sonicator for 10 cycles (30 sec ON followed by 30 sec OFF). Debris were removed by centrifugation at 15000 g for 15 min at 4 °C. Supernatant was used for incubation with the affinity resin. An aliquot of 1 ml TALON affinity resin (Clonetech) was prepared by eliminating ethanol with centrifugation at 1000 rpm for 5 min. Afterwards the resin was equilibrated with 10 mM KPO4, 150 mM NaCl, 5% glycerol and 5 mM Imidazole, PMSF at 0.2 mM and protease inhibitors were added just before incubation with the lysate. The equilibrated resin was centrifuged and supernatant discarded. The lysate was directly mixed with the resin and incubated for 30 min at 4 °C. The entire volume was loaded on a column for gravity flow purification at 4 °C. After a first passage the collected flow was loaded again on column and left flowing. Column was washed twice with 10 mM KPO4, 300 mM NaCl, 10% glycerol, 10 mM Imidazole and 0.2 mM PMSF together with the addition of protease inhibitors. After washing, fractions were eluted in 1 mL of elution buffer (10 mM KPO4, 300 mM NaCl, 10% glycerol, 300 mM imidazole, 0.2 mM PMSF and protease inhibitors). Aliquotes from eluted fractions were taken for western blot analysis. To remove imidazole, the eluted fractions were desalted using desalting columns (GE-Healthcare) in 10 mM KPO4, 150 mM NaCl, 10% glycerol with 0.2 mM PMSF with protease inhibitors. Eluted fractions were aliquoted and immediately frozen in liquid nitrogen.

Primer extension assays

For TNT reactions, an aliquot was used for primer extension assay of a template containing a thymidine dimer (CPD) at position +13/+14: 5' CTCGTCAGCATCT-TCATCATACAGTCAGTG 3' annealed with a 5' FAM labelled 13mer 5' CACTGACTGTATGATG 3'. TNT products were quantified by western blot analysis while purified His tagged proteins were quantified by Comassie stain. Comparable amounts of proteins were used for the primer extension assays. Primer extension assays were performed in 10 µl of replication buffer (40 mM Tris–Cl pH 8, 5 mM MgCl2, 10 mM DTT, 0.25 mg/ml acetylated BSA, 60 mM KCl, 2.5% glycerol).

dNTPs or NTPs were used at a 100 μM concentration of each nucleotide. All reactions were incubated at 37°C for 15 min and stopped with 10 μl of 2× loading buffer, (98% deionized formamide, 10 mM EDTA pH 8, 0.025% xylene cyanol and 0.025% bromophenol blue). Samples were boiled at 95°C for 5 min and rapidly chilled on ice. Reaction products were resolved on a 15% acrylamide-7 M urea gel and then imaged on a Typhoon imager (GE HealthCare).

Post replication repair assay

Cells were seeded and the day after irradiated with 8 J/m^2 as previously described (Bienko M et al. 2010). After irradiation, cells were incubated in 2 ml of DMEM 10% FBS supplemented with 0.3 mg/ml caffeine for 30 min before the addition of 1.85 MBq of ^{3}H-thymidine. After irradiation cells recovery occurred in fresh media supplemented with 10 μM thymidine and 10 μM deoxycytidine for 150 min. At the end of the chase period, cells were suspended in PBS containing 0.2 g/L of ethylenediaminetetraacetic acid (EDTA). After that cells were lysed in 0.2 mL of lysis buffer (1% SDS, 0.75 M NaOH, 0.75 M NaCl, 20 mM EDTA) and layered on top of 5 ml of a 5–20% sucrose gradient (in 0.1 M NaOH, 0.1 M NaCl). Gradients were exposed to visible light for 1 hour and then centrifuged at 38500 rpm for 70 min. In the end, 25 fractions were spotted on Whatman grade 17 paper, followed by precipitation in trichloracetic acid (TCA) and washed twice in 70% ethanol before being counted on a Perkin Elmer scintillation counter. The weight-average molecular weights of the distributions were calculated as omitting the top and bottom three fractions.

Purification of S9.6 antibody

The HB-8730 hybridoma cell line was purchased from ATCC. Cells were cultured according to manufacturer's instructions. The supernatant was collected and used for purification by affinity chromatography. A Sepharose protein G resin (GE-Healthcare) was used for antibody purification. Resin was diluted into cold PBS 1x and left sediment in 10 mL purification column. Once thawed overnight at 4°C, supernatant was buffered with 1/10 of volume of PBS 10x. Sample was loaded on column and flow-through was saved for a second round of flow. Column was washed 5 times with PBS 1x.

A pre-elution step was performed with 10 mM phosphate buffer pH 6.8. After that, elution was performed with 200 mM glycine pH 2.5 in 1/10 of elution volume in 1.5 M Tris pH 8.8. Aliquotes of eluted fractions were used for analysis by TGX gel (Bio-Rad).

Detection of genomic embedded ribonucleotides

Detection of ribonucleotides in genomic DNA was performed as previously described (Meroni A et al. 2017). In brief, genomic DNA was extracted from cells using Macherey-Nagel Nucleospin Tissue kit according to manufacturer's instructions. 1 μg of genomic DNA was used for *E. coli* recombinant RNase HII (NEB) digestion. Genomic DNA was either treated or mock treated with 1 U of RNase HII at 37 °C for 2 h and 30 min shaking at 550 rpm. After that, DNA was precipitated with 1/10 volume of sodium acetate 3 M pH 7 and 2 volumes of cold ethanol. Samples were left precipitating overnight at -20 °C. Pellets were obtained by centrifugation at 13200 rpm at 4° C for 30 min, followed by wash with 1 mL of cold ethanol 70% and a further spin at 13200 rpm at 4 °C for 15 min. Pellets were left drying and finally resuspended into 25 μL of ddH2O overnight at 4 °C. Samples were run on a 1% agarose gel in TAE 1x and then normalised by densitometric analysis for nick translation reaction. Nick translation reactions were performed in the presence of 15 μCi of α-^{32}dCTP with 20 μM of each of the remaining nucleotides and 5 U of DNA polI (NEB). Samples were incubated at 16 °C for 30 min and reactions stopped with 5% glycerol, 20 mM EDTA and Bromophenol Blue. Reactions were run on 1% agarose gel in TAE 1x and ethidium bromide signal was quantified. Gel was treated with 30% TCA for 30 min shaking and then dryed on paper overnight at room temperature. Radioactive signal was detected at Typhoon imager (GE-Healthcare) and quantified by densitometric analysis with ImageQuant software (GE-Healthcare). Detection with fluorescent nucleotide analogues after RNase HII treatment was performed as before. Nick translation reactions were done by stopping RNase HII reaction on ice and then incubating with DNA polI and the Atto647 NT labelling mix (Jena Bioscience). After labelling, genomic DNA was run on 1% agarose gel in TAE 1x and fluorescent signals of both EtBr and Atto647 fluorophore was detected at Typhoon imager (GE-Healthcare).

Biotinylation of cDNA by PCR

For biotinylation of cDNA, GAPDH locus was amplified with a nucleotide mix containing 75 μM Biotin-16-dCTP (Jena Bioscience) and 50 μM unlabelled dCTP, each of the remaining nucleotide was at an equimolar concentration of 125 μM, control samples were amplified with unlabelled dCTP in the nucleotide mix. The GAPDH locus was amplified with specific primers (Table 1, primers 3 and 4). Amplification was obtained using GoTaq PCR reagents (Promega) according to manufacturer's instructions at an annealing temperature of 60 °C with 25 cycles of amplification. Detection was performed by adding 2 μg of Streptavidin conjugated Alexa Fluor 647 (Life Technologies). Samples were incubated at room temperature for 1 h and then run on a 1% agarose gel stained with EtBr. Fluorescent signal was detected with Typhoon Imager (GE-Healthcare).

Nicking and biotinylation of genomic DNA

Creation of nick into genomic DNA was obtained by treating 5 μg of DNA with 50 U of Nt. BspQI nickase enzyme (NEB) at 50 °C for 1 h. Reactions were stopped by chilling on ice for 30 min. Sample was splitted in two and either labelled 100 μM of Biotin-16-dCTP or with unlabelled dCTP in a nick translation reaction as previously described. Genomic DNA was precipitated and resuspended in ddH_2O overnight. Samples were sonicated for 10 cycles (30 sec ON followed by 30 sec OFF). After sonication samples were incubated with MyOne C1 streptavidin beads (Life Technologies) according to manufacturer's instructions. Each sample was splitted: half volume was eluted with 95% formamide 10 mM EDTA pH 8, the other half was used to test direct PCR amplification from beads. GAPDH locus was amplified as previously described. Each purification step was monitored by agarose gel analysis.

Amplification of common fragile sites

CFS were amplified from genomic DNA or sonicated fragments by PCR with GoTaq (Promega) according to manufacturer's instructions. Each of the target CFS, FRA7H.1, FRA7H.2, FRA16D and FRA3B was amplified with the indicated primers (Table 1, respectively from 5 to 12).

Knock-down of RNase H2A

For lentiviral infection, cells were seeded and then incubated with viral supernatant encoding for small hairpin RNA constructs against either a scramble sequence (shScramble) or RNase H2A subunit (shRNase H2A) for 48 hours as previously described (Pizzi S et al. 2015). Aliquotes were used to check silencing efficiency by total protein extraction and western blot analysis with a specific antibody against RNase H2A subunit (Abcam). Efficiency of silencing was determined by densitometric analysis of blots. The remaining samples were used for genomic DNA extraction as previously indicated. Genomic DNA was used for detection of genomic embedded ribonucleotides as previously described.

Silencing of RNase H2A by siRNA was performed using a smart pool mix against RNase H2A transcript and a non targeting control pool (Dharmacon). siRNAs were transfected at 50 nM concentration for 48 hours using HiPerfect transfection reagent (QIAGEN) according to manufacturer's instructions. Efficiency of silencing was determined as above described.

Genome editing by CRISPR/Cas9

To target lysine 164 in the PCNA locus, we designed a custom made all in one Cas9 and guide RNA (gRNA) expression plasmid (Sigma Aldrich). gRNA was designed and validated by the supplier to target the exon 5 region at the level of K164. As a donor homology, ssDNA oligonucleotides were used (Table 1, primers 13 and 14). Either XP30RO or MRC5 cells were transfected with both CRISPR/Cas9 and oligonucleotides using Viafect (Promega). Cells were sorted and seeded at low density to obtain single colonies in DMEM 10% FBS. After 15 days of subculturing, surviving colonies were picked and expanded. Once at confluency, cells were screened by HU treatment followed by western blot analysis of whole cell extracts as previously described. As a second attempt, we designed a custom made all in one Cas9 and guide RNA (gRNA) expression plasmid (Sigma Aldrich) targeting instead the intronic region between exon 5 and exon 6 of the PCNA locus. The pDonor-D09 (Genecopoeia) plasmid was used as homology donor. Once obtained, either wild type or K164R homology constructs were simultaneously transfected into XP30RO or MRC5 cells using Viafect

(Promega). After 24 hours cells were seeded at low density to obtain single colonies and puromycin selective agent was added at 2 μg/mL concentration. After one week, positive selected clones were counterselected to remove cells in which unscheduled recombination events had occurred by adding ganciclovir at 0.5 μg/mL concentration for one week. Surviving colonies were picked and expanded. Once at confluency genomic DNA was extracted as previously indicated and colonies were screened by PCR. Screening was performed by checking for the presence of puromycin resistance cassette inserted in the genomic locus of PCNA. A forward primer in the genomic locus was used (Table 1, primer 21), while two reverse primers were tested for the puromycin gene (Table 1, primers 21, 22 and 23). Presence of the mutation was assessed by sequencing PCR products.

4. Results

1. Tracking DNA polη

1.2 Generation of F18A mutants and polymerase activity analysis

Mutations of the steric gate residues of DNA polymerases and ribonucleotides sequencing have been established as a reliable strategy to track polymerases along the genome. The first aim of this work was to identify the steric gate residue in DNA polη. Phenylalanine 35 (F35) was already identified as the steric gate in the yeast orthologous polymerase (Donigan K et al. 2015). Mutation of the residue led to higher efficiency of ribonucleotides incorporation *in vitro*. Interestingly, expression of F35A in RNase H2 deficient strains caused a higher UV sensitivity and an increased mutagenic spectrum (Donigan K et al. 2015). We looked for the steric gate residue in human DNA polη by sequence conservation. The steric gate of another TLS polymerase, DNA polι, was already characterised in humans (Donigan K et al. 2014). By performing sequence alignment with the addition of this TLS polymerase, we found that the steric gate residue of DNA polη is well conserved between organisms and can be identified as phenylalanine 18 (F18) in humans (Fig. 1). After we identified the steric gate residue *in silico*, its role was confirmed by a work that characterised its mutation *in vitro* (Su Y et al. 2016). F18A mutation in human DNA polη caused a higher efficiency in incorporating NTPs vs dNTPs, while maintaining base selectivity also in the presence of DNA lesions. Crystal structure of the polymerase showed that F18 clashes with the 2'-OH group of incoming ribonucleotides increasing the distance with the 3'-OH template and thus slowing the nucleotidyl transfer reaction.

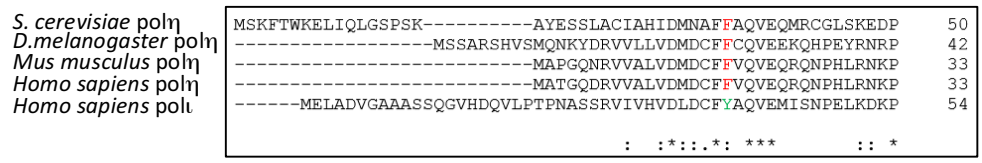


Fig. 1 The steric gate residue is conserved in DNA polη
ClustalOmega sequence alignments showing that the steric gate residue of polη is conserved. A conserved phenylalanine in position 35, 27 or 18 acts as a steric gate in *S. Cerevisiae*, *D. melanogaster*, *M. musculus* and human respectively. As a comparison, in DNA polι, another Y family polymerase, the steric gate changes to tyrosine 39.

Convinced by these preliminary data and by our *in silico* analysis, we mutated the F18 residue in the human DNA polη to alanine (F18A). In detail, we mutated plasmids encoding for DNA polη to generate both eGPF polη constructs for *in vivo* expression in mammalian cells and His-DNA polη constructs for *E. coli* expression. Any mutation in the catalytic site of the polymerase may affect its elongation activity or, in the case of DNA polη, the damage bypass capability. As a preliminary attempt to verify the catalytic activity of the F18A mutant, we performed an *in vitro* coupled transcription and translation reaction (TNT). This system takes advantage of engineered reticulocytes lysates to transcribe and translate exogenous cDNA under the T7 promoter *in vitro*. The final product of the reaction is a functional protein, in our case DNA polη. We performed a TNT reaction to produce His-tagged DNA polη either wild type or F18A mutated (Fig. 2A). Polymerases were used for primer extension assays to test catalytic activity in bypassing a CPD lesion (Fig. 2B).

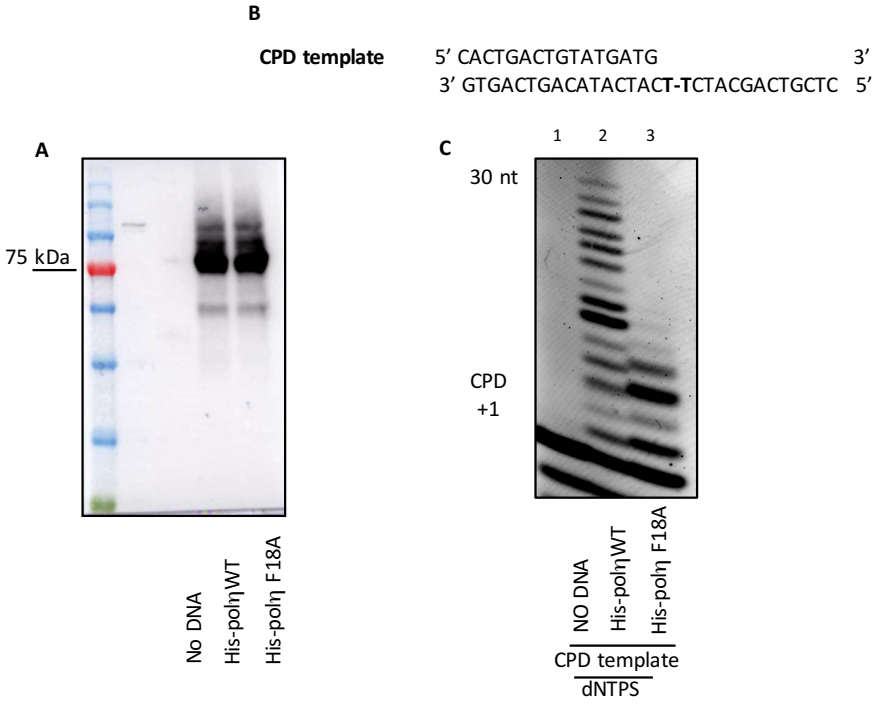


Fig. 2 TNT reaction can produce DNA polymerase η *in vitro* for primer extension assays

A Production of DNA polymerase η by TNT was assessed by western blotting using a specific antibody against polη.
B CPD template used in the reactions.
C Primer extension assays with TNT reactions products. Crude reticulocytes lysate was used as a control to exclude other secondary elongation activities (lane 1).

Polη is required for such a bypass and, as can be observed in lane 1 of Fig. 2B, the reticulocyte lysate is not competent for the elongation of the substrate. Thus the use of the specific polη substrate allowed us to exclude elongation activities by other polymerases possibly present in the reticulocytes lysate. Interestingly, both wild type and F18A polymerases produced *in vitro* were able to bypass the CPD at +1 position (Fig. 2C lane 2 and 3). However, while the wild type polymerase extended completely the primer, the steric gate mutant had a lower processivity (Fig. 2C lane 3). In addition to this, it is worth noticing that primers elongated by the mutant polymerase presented a shift in the electrophoretic run (Fig. 2C lane 3).

It is well known that the presence of ribonucleotides in DNA cause such electrophoretic shift due to the presence of the additional 2'-OH group compared to dNTPs (Crespan E et al. 2016). Even though we used dNTPs as nucleotides mix in primers elongation, the reticulocyte lysate contains a large excess of ribonucleotides for the transcription reaction. Thus, we can hypothesize that the mutant polymerase incorporates NTPs residual from the TNT mix.

1.3 Purification of His-polη F18A mutant

TNT reactions provide a fast method to obtain the protein of interest *in vitro*. However, subsequent reactions cannot be precisely controlled due to the presence of residual reticulocytes components, as seen by the presence of NTPs. To have a higher control of the reactions conditions we purified the mutant DNA polymerase. A codon optimized plasmid carrying the DNA polη F18A cDNA was used to transform BL21-λde3 Codon+ *E. coli* competent cells. Transformed bacteria were pre inoculated for an overnight culture at 30 °C. Growth rate was calculated and cultures diluted to an optical density at 600 nm (OD600) of about 0.1 into 3 liters of culture medium. At OD600 of 0.6 the culture was chilled on ice for 20 minutes followed by induction with IPTG 0.5 mM. After induction, culture was grown at 12 °C for 40 hours. Pellets were resuspended and lysed with lysozyme followed by sonication. Bacterial cells suspensions were centrifuged to remove cell debris and the lysate was incubated with a pre equilibrated TALON affinity resin. The resin contains cobalt to bind with high affinity and high specificity the Histidine tag of target proteins. The lysate incubated with the resin was packed in a column and circulated by gravity. After washes, fractions were eluted with 300 mM imidazole. The first two fractions should contain the highest concentrated proteins and thus were used in a desalting step to remove imidazole and lower the concentration of salts. Aliquotes from both eluted fractions and desalted samples were used for western blot analysis (Fig. 3). As observed by TGX gel stain, we were able to purify polη at over 90% purity (Fig. 3 lanes from 1 to 7). The purified product was quantified at the concentration of 280 ng/microL by densitometric analysis with a bovine serum albumin (BSA) scale (Fig. 3 compare lane 6 with lanes from 8 to 11).

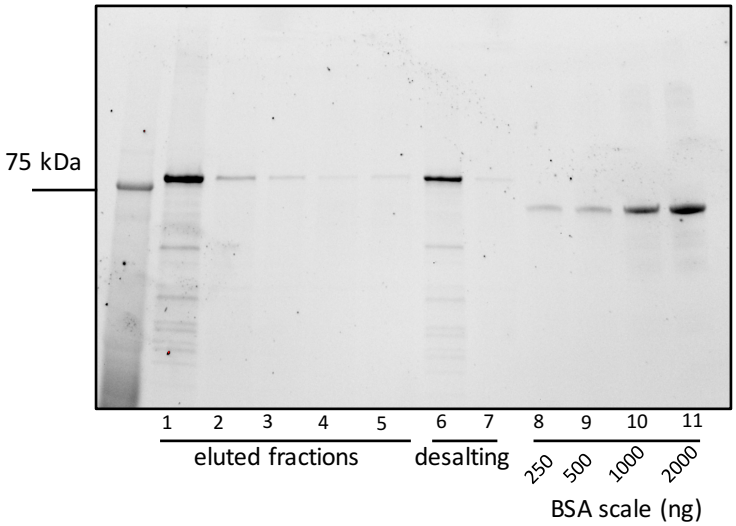


Fig. 3 Purification of His-tagged DNA polymerase η F18A

Each eluted fraction was analysed by running samples on a prestained TGX gel. Purity of the sample can be assessed by the presence of a predominant band for His-polη. Concentration has been confirmed by densitometric analysis comparing fractions with a BSA scale.

1.4 His-polη F18A incorporates ribonucleotides *in vitro*

Having purified the mutant polymerase, we wanted to test both its catalytic activity and the propensity to elongate primers with ribonucleotides (NTPs) compared to deoxyribonucleotides (dNTPs) in a more controlled reaction environment. With this aim, we used purified polymerases in primer extension reactions with different templates (Fig. 4A) and different nucleotide substrates. In the presence of an undamaged template extension of the primer was efficient and the wild type polymerase terminated the reaction using dNTPs (Fig. 4B lane 1). The mutant polymerase was able to elongate the primer, although it showed a reduced processivity in the same conditions (Fig. 4B lane 2). The same undamaged template was used as a substrate for polymerases in the presence of NTPs instead of dNTPs to test the capability of ribonucleotides incorporation. Although both polymerases, WT and F18A, showed a remarkable reduction of elongation efficiency (Fig. 4B lane 3 and 4), the F18A polymerase was able to extend the substrate with NTPs more efficiently that the WT (Fig. 4B lane 4). After testing the elongation efficiency of the polymerases we decided to verify the CPD bypass capability with either of the two nucleotides (Fig. 4C). Again the wild type polymerase was able to bypass the CPD at +1 position and fully extended the primer with dNTPs, while the F18A mutant bypassed the lesion but showed once more a lower processivity (Fig. 4C lane 1 and lane 2). More interestingly the mutant polymerase bypassed the lesion with NTPs while the wild type showed a very low efficiency in elongating past the damaged bases with the same nucleotides (Fig. 4C lane 3 and lane 4). Taken together this data shows that mutation of F18 in human DNA polη stimulates ribonucleotides incorporation at the expense of its processivity. In such scenario, we wanted to firmly confirm that the mutation was not affecting the catalytic function of the polymerase. For this aim, we took advantage of different experimental conditions while performing primer extension assays. Hence, we substituted magnesium with manganese in the reaction buffers and performed the same assays on undamaged templates. The precise effects of manganese on polymerase reactions are still under investigation, but it has long been known as an enhancer of polymerases catalytic activity.

In fact, it has been hypothesised that the wider atomic radius of manganese may enlarge polymerases catalytic site, thus favouring the entrance of nucleotides for DNA elongation. In this conditions, both polymerases fully elongated undamaged primers in the presence of either dNTPs or NTPs, thus finally excluding the possibility that mutation of F18 would completely abolish the polymerases activity (Fig. 4D). Notably, polymerases elongated the primers giving rise to products longer than the expected 30 nucleotides (Fig. 4D lanes from 1 to 4). We hypothesise that this phenomenon may be caused by the presence of manganese that strengthens catalytic activity to the point of causing primer slippage. As a consequence, slippage of primers on template causes addition of bases that are detectable during electrophoresis as longer products. Remarkably in all reactions, as previously observed in primer extensions by TNT products, the incorporation of ribonucleotides caused an electrophoretic shift clearly visible by comparing the products where NTPs substituted dNTPs in the reaction mixes (Fig. 4B lanes 3 and 4, Fig. 4C lanes 3 and 4, Fig. 4D lanes 3 and 4). This mobility shift could also cause the accumulation of products of increased apparent (>30nt) length.

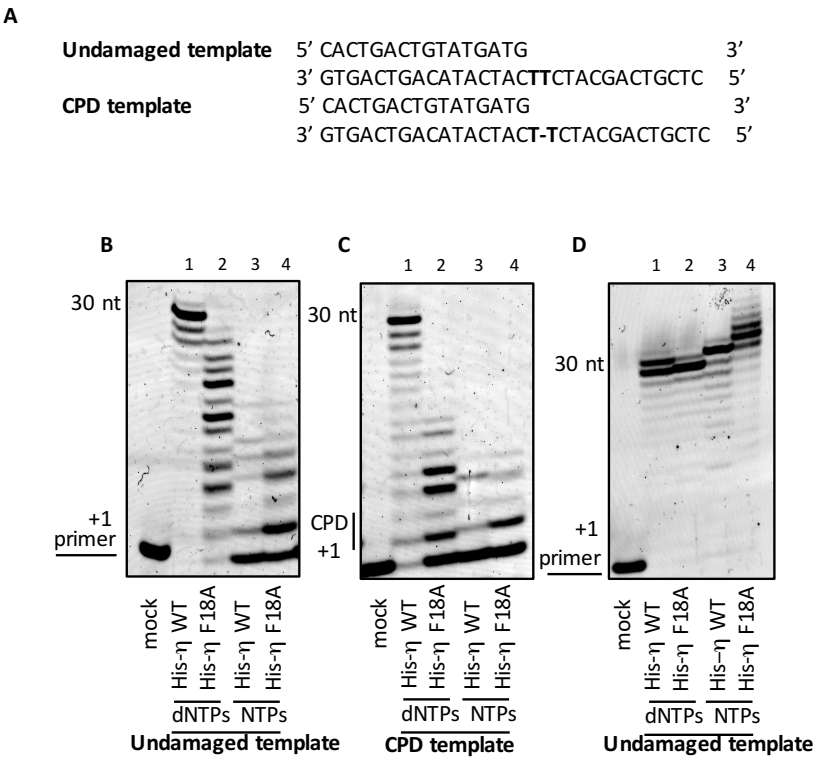


Fig. 4 Primer extension assays

A Annealed templates used in the assays. 30 mer templates were either undamaged or contained a CPD at +1. Primers were 5' labelled with FAM.

B Elongation of undamaged primers by purified polymerases either with dNTPs or NTPs.

C Bypass of the +1 CPD lesion by purified polymerases either with dNTPs or NTPs

D Elongation of undamaged primers by purified polymerases in reactions containing manganese instead of magnesium, with either dNTPs or NTPs.

1.5 F18A mutant cells have an increased UV sensitivity

Since the mutant polymerase was functional *in vitro*, we wanted to verify the effects of steric gate mutation *in vivo*. At first, we verified the correct subcellular localisation of the polymerase, and its capability to be recruited to replication factories, thus forming discrete nuclear foci. We transiently transfected eGFP-DNA polη wt or F18A in MRC5 cells. Cells were UV irradiated with a high dose of UV-C light (25 J/m^2) and fixed after 6 hours. Under these conditions the majority of cells are found blocked in S phase of the cell cycle and thus it is possible to appreciate discrete nuclear foci of DNA polη by immunofluorescence. The steric gate mutant showed a correct nuclear localization and formed visible foci as the wild type variant (Fig. 5). This was the confirmation that mutant polymerase showed a similar behaviour as the wt variant.

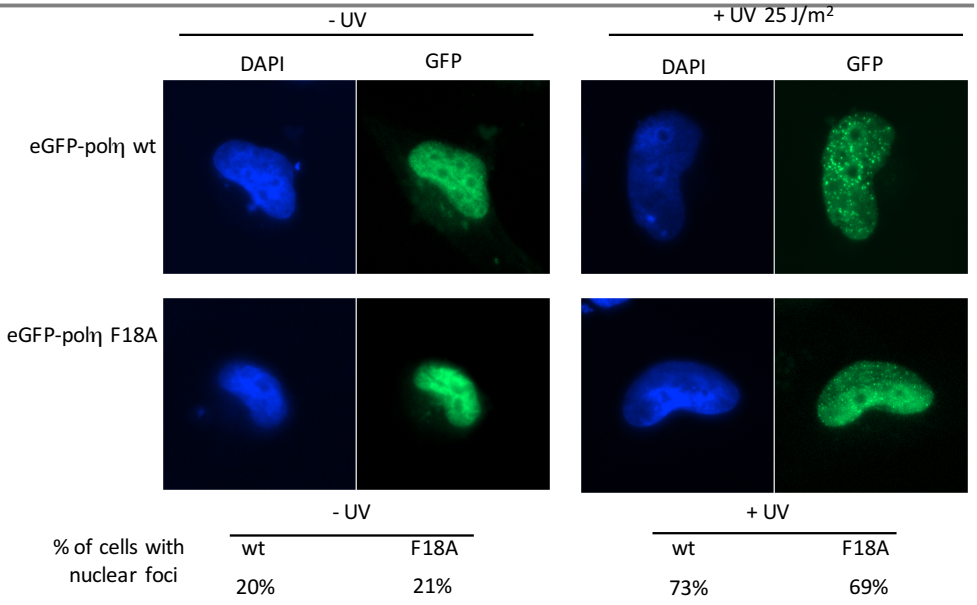


Fig. 5 Localisation of eGFP-polη variants in mammalian cells

Correct localisation of eGFP-polη variants (wt or F18A) was assessed by epifluorescence after transient transfection.
Representative images of polη localisation are shown: diffused in the nucleus (left) and focally accumulated (right) for both WT and F18A. About 100 nuclei were quantified for the analysis. Quantitation of the foci distribution is shown at the bottom in the absence and presence of UV irradiation.

In light of this, we decided to establish stable cell lines with the DNA polη variant transgenes. We took advantage of XP30RO cell lines from Xeroderma Pigmentosum Variant (XPV) patients. These cells carry a frameshift mutation in the first exon of the DNA polη gene and thus produce a non functional DNA polymerase and are considered to be polη null. We complemented these cells with the previously mentioned construct coding for a N-terminally eGFP tagged DNA polymerase η and established XP30RO-eGFP polη wt and XP30RO-eGFP polη F18A stable cell lines that were used for characterisation of the mutant polymerase *in vivo*. Cells were transfected and seeded to obtain single colonies in the presence of G148 as selective agent.

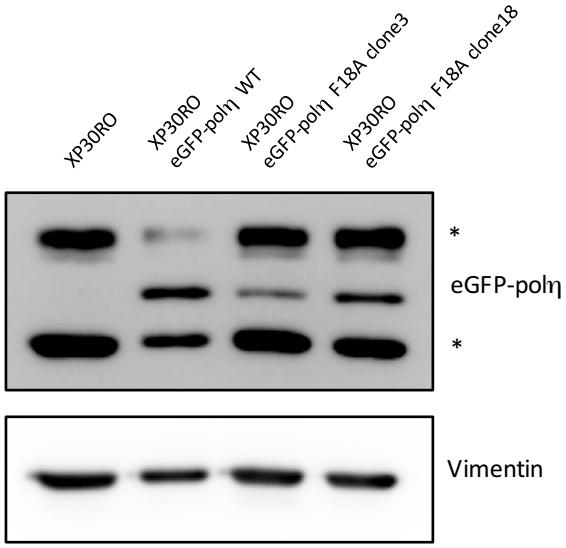


Fig. 6 Detection of eGFP-polη in stable cell lines

After isolation of single colonies, cells were screened by western blot analysis using an antibody against DNA polη. Each clone was compared to XP30RO cells and XP30RO cells complemented with with the wild type eGFP-polη variant. The asterisk (*) indicates an aspecific band identified by the antibody.

Surviving clones were screened for the presence of DNA polymerase η by western blot (Fig. 6). Two independent clones were isolated, namely clone 3 and 18 (Fig. 6 lanes 3 and 4). Subsequent monitoring of eGFP expression by FACS revealed an unstable expression of the transgene in clone 18 and thus these cells were discarded for further analysis (data not shown). To verify the possible effects of the transgene on cell growth, we followed cell duplication by counting cell number at different timepoints. No significative differences were observed at each time point between the different cell lines, thus excluding the possibility that the mutant polymerase could impair cell growth (Fig. 7).

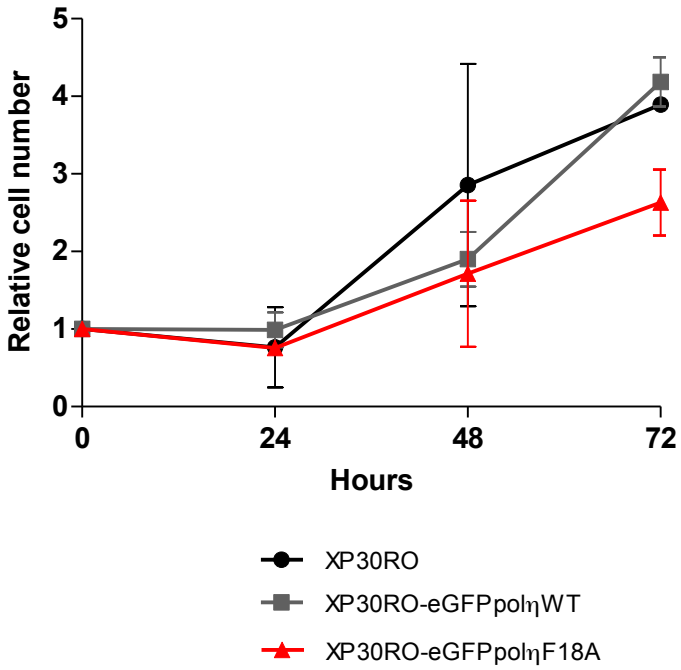


Fig. 7 Cell growth of isolated cell lines

Cells were seeded at equal numbers and then counted at the indicated timepoints. Each count was normalised to the initial number and then expressed as relative cell number. Error bars represent S.E.M. (n=2)

The function of DNA polη is crucial for cell viability in the presence of UV damage on DNA, thus we verified the effect of the mutant polymerase on bypass of UV lesions at long timepoints. A clonogenic survival assay was performed, where 12 h after seeding the cells were irradiated at increasing UV doses before being allowed to form colonies. The number of survived colonies was finally scored 15 days after irradation. As expected, XP30RO cells had the highest sensitivity showing less than 1% survival at the maximum UV dose of 6 J/m^2 (Fig. 8).

Conversely, cells complemented with the wild type polymerase are able to bypass the lesions and thus have the highest survival percentage. In contrast, F18A mutant cells show a more sensitive phenotype reducing their survival. A similar phenotype was observed in yeast where the presence of ribonucleotides in the genome inserted by the F35A mutant of DNA polη caused a higher UV sensitivity than its matched WT control (Donigan KA et al. 2015) when RNase H2 was present. The phenotype was explained by the possible conversion of genomic ribonucleotides inserted by the polymerase into more toxic intermediates. For example, embedded ribonucleotides may be recognised and cleaved by RNase H2 when the RER pathway is active. Processing of ribonucleotides may lead to the formation of gaps in DNA that further damage cells and affect the survival after UV damage.

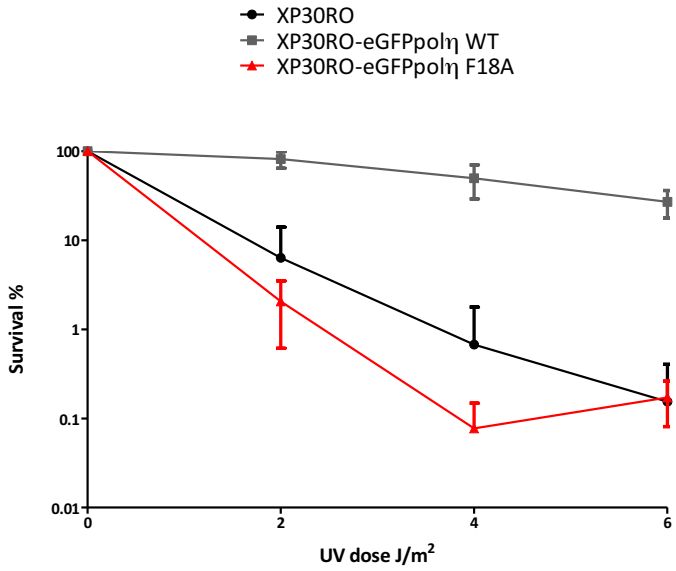


Fig. 8 UV sensitivity of different cell lines

Cells were seeded and UV irradiated at increasing doses. Survival colonies were counted and expressed as percentage of survival. Error bars represent S.E.M (n=3)

The presence of long term effects on the survival of F18A cells challenged with UV led us to analyse in more details the progression of cell cycle after UV irradiation. Since XP30RO cells cannot bypass UV damage, they are found arrested in early stages of S phase after irradiation. Complementation of the cells with the wt DNA polη allows damage bypass and thus progression through cell cycle. With this in mind, we UV-irradiated the cells and after fixing, we stained them with propidium iodide (PI). PI intercalates into DNA and FACS analysis allowed us to follow the cell cycle progression upon irradiation by monitoring the quantity of DNA in the cells (Fig. 9). As expected, we found XP30RO cells accumulated in early S phase of the cell cycle following UV irradiation. In comparison, cells complemented with the wt variant of DNA polη were found in the middle to late stages of S phase.

F18A mutant cells had instead an intermediate phenotype with the majority of cells accumulated at later stages of S phase if compared to the XP30RO cells but at earlier phases if compared to the DNA polη wt cells.

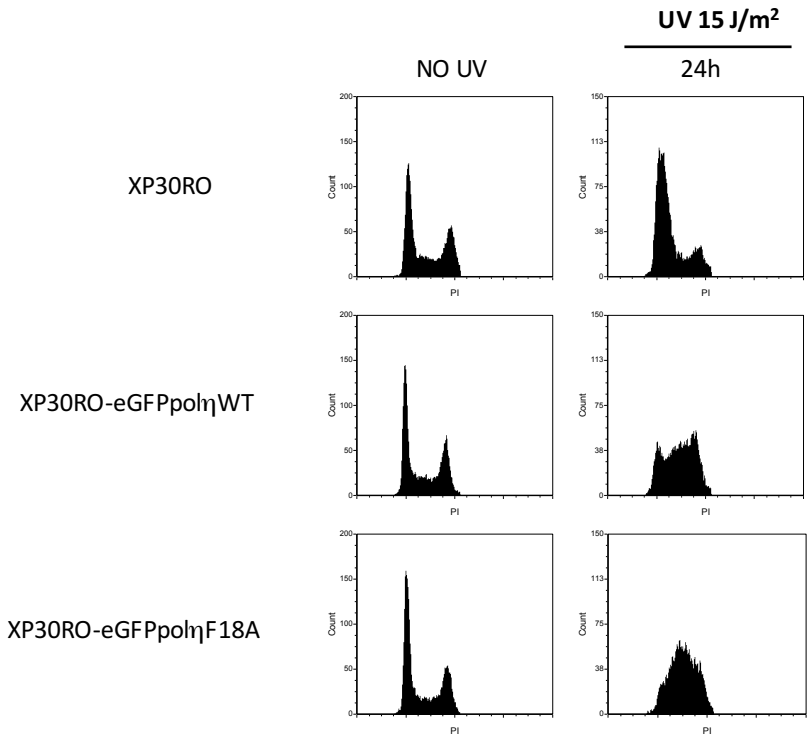


Fig. 9 Cell cycle progression after UV irradiation

Cells were challenged with UV irradiation and fixed after 24 hours. Cell cycle distribution was analysed by FACS. Y axis scale was reduced in UV treated samples to increase readability of the plots.

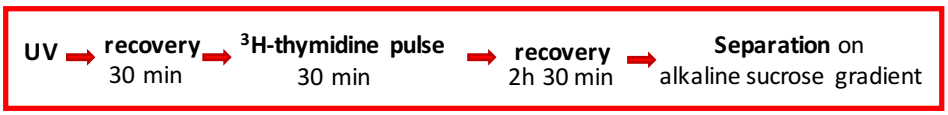
Thus, even at earlier timepoints with respect to the clonogenic assay, the F18A mutation of DNA polη influences UV damage bypass and therefore cell cycle progression. This deficiency in UV damage bypass confirms our previous observations and the data obtained in yeast (Donigan K et al. 2015). In *S. cerevisiae* strains lacking NER activity, DNA polη is the sole UV damage repair mechanism and thus the steric gate mutant bypass damages by inserting ribonucleotides. In this context, the presence of RNase H2 activity confers cells a higher UV sensitivity. In fact, when damages are bypassed with ribonucleotides they are subsequently removed by RER pathway leaving gaps on DNA. We speculate that the UV sensitivity of the F18A human cells may be attributed to a similar pathway. Differently from the yeast strains, NER is active in our cell lines. However, cells still rely on polη activity to repair UV damages as it is evident by the sensitivity of XP30RO cells (Fig. 8 black line). Nevertheless, F18A mutant cells are capable of progressing through S phase and thus can be used as a tool to track polymerase activity *in vivo*.

1.6 Analysis of Post Replication Repair

A more direct analysis of UV damage bypass and in particular post replication repair is made possible by a specific assay. The so called post replication repair assay, detects nascent DNA by following incorporation of a radioactive nucleotide analogue (Fig. 10A). Cells are UV irradiated and pulsed with ^{3}H-thymidine that is incorporated into nascent DNA. This is chased over time in the absence of labelled nucleotides. Deficiency in UV damage repair will cause replication fork slowing or stalling and thus the production of short nascent DNA filaments labelled with ^{3}H-thymidine. On the contrary, an efficient bypass of damaged DNA will produce longer nascent filaments by the active replication fork. Separation of nascent DNA by means of molecular weight is carried out by centrifugation in an alkaline sucrose gradient. Detection of radioactive signal along the gradient is a direct measurement of nascent DNA length and thus can be correlated to bypass capacity of cells. In a pulse and chase experiment XP30RO cells had a peak of low molecular weight fragments indicating the presence of short stretches of nascent DNA and thus a defect in damage bypass (Fig. 10B grey line).

In contrast, XP30RO cells complemented with the wild type polymerase showed a peak of high molecular weight fragments indicating an efficient elongation after damage bypass (Fig. 10B black line). Cells complemented with the F18A mutant had a peak indicating the presence of low molecular weight fragments, leading us to hypothesise a possible defect in post replication repair after UV irradiation (Fig. 10B red line). However, it should be considered that in this assay DNA is separated in an alkaline gradient. Alkali conditions are known to cause breaks at the level of ribonucleotides due to their higher reactivity. Thus, it is reasonable that in this case the low molecular weight fragments we are detecting in F18A cells could be an artefact given by the reaction of incorporated NTPs with the alkaline gradient.

A



B

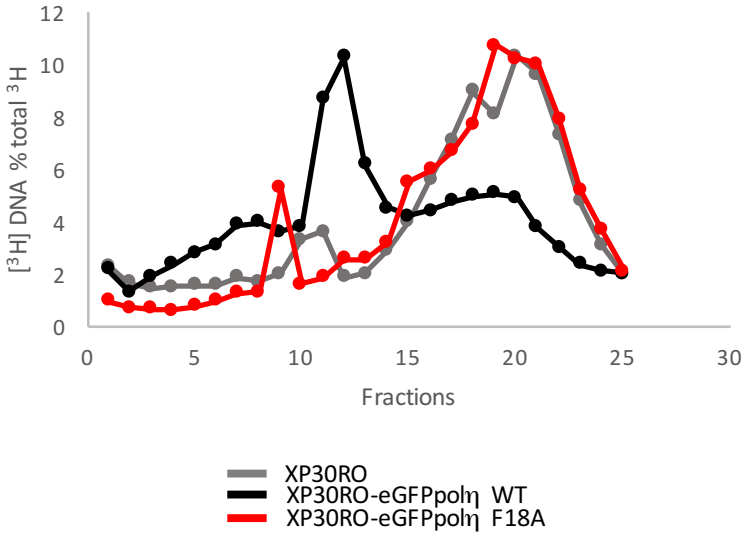


Fig. 10 Post Replication Repair Assay

A Schematic workflow of the assay

B Dimension of nascent DNA is correlated to damage bypass capability. The sucrose gradient is spotted as separate fractions from the highest molecular weight fractions (High MW) to the lowest molecular weight fractions (Low MW). Radioactive signal of each fraction is plotted as percentage of ^{3}H-thymidine over total ^{3}H.

1.7 F18A cells accumulate DNA:RNA hybrids detected by the S9.6 antibody

After the initial characterisation of the mutated cell lines, we decided to have a more direct proof of ribonucleotides incorporation in DNA by the F18A mutant polymerase. With this aim we purchased an hybridoma cell line to have a large scale production of the S9.6 antibody. This antibody has been extensively characterised for its affinity to DNA:RNA hybrids (Bugoslawski SJ et al. 1986; Garcia-Rubio M et al. 2018). The antibody shows a sub-nanomolar binding to DNA:RNA stretches with a minimum length of 6 bp and has been recently used to detect DNA:RNA hybrids formation *in vivo* (Phillips DD et al. 2013; Garcia-Rubio M et al. 2018). We subcultured the hybridoma cells to have a sufficient volume of supernatant that was used for affinity purification of the antibody (Fig. 11). The purified S9.6 antibody was used for immunofluorescence to detect the nuclear signal of DNA:RNA hybrids in our stable cell lines. Staining of cell lines carrying either the wt or the F18A allele revealed a different accumulation of hybrids in the nucleus (Fig. 12A). Despite the cytoplasmic signal that is detected by the antibody and documented in other works (Zhang ZZ et al. 2015; Hartono SR et al. 2018) we were able to quantify with high level of significance an increase of about 2 folds in the nuclear S9.6 signal in F18A cells compared to the wild type cells (Fig. 12B).

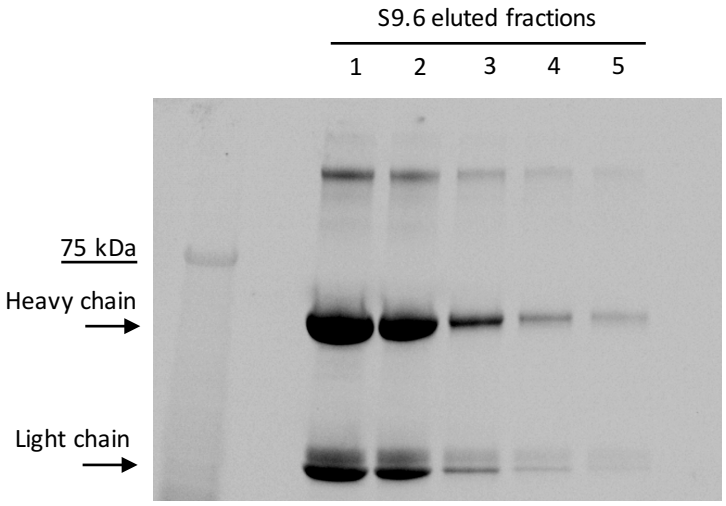


Fig. 11 Purification of S9.6 antibody from a hybridoma cell line

Supernatant from a hybridoma cell line was used to purify the antibody by gravity flow with a Protein G Sepharose resin. Eluted fractions were qualitatively analysed by running a TGX gel to check for the presence of the eluted antibody. Each fraction was subsequently quantified by Comassie stain with a BSA scale reference.

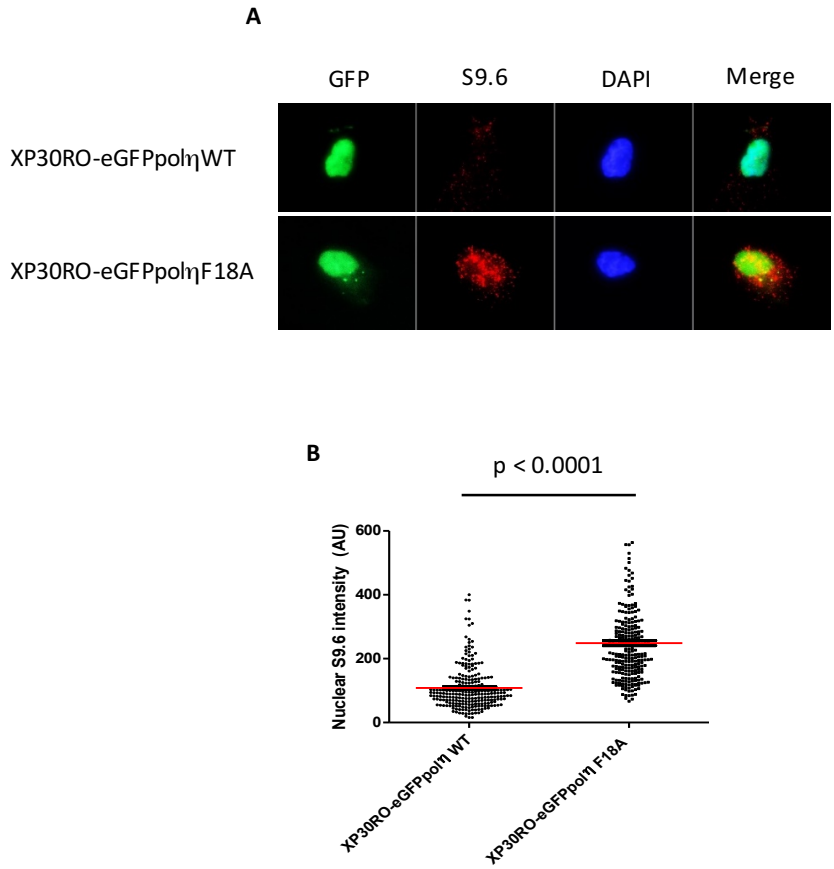


Fig. 12 Immunofluorescence detection of DNA:RNA hybrids

A Immunofluorescence detection of nuclear hybrids with purified S9.6 antibody. Cells were fixed and stained with S9.6 primary antibody followed by staining with fluorescent secondary antibody.
B S9.6 nuclear signal of GFP positive cells was quantified using a specific CellProfiler pipeline. Relative fluorescence signal for each cell is here reported as arbitrary units.

It should also be considered that the S9.6 is capable also of recognizing RNA stretches paired with DNA filaments namely R-loops (Garcia-Rubio M et al. 2018). The specificity of the antibody and the increase in nuclear signal were confirmed by a pre-treatment of the cells with recombinant RNase HII, before processing for immunofluorescence. In particular, prior to fixation the cells were permeabilised and treated in situ with RNase HII to remove both DNA:RNA hybrids and genomic embedded ribonucleotides. After treatment with RNase HII we could appreciate a reduction of the S9.6 signal in both the wt and F18A cells (Fig. 13). While the antibody has a broader specificity towards R-loops, dsRNA structures and genomic embedded ribonucleotides, the enhanced nuclear signal was directly correlating with the expression of the F18A mutant of the polymerase. This result made us confident that the increase we were detecting was specifically caused by ribonucleotides inserted by the steric gate mutated polη.

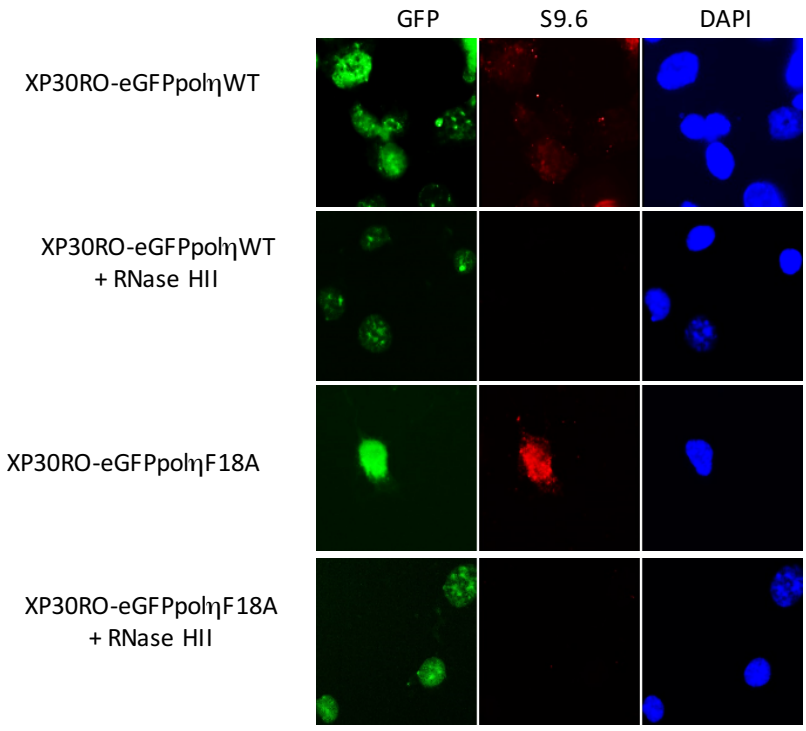


Fig. 13 Detection of S9.6 specificity towards DNA:RNA hybrids

Slides were permeabilised and pre treated with recombinant RNase HII before fixation. After fixation cells were stained with S9.6 antibody and nuclear signal was quantified with a specific CellProfiler pipeline.

1.8 Load of genomic ribonucleotides is higher in F18A cells

Accumulation of ribonucleotides embedded in genomic DNA can be detected more directly and at higher level of resolution with a specific assay. Recombinant prokaryotic RNase HII recognises and cleaves NTPs embedded into DNA. Treatment of genomic DNA purified from cells with RNase HII produces nicked genomic DNA at the level of ribonucleotides. Nick translation reactions allow to fill the gaps left by RNase HII and the use of a detectable nucleotide analogue permits quantification of the ribonucleotides originally present in DNA. We decided to use this assay to quantify the accumulation of genome embedded ribonucleotides in our cell lines. At first, we took advantage of a radioactive nucleotide analogue, ^{32}P-αdCTP for nick translation reaction and subsequent quantification. Comparison of radioactive signal between mock treated and RNase HII treated samples allows to monitor the background incorporation due to nicks generated by genomic DNA extraction and processing. Quantification of radioactive signal after gel electrophoresis of genomic DNA revealed a significant accumulation of ribonucleotides in the F18A mutated cells when compared to the wild type or XP30RO parental cell line (Fig. 14 compare lane 6 with lane 4 and lane 2). Quantification of the radioactive signal showed an almost 2-fold increase when compared to the wt cells, confirming the observations obtained by staining with the S9.6 antibody. This is a direct indication of the presence of ribonucleotides specifically incorporated into genomic DNA by the steric gate mutant of DNA polη. While being extremely sensitive, this assay resulted to be laborious and samples had to undergo a considerable number or passages.

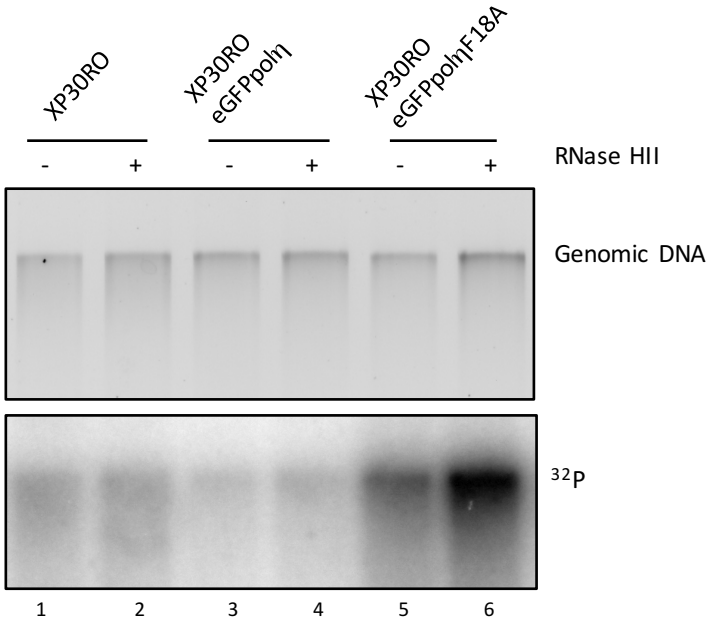


Fig. 14 Detection of genomic ribonucleotides

After digestion with RNase HII and nick translation reaction with α^{32}P-dCTP, samples were run on agarose gel and radioactive signal was detected at the phosphoimager.

We wanted to improve this assay and be able perform it more quickly and in a safer experimental setting, avoiding the use of radioactive nucleotides. To pursue this goal, we decided to substitute radioactive dCTP with a fluorescently labelled nucleotide analogue for nick translation reactions. After setting the right reaction conditions, we performed the same assay with the fluorescent analogue. Detection of the signal from dUTP-Atto647 again revealed a higher and significant accumulation of ribonucleotides in the DNA polη steric gate mutant cells, thus confirming previous results (Fig. 15 compare lane 6 with lane 4 and lane 2).

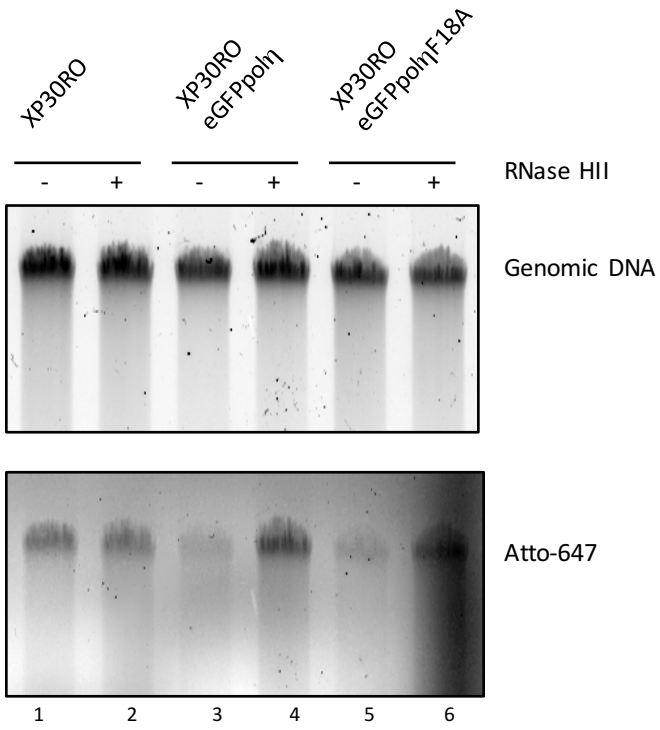


Fig. 15 Detection of genomic ribonucleotides with a fluorescent analogue

After digestion with RNase HII and nick translation reaction with Atto647-dUTP, samples were run on agarose gel and fluorescent signal was detected.

1.9 Ribonucleotides can be isolated from genomic DNA of F18A mutant cells

Since the final aim is to track the activity of the polymerase along the genome, we wanted to be able to isolate DNA fragments where ribonucleotides were inserted by DNA polη F18A. Once again the use of a different nucleotide analogue during nick translation reaction makes possible to label and subsequently isolate DNA fragments. We decided to use biotinylated nucleotide analogues, in particular biotin-dCTP. As a preliminary step, we tested biotinylation of DNA during a PCR amplification. As a proof of concept, we decided to amplify GAPDH from cDNA in the presence of biotin-dCTP. After PCR reactions, we detected biotinylated DNA by incubation with fluorescently labelled streptavidin and electrophoresis on an agarose gel. Amplification of GAPDH gene in the presence of biotin-dCTP resulted in the partial biotinylation of the PCR product as detected by fluorescent signal of the conjugated fluorophore (Fig. 16 lane 4). This experimental conditions were set as a proof of principle for our subsequent biotinylation of genomic DNA. In that case we would expect a more efficient insertion of biotinylated nucleotides due to the higher frequency of gaps present in DNA.

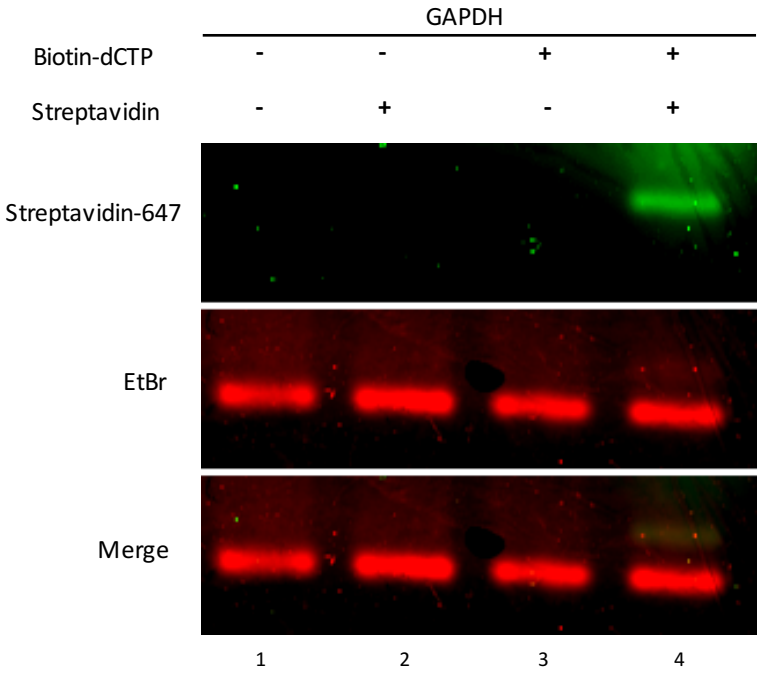


Fig. 16 Biotinylation of GAPDH locus with biotin-dCTP and fluorescence detection

GAPDH locus was amplified from cDNA by PCR either with dNTPs or with a dNTPs mix containing biotin-dCTP. Samples were incubated with a fluorescent conjugated streptavidin and run on gel. Fluorescent signal was detected at Typhoon Trio imager.

Given these results we were confident that our biotin-dCTP substrate could be used in nick translation reactions. We moved to genomic DNA and decided to test the experimental workflow by initially nicking purified gDNA. We took advantage of a recombinant nickase, Nt.BspQI that generates nicks at high frequency in genomic DNA recognising a highly frequent consensus.

After incubation with the nickase, nick translation reactions were performed in the presence of biotin-dCTP, followed by sonication to obtain smaller genomic fragments of around 200 bp (Fig. 17A, lanes 1 and 2). Fragmented genomic DNA was incubated with paramagnetic streptavidin beads and finally DNA was eluted. We observed that only samples that had been filled by DNA polI in the presence of biotin-dCTP could be eluted from the beads (Fig. 17A, compare lanes 5 and 6; and lanes 7 and 8). Exclusive elution of biotinylated samples led us to confirm that binding was specific.

However, the final outcome of this assay would be to amplify and sequence all the genomic regions that were filled with ribonucleotides by DNA polη, thus marking its activity along the genome. With this in mind, we decided to test the possibility to amplify specific genomic loci after biotinylation and DNA elution from beads. As a proof of principle, we successfully amplified GAPDH locus directly from the beads (Fig. 17B).

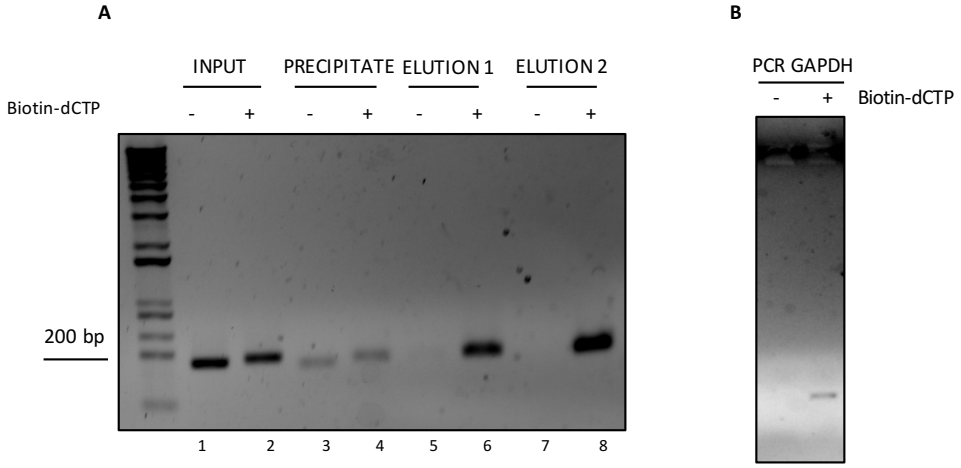


Fig. 17 Elution of biotinylated genomic DNA and direct amplification of GAPDH locus

A Biotinylated or not biotynilated control genomic DNA was sonicated, precipitated and incubated with paramagnetic streptavidin beads. Two subsequent elution steps were performed. Expected lengths of PCR products are of 133 bp.
B Eluted samples were used in a PCR reaction to amplify GAPDH locus.

The activity of DNA polη is required for replication past difficult to replicate regions, such as CFSs (Bergoglio V et al. 2013). These loci were the first DNA polη target that we decided to isolate in our experimental setting. To begin we tested amplification of all the fragile sites previously identified as DNA polη substrates (Bergoglio V et al. 2013). FRA7H.1, FRA7H.2, FRA16D and FRA3B were successfully amplified from genomic DNA (Fig. 18A) and FRA7H.1 was also amplified from sonicated genomic DNA fragments (Fig. 18B).

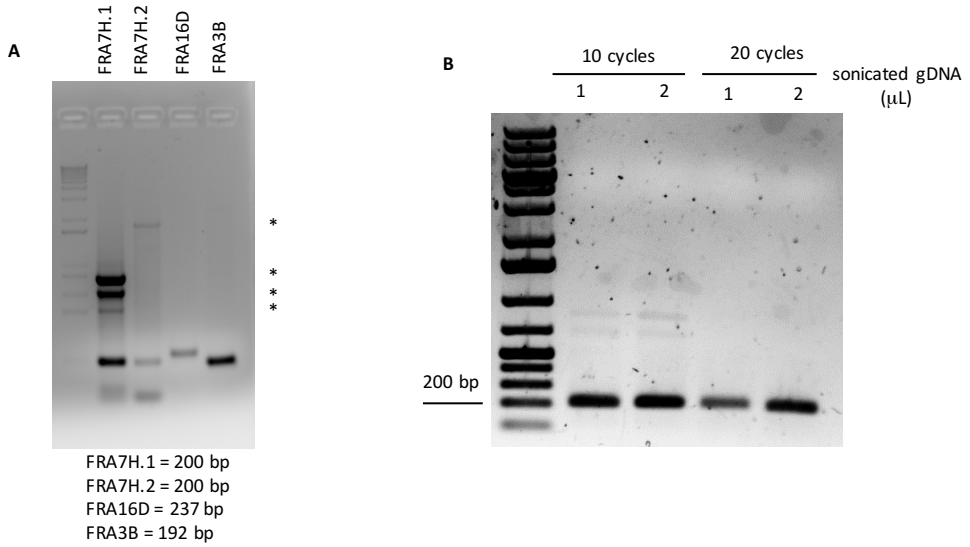


Fig. 18 Amplification of fragile sites from genomic DNA

A Genomic DNA was used a template to amplify fragile sites that were already identified as DNA polη substrates. Expected lengths of PCR products are listed below the figure.
* indicates aspecific products.

B Amplification of FRA7H.1 from sonicated gDNA. Different sonication cycles were tested.

To summarise our preliminary data, we demonstrated that we could nick, biotinylate and detect genomic DNA. Moreover, we could amplify fragile sites from sonicated DNA that is the final substrate of the assay after binding with paramagnetic streptavidin beads. Thus we had established all experimental conditions for the final assay to nick specifically at the level of ribonucleotides embedded in genomic DNA by DNA polη F18A. To isolate genomic loci where DNA polη F18A inserted ribonucleotides, we treated genomic DNA from cells with RNase HII and then biotinylated the nicked DNA. Sonication produced gDNA fragments that were incubated with streptavidin beads and after washing, beads were used as a PCR substrate to amplify fragile sites. While FRA7H.1 was efficiently amplified in the input samples, we could not detect any amplification product from beads (Fig. 19 lanes from 1 to 6).

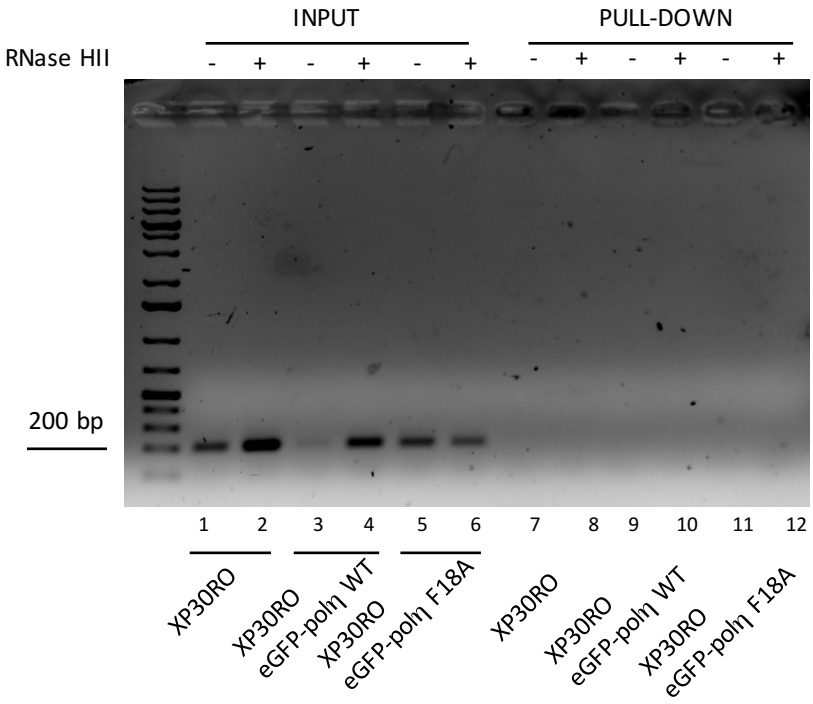


Fig. 19 Amplification of fragile sites from streptavidin beads

Amplification of FRA7H.1 after treatment with RNase HII and biotinylation. After binding beads were directly used as a PCR substrate. An aliquote of sonicated genomic DNA was used as an input sample.

We hypothesise that further improvements of the assay are necessary to enrich our samples for the desired genomic targets. Indeed, at present DNA polη has been detected in CFSs only in the presence of replication stress, such as after aphidicoline treatment. Thus, we cannot exclude that such conditions may be necessary to be able to identify DNA polη activity in these sites.

1.10 Inactivation of the RER pathway in the steric gate mutants

All of the previous experiments were performed in the presence of an active RER pathway in the cell lines. For instance, the activity of RNase H2 is removing ribonucleotides from genomic DNA leading us to underestimate the quantity of genomic NTPs. Having a more precise quantitation of ribonucleotides load by DNA polη requires the inactivation of the RER pathway. As a first attempt to remove RNase H2 activity in our cell lines we used lentiviral infection with small hairpin RNAs (shRNAs) against either the RNase H2A subunit or a control scramble construct. Efficiency of silencing in cell lines was assessed by western blot analysis (Fig. 20A). Quantification of the residual RNase H2A protein revealed an unexpected increase of the subunit in XP30RO cells knocked down with shRNase H2A compared to control (Fig. 20A compare lane 1 and lane 4), while about 50% residual protein was present in wild type completemented cells (Fig. 20A compare lane 2 and lane 5). Interestingly we detected only 30% of residual protein in F18A complemented cells (Fig. 20A compare lane 3 and lane 6). The F18A cells had the most efficient reduction in RNase H2A and thus we decided to assess the presence of ribonucleotides in their genomic DNA. Genomic DNA from lentiviral infected cells was purified and treated with RNase H2 followed by nick translation with fluorescent nucleotide precursors as described before (Fig. 20B). Detection of fluorescent signal in shRNase H2A samples showed a 2x increase in ribonucleotides content when calculating the ratio between RNase H2 digested vs untreated samples. This result further confirms our previous analysis showing an increase in genomic ribonucleotides by the F18A mutant. Thus, reduction of RNase H2A by lentiviral infection increased the amount of detectable ribonucleotides as expected.

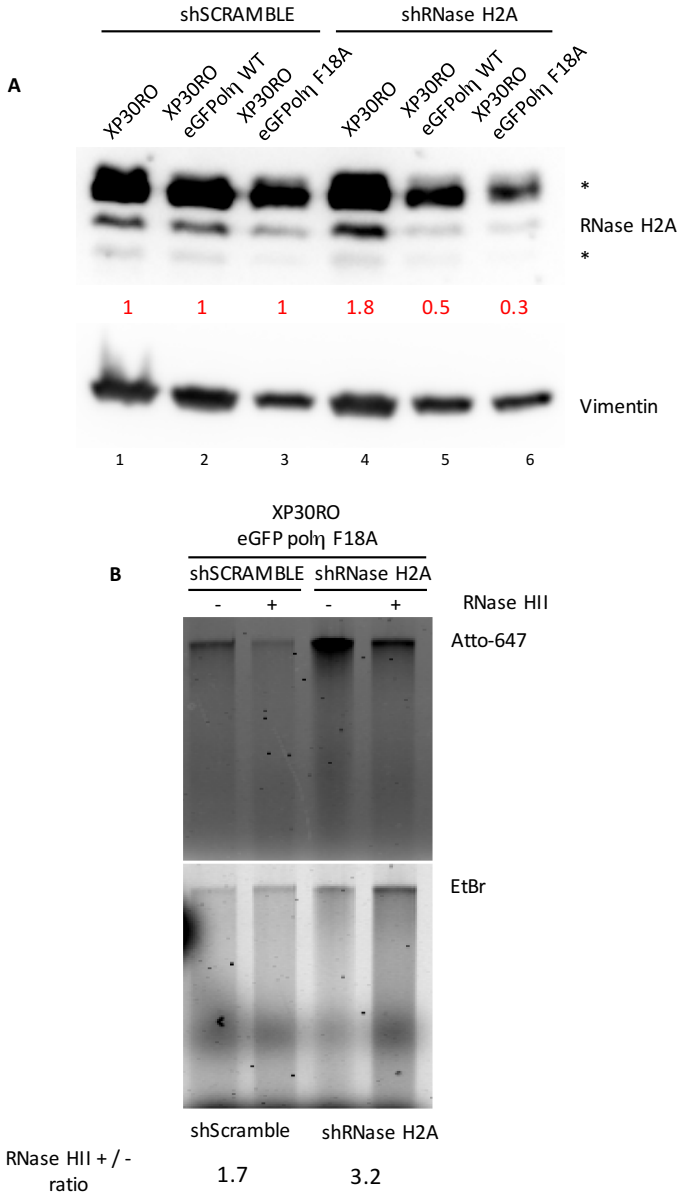


Fig. 20 Knock down of RNase H2A by lentiviral infection

A Efficiency of silencing was assessed by western blot for both shScramble and shRNase H2A, using an antibody against H2A subunit. Quantification by densitometric analysis is reported below the blot.
The asterisks (*) indicates an aspecific band identified by the antibody.

B After digestion with RNase HII and nick translation reaction with Atto647-dUTP, samples are run on agarose gel and fluorescent signal is detected. Ratios of treated vs untreated samples are reported.

Although lentiviral infection led to a significant reduction of the RNase H2 protein, we wanted to have a more efficient method to inactivate the RER pathway. For this reason, we decided to silence the transcript for RNase H2A by siRNAs. After an initial setup of the optimal conditions, we were able to silence more than 90% of the RNase H2A subunit in all of the cell lines, as detect by western blotting with an antibody against this subunit (Fig. 21). A novel clone expressing the steric gate mutation for DNA polη was isolated and will be used for future analysis. This clone showed a higher and more stable expression of the transgene at levels comparable to the wt transgene in the control cell line (data not shown). This RNase H2A deficient background will be useful for future experiments to have a more precise quantitation of ribonucleotides inserted by the mutant DNA polη.

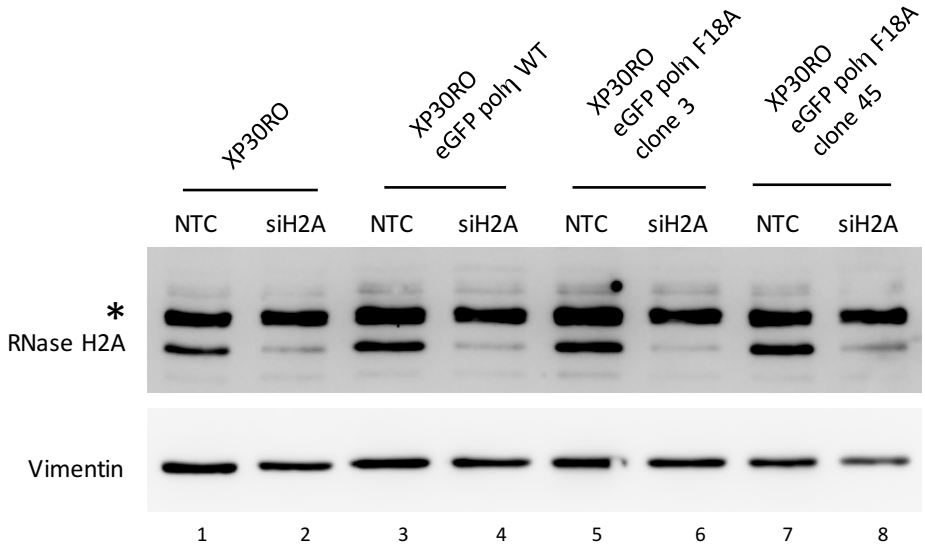


Fig. 21 Knock down of RNase H2A by siRNA

Cells were silenced either with a NTC scrambled siRNA or with a smartpool against the H2A subunit of human RNase H2. Efficiency of silencing was tested by western blot in all of the cell lines. The asterisk (*) indicates an aspecific band identified by the antibody.

2. Genomic editing of PCNA K164

2.1 CRISPR/Cas9 of PCNA K164 with ssDNA oligos for homologous recombination

Previous works on PCNA K164 have employed different model organisms showing conflicting behaviours of cell lines carrying the K164R mutation. This in turn puzzled researchers in the field about the role and importance of this residue in DNA damage tolerance. To date we still lack a mammalian model to study the importance of this residue on PCNA. For these reasons we decided to establish a mammalian cell line carrying the K164R mutation on the PCNA genomic locus. In recent years the CRISPR/Cas9 system has demonstrated to be a powerful tool to edit precisely genomic loci and generate useful model organisms. We took advantage of this system to target the K164 residue in mammalian cell lines thus abolishing post-translational modifications. We purchased a verified gRNA plasmidic system that carries both the gRNA and a GFP-fused Cas9 enzyme (Fig. 22B). CRISPR/Cas9 system has been extensively used to generate precise knockouts, however precise editing of a single allele, also called knock-in, is still considered a challenging task. Generation of point mutations in desired alleles requires a homologous recombination intermediate and thus there is the necessity of a homologous donor DNA sequence. Many works have demonstrated that ssDNA homologous sequences are sufficient to have a successful recombination and acquisition of the mutated allele (Liang X et al. 2017). For this reason, we decided to use ssDNA oligonucleotides as donor DNA sequences (Fig. 22A). Two different oligonucleotides were chosen: one targeting the coding strand and a complementary sequence targeting the opposite strand. Both oligonucleotides carried the K164R mutation, a PAM inactivating mutation to prevent undesired cut by Cas9 and finally a silent mutation to inactivate the ApoI restriction site, useful for subsequent screening.

A

5'-ATA TGC CGA GAT CTC AGC CAT ATT GGA GAT GCT GTT GTA ATT TCT TGT GCA AGG GAC GGA GTG AAG TTT TCT GCA ACT GGA GAA CTT GGA AAT GGA AAC A-3'

5'-TGT TTC CAT TTC CAA GTT CTC CAC TTG CAG AAA ACT TCA CTC CGT CCC TTG CAC AAG AAA TTA CAA CAG CAT CTC CAA TAT GGC TGA GAT CTC GGC ATA T-3'

B

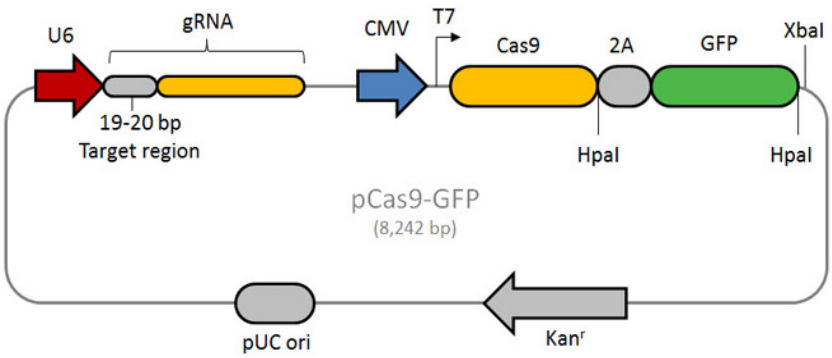


Fig. 22 DNA substrates for CRISPR/Cas9 targeting

A Sense and antisensedonor homology single stranded oligonucleotides with the indicated modifications. In green the PAM mutation, in red the K164R mutation and in blue the ApoI mutation.

B gRNA design and verification was performed by SIGMA. The plasmid contains the designed gRNA cloned together with the Cas9 endonuclease fused with GFP to detect transfected cells and the presence of Cas9.

We chose to target two cell lines: the human transformed embryonic fibroblasts MRC5 and the XP30RO transformed fibroblasts from XPV patients. The difference between these two cell lines lies in either the presence or absence of the DNA polymerase η respectively. After transfecting cells with the gRNA/Cas9 construct, GFP positive cells were sorted by FACS and seeded to obtain isolated colonies. Screening of surviving colonies was performed by treating cells with hydroxyurea (HU). HU is a ribonucleotide reductase inhibitor and slows down the replication fork. This slow down causes ubiquitylation of PCNA on K164. In fact, if cells were correctly targeted we would expect a reduction or the total absence of PCNA ubiquitylation upon HU treatment due to successful targeting of the locus. Thus, colonies were screened by western blot for PCNA ubiquitylation. Only few examples of the total clones screened are here reported (Fig. 23). Clones that did not present monoubiquitylation were reconfirmed by a second treatment in the same conditions (Fig. 23E). After the second confirmation screening, a single colony of XP30RO cells still presented a drastic reduction in PCNA ubiquitylation after HU treatment, remaining clones were ubiquitylated and thus discarded (Fig. 23E lane 6). For the identified putatively positive clone, we amplified the genomic locus for PCNA K164 and sequenced the PCR products. Unfortunately, although showing a non ubiquitylated phenotype, the locus resulted to be wild type (Fig. 24). Overall we screened 94 clones comprising both cell lines but did not obtain any successfully targeted clone.

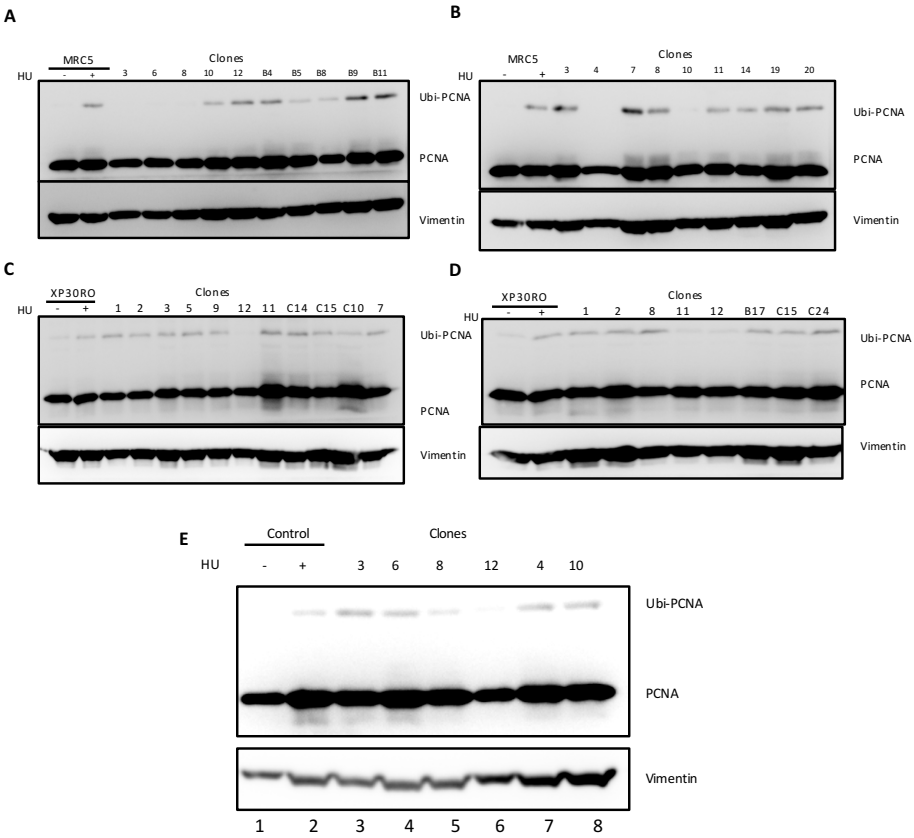


Fig. 23 Screening of targeted cells

Clones were screened by western blot for PCNA ubiquitylation after hydroxyurea treatment. Controls in lane 1 and 2 for each gel.

A MRC5 cells targeted with sense oligonucleotide.
B MRC5 cells targeted with antisense oligonucleotide.
C XP30RO cells targeted with sense oligonucleotide.
D XP30RO cells targeted with antisense oligonucleotide.
E Secondary screening for putatively positive clones.

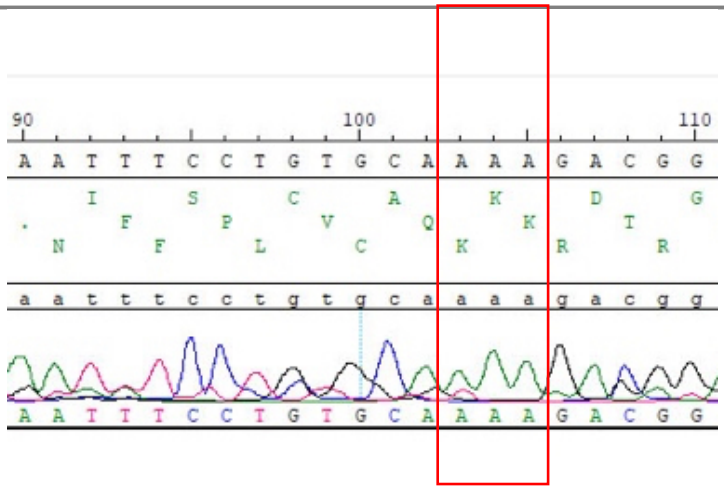


Fig. 24 Sequencing of PCNA K164 locus in CRISPR targeted cells

Genomic DNA was extracted from XP30RO clone 12 and the PCNA K164 locus amplified by PCR. The amplification product was sequenced and the chromatogram is here reported. The site of interest is here highlighted.

2.2 CRISPR/Cas9 of PCNA K164 with plasmidic DNA for homologous recombination

Given the unsuccessful outcome of the previous editing we decided to change our targeting strategy. To reduce the presence of false positive clones and thus be able to reach a higher efficiency of screening we decided to insert a selectable marker into the targeted locus. The only way to have a selectable cassette as donor DNA is to use a donor plasmid containing homologous sequences for recombination with the target locus. For this aim we substituted DNA oligonucleotides with a donor plasmid containing two homology arms that flank the selectable marker (Fig. 25).

Insertion of the marker should not affect the coding sequence of the locus, so in this case we targeted insertion into the intron between exon 5, that contains the K164 residue, and the flanking exon 6 (Fig. 25). For this reason, we had to change our gRNA to target the desired intronic region. Cutting by Cas9 in the region would stimulate homologous recombination using the homology regions in the donor plasmid. If the donor plasmid is mutated homologous recombination would insert the nucleotide variant together with the selectable marker. The plasmid also carries the thymidine kinase gene, that becomes toxic after treatment with ganciclovir. Consequently, a secondary negative selection with ganciclovir excluded all cells that may have integrated the entire donor construct and not only the regions flanked by the homology arms. In addition, subculturing clones in the presence of the selective agent would kill cells where homology recombination was not successful thus reducing the number of false positive clones. In the end, we also changed our screening protocol and clones were screened by PCR checking the presence of the selectable marker cassette in the PCNA intronic locus. Subsequent sequencing of the amplification products would allow genotyping of the clones (Fig. 25). The first step of the workflow required the insertion of the homologous sequences into the donor plasmid. Starting from genomic DNA we amplified about 1130 bp around the K164 locus in the 5th exon and the same length of bp in the 6th exon. Amplification was performed to insert specific restriction sites (see Materials and Methods) and homology sequences were cloned into the donor plasmid. The final product is a donor plasmid that carries the selectable marker and homology arms with homologous sequences from exon 5 and exon 6 of PCNA. After cloning the donor plasmid, we mutated the K164 in exon 5 by site directed mutagenesis to have both the wild type and K164R donor sequences.

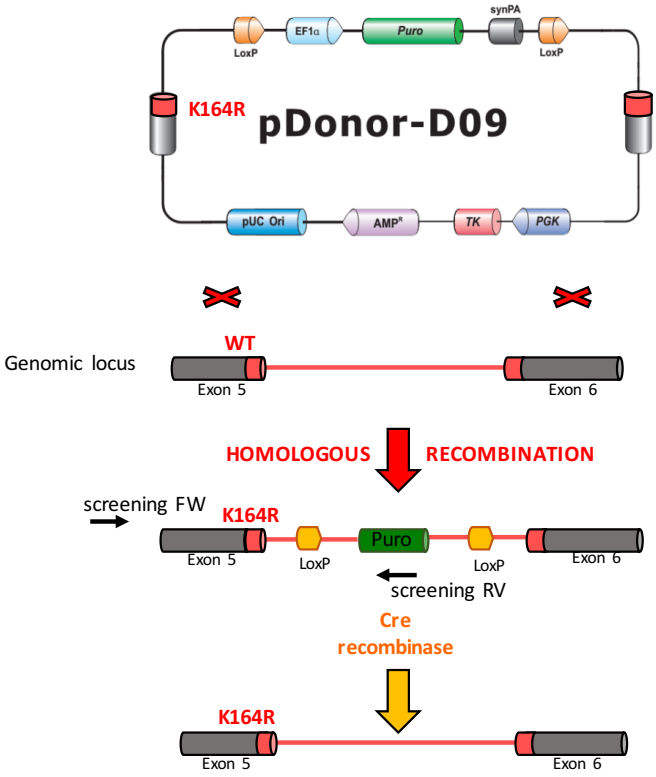


Fig. 25 Strategy to target PCNA k164 locus

The pDonor-D09 plasmid carries a puromycin resistance gene. The puromycin cassette can be inserted in the desired locus by flanking homologous sequences. Thus once inserted in the plasmid, homology arms for PCNA would target the mutation and the resistance cassette to the correct locus. Targeted cells are screened by PCR with a forward primer 5' to exon 5 and a reverse primer on the puromycin gene. The presence of LoxP sites flanking the resistance cassette allow their excision to have a final scarless locus.

2.3 Isolation of a K164R heterozygous clone

MRC5 and XP30RO cells were transfected with the gRNA/GFP-Cas9 constructs and the donor plasmid for homologous recombination with either the wild type or the K164R allele. GFP positive cells were seeded to obtain single colonies and grown with puromycin to select for positive recombination events, before negative selection in ganciclovir. Colonies were screened by PCR looking for the presence of the puromycin cassette in the PCNA locus (Fig. 26A). Three clones from MRC5 cells showed amplification products, but only one of them could be correctly amplified with the two screening primers (Fig. 26B). Clone number 36 was further characterised but presented incorrect amplification products of the locus due to possible unscheduled rearrangements and thus was discarded. A subsequent screening amplifying the entire region from exon 5 to exon 6, identified another positive colony, clone number 122 (Fig. 26C lane 3). As can be seen from PCR analysis this clone presented the parental locus with the addition of the longer amplification product given by insertion of the puromycin cassette. PCR products of the amplicon from clones number 122 and 82 were sequenced. (Fig. 26D). As evident by the chromatogram we could detect the presence of a wild type coding allele for clone 122 and a heterozygous sequence for clone 82. Since products were amplified using a primer for the puromycin resistance, we hypothesize a phenomenon, where, in clone 82, only a part of the homology harms were used for recombination. In fact, in these cells two alleles were targeted, one carrying the WT gene and the other the K164R mutant. Further analysis of the locus by amplification of the region between exon 5 and exon 6 revealed the presence of both the parental and the puromycin resistance locus (Fig. 26C lane 2). Having simultaneously the presence of two targeted alleles and an additional parental locus, we presumed that more than two copies of the PCNA gene may be present in our cell lines.

To verify this hypothesis we sent our cells for a karyotipic analysis. MRC5 paternal cell lines showed the presence of 3 copies of the PCNA locus (data not shown, Courtesy of Catherine Green, University of Oxford). The amplified karyotype explains the presence of a non targeted locus as shown by PCR analysis (short band in Fig. 26C lane 2) alongside a longer amplicicons carrying the puromycin cassete and both WT and K164R mutated exon 5 (as monitored by sequencing).

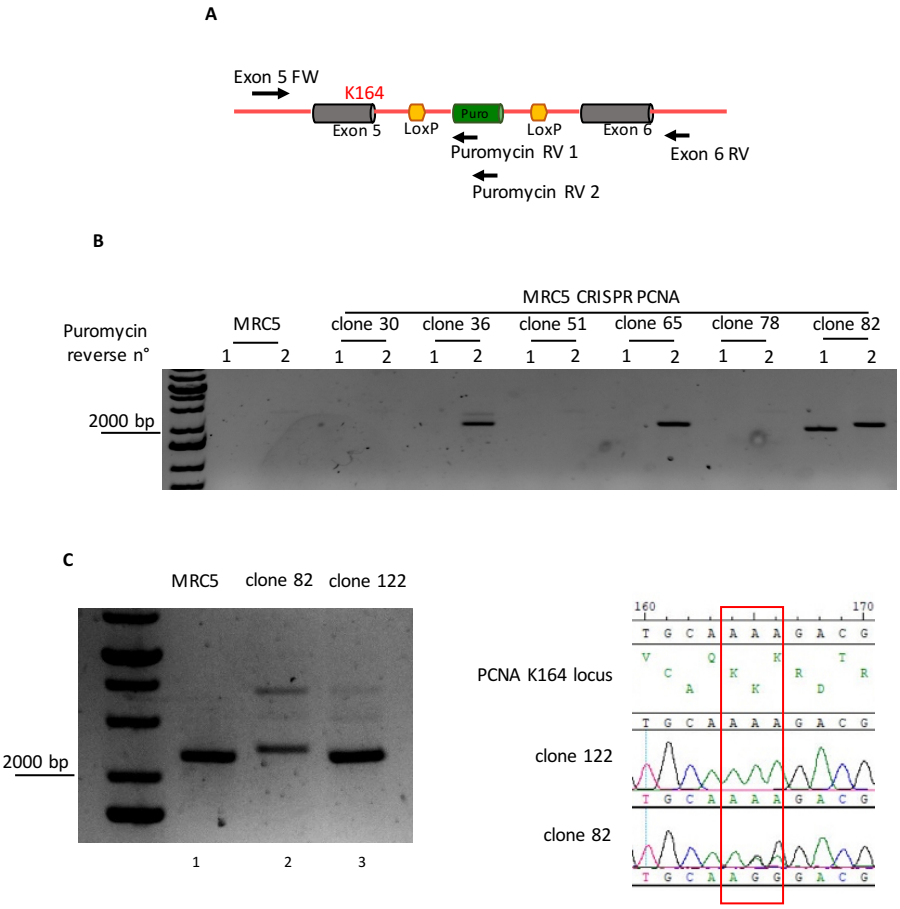


Fig. 26 Screening of PCNA CRISPR targeted cells

A Schematic representation of the screening strategy. The genomic locus is targeted by a forward primer mapping 5' the exon 5 and two different reverse primers inside the puromycin resistance gene.

B Results of PCR screening of surviving colonies. Both reverse primers mapping inside the puromycin resistance have been tested. MRC5 genomic DNA is used as negative control.

C Results of PCR screening using primers to amplify the region from exon 5 to exon 6. Presence of the puromycin cassette should be detected as a higher electrophoretic band compared to the parental locus.

D Sequencing results of the amplification products from clone 122 and 82. K164 locus is highlighted in the figure showing the presence of wild type alleles for clone 122 and heterozygous alleles for clone 82.

Considering the complex genomic rearrangements and taking into account that our strategy targets the intronic region of the PCNA gene, we also decided to control the correct maturation of transcripts in the CRISPR/Cas9 targeted cells. We extracted total mRNA from cells, retrotranscribed the samples and finally amplified the target region from exon 5 to exon 6. Only one amplification product was present, indicating that no alternative splicing was occurring between exon 5 and exon 6 (Fig. 27A). The amplification product was sequenced and (Fig. 27B) confirmed, as can be seen from the chromatograms, the expression of both WT PCNA but also PCNA K164R. This made us confident that we had indeed established a heterozygous cell line and the transcript was maturating correctly, eventually to be translated as a functional protein.

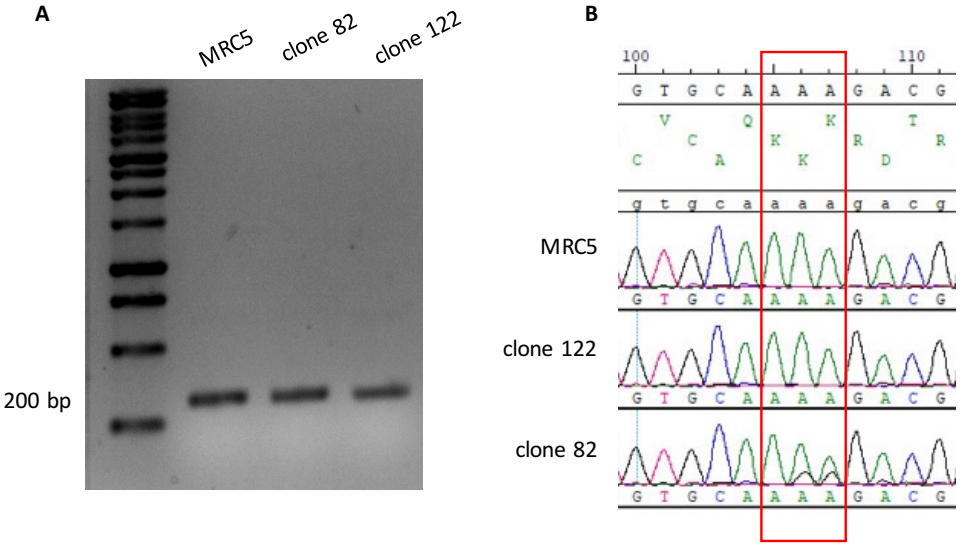


Fig. 27 Analysis of PCNA transcripts by cDNA sequencing.

A Total RNA was isolated from cell lines and cDNA was obtained. PCNA transcript was amplified with primers spanning from exon 5 to exon 6 to comprise the K164 locus and the region targeted by the CRISPR/Cas9.

B Chromatograms from cDNA sequencing of each cell line are here reported. The K164 is highlighted in the figure showing a wt transcript for parental cell line and CRISPR targeted wt cells. The K164R mutation previously identified at genomic locus is retained in the transcript of clone 82.

2.4 K164R cells have a reduced PCNA ubiquitylation and a slower progression through cell cycle in the presence of hydroxyurea

Having obtained a heterozygous clone and its targeted wild type control, we decided to start their characterisation. At first we checked the response of cell lines to HU treatment. To begin, we analysed PCNA ubiquitylation by western blotting. To do so we treated cells with 1 mM HU for 24 hours and prepared whole cell extracts for SDS PAGE analysis. Comparing ubiquitylation of PCNA between MRC5, and MRC5 PCNA K164R heterozygous cells and MRC5 PCNA wt targeted cells, a considerable reduction of PCNA ubiquitylation can be appreciated both before and after HU treatment (Fig. 28).

Quantification of the PCNA monoubiquitin signal revealed a reduction of the modification when comparing PCNA wt and PCNA K164R heterozygous both in untreated and HU treated samples (Fig. 28 compare lanes 1, 3 and 5 and lanes 2, 4 and 6). A reduction of about 1/3 was present in the K164R cell lines both in untreated and HU treated cells. (Fig. 28 lanes 3 and 4). This is consistent with a possible reduction of the PCNA subunits where K164 cannot be efficiently ubiquitylated as a consequence of CRISPR targeting.

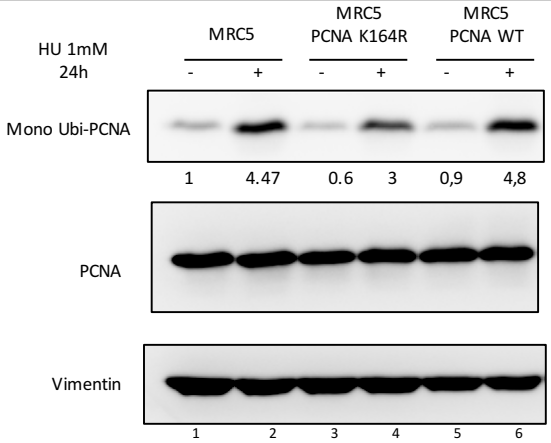


Fig. 28 PCNA ubiquitylation analysis after HU treatment

Ubiquitylation of PCNA was tested in MRC5 parental cell line, MRC5 CRISPR PCNA wt and MRC5 CRISPR PCNA K164R heterozygous clone by treatment with 1 mM HU for 24 hours followed by western blot. Intensity of Ubi-PCNA was detected by densitometric analysis with a specific Ubi-PCNA antibody and normalised on PCNA content. Values are reported below the blot.

Since we noticed a reduction in the ubiquitylation of PCNA in the heterozygous cell line, we decided to assess the progression through cell cycle of the mutated cells. Cell cycle progression is affected following HU treatment by the depletion of dNTPs pools. In particular, replication forks slow down due to scarceness of substrates for replication. PCNA ubiquitylation has been hypothesised to be a central event to preserve S phase progression and avoid replication fork stalling. To test this hypothesis in our cell lines, we decided to follow cell cycle progression upon HU treatment. After treating samples with low doses of HU for 24 hours, cells were stained with propidium iodide and analysed by FACS analysis. Untreated samples showed a normal and comparable progression through cell cycle with the majority of cells in the G1 phase after 24 hours. At 24 hours past HU treatment, MRC5 cells presented a block at the early stages of S phase comparable to the MRC5 PCNA wt targeted cells (Fig. 29). Surprisingly the heterozygous K164R cells showed a distinct phenotype with a higher number of cells blocked at even earlier steps of S phase (Fig. 29).

Thus while progression through unperturbed cell cycle is unaffected in the K164R heterozygous cells, following conditions that perturb the replication fork they show a slower entry or progression in S phase.

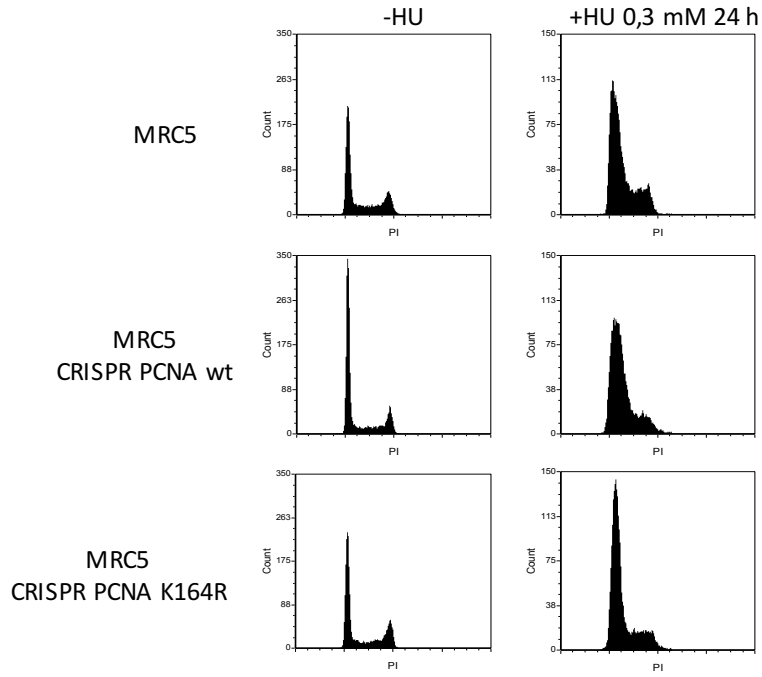


Fig. 29 Cell cycle progression after HU treatment

Cells were treated with 1 mM HU and fixed at 24 hours. Cell cycle distribution was analysed by propidium iodide stain and detected by FACS. Y axis scale was reduced in UV treated samples to simplify readibility of the plot.

2.5 Progression through cell cycle is faster in K164R cells after UV irradiation

The role of PCNA ubiquitylation is fundamental also in the bypass of UV lesions, to permit translesion synthesis. In fact, blockages of the replication fork, encountering UV damage, can be rescued by TLS polymerases that are recruited after PCNA ubiquitylation. We decided to test the response of our cell lines also in this case and thus we irradiated the cells with UV-C and checked PCNA monoubiquitylation by western blotting. (Fig. 30). Even in unirradiated samples a reduction of PCNA ubiquitylation was detectable between heterozygous K164R cells and wild type targeted cells (Fig. 28 compare lanes 1 and 3 with lane 5), indicating a lower abundance of the modification in unperturbed conditions. PCNA ubiquitylation was considerably reduced at more than a half upon UV treatment in the heterozygous cells (Fig. 30 compare lane 2 and lane 4 with lane 6). Thus, even being able to target just one allele we already had a strong phenotype in terms of PCNA ubiquitylation in response to UV damage.

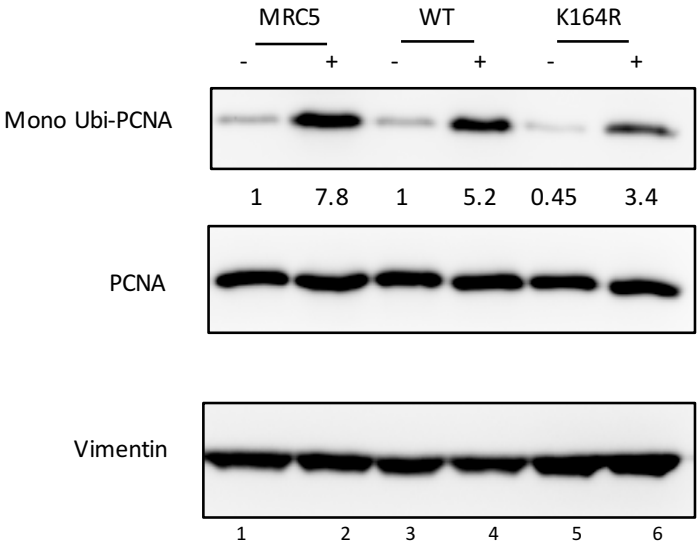


Fig. 30 PCNA ubiquitylation analysis after UV treatment

Cells were irradiated at 15 J/ m^2 of UV-C to cause replication fork arrest and PCNA ubiquitylation. Samples were taken after 24 hours. Ubiquitylation of PCNA was assessed by western blot with a specific Ubi-PCNA antibody.

Also in the case of UV irradiation, damage on DNA blocks the replication fork and affects cell cycle progression. We tested the role of PCNA ubiquitylation also in this case, following cell cycle in our targeted cell lines. Cells were UV irradiated and then stained with propidium iodide at 24 hours post irradiation (Fig. 31). All untreated cells showed a normal progression through cell cycle as previously shown. On the other hand, MRC5 and MRC5 wild type targeted cells showed a similar profile with cells blocked mostly at the early S phase. Interestingly, heterozygous K164R cells had a faster progression through S phase with less cells at in early S phase and more in to mid-to late S phase. Again, even in the presence of a single targeted locus, a clear phenotype was detectable in K164R cells.

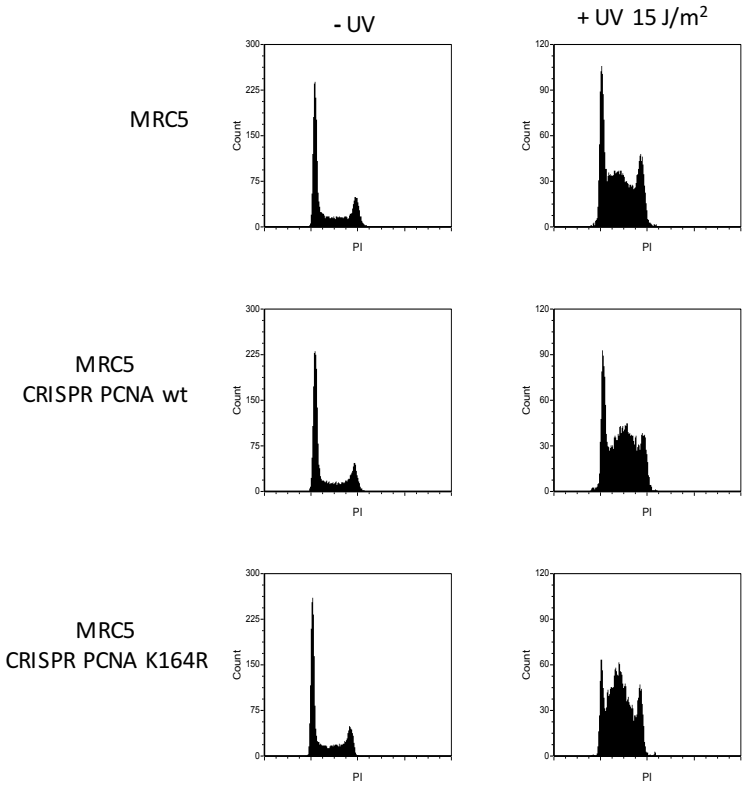


Fig. 31 Cell cycle progression after UV irradiation

Cells were treated with 15 J/m^2 UV-C and fixed at 24 hours. Cell cycle distribution was analysed by propidium iodide stain and detected by FACS. Y axis was reduced in UV treated samples to simplify readibility of the plot.

To summarise these results, following treatments that either slow or block replication fork, monoubiquitylation of PCNA was consistently reduced confirming the reduction of target wt subunits. Surprisingly, heterozygous K164R cells had a clear distinct phenotype when compared to wild type counterpart and parental cells. In the case of fork slow down by HU, heterozygous mutated cells showed a more pronounced block at earlier stages of S phase compared to the other cell lines. On the contrary, upon UV irradiation, K164R heterozygous cells showed a faster progression through S phase than the wild type counterpart.

These different phenotypes may account for different necessities in terms of PCNA ubiquitylation in the two different scenarios and thus require further analysis. In fact, upon UV irradiation, PCNA ubiquitylation has been demonstrated fundamental to have a maximal UV tolerance in mice (Hendel A et al. 2011). However other bypass mechanisms that do not rely on PCNA ubiquitylation on K164 should not be excluded, since knock down of other players (Rev3L, PolH and Rev1) caused each a further increase in UV sensitivity. This data may be consistent with our results where partial loss of PCNA K164 ubiquitylation does not impede progression through S phase.

3. Translesion synthesis and G4 DNA

3.1 Stabilisation of G-quadruplex DNA affects cell viability in the absence of DNA polymerase η

The needs for a TLS mechanism in replicating past non B-DNA structures, such as g-quadruplexes is yet to be defined *in vivo* in mammalian cells. The use of G4-stabilising compounds has expanded this field of research and given the possibility to study G4 dynamics *in vivo*. DNA polη was already shown to be able to replicate past complex DNA structures. This capability is provided by its peculiar structural features (Eddy S et al. 2015). Given these evidences, we decided to investigate the possible involvement of the TLS mechanism in replication past g-quadruplex DNA. To this end we took advantage of pirydostatin (PDS), a compound that was isolated as a small highly selective G4 binding molecule and has been exploited to study the presence of g-quadruplex in mammalian cells (Rodriguez R et al. 2012; Zimmer J et al. 2016). In particular, the compound is classified as a G4 stabilizing compound both *in vitro* and *in vivo* (Muller S et al. 2010; Koirala D et al. 2011). Its binding to g-quadruplex DNA *in vivo* prevents the resolution of the structure causing replication fork stalling and inducing DSBs (Rodriguez R et al. 2012; Zimmer J et al. 2016; Moruno-Manchon J et al. 2017). Since we were interested in dissecting TLS dynamics upon PDS treatment, we used XP30RO and XP30RO-eGFPpolη complemented cell lines. Eventual differences between cell lines may be correlated to the activity of polη and thus to replication of g-quadruplex DNA via a TLS mechanism.

To begin, we analysed viability of the two cell lines upon treatment with increasing doses of PDS. At the highest dose of PDS (10 μM), a significative difference between cell lines was evident (Fig. 32). In particular, XP30RO cells showed about 50% reduction in cell viability compared to the complemented cell line at 24 hours from treatment. Summarising, in the absence of DNA polη the presence of unresolved g-quadruplex reduces cells viability through a yet to be defined mechanism.

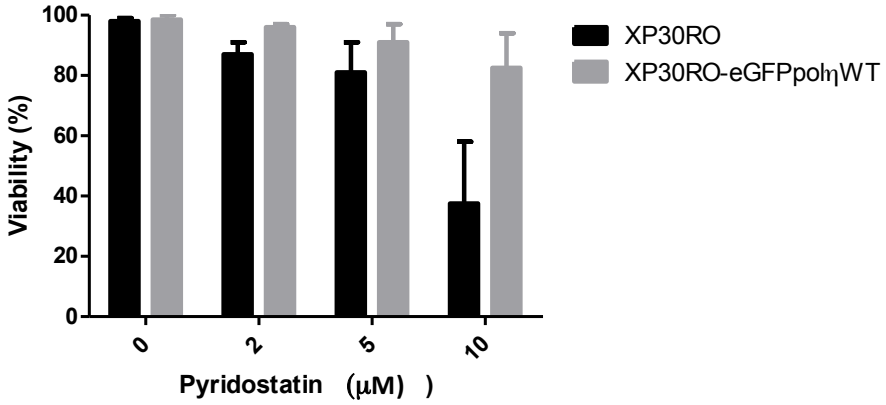


Fig. 32 Analysis of cell viability after PDS treatment
Equal number of cells were seeded and treated with the indicated doses of pyridostatin. After 24 hours cells were treated with Trypan Blue and counted with an automated cell counter. Each count was normalised to the initial values and viability expressed as percentage of living cells.

3.2 Unresolved G4 structures activate S phase checkpoint in TLS proficient cells

To analyse more in details, the effect of G4 stabilisation in the two cell lines, we decided to dissect the DDR pathway. A possible explanation of the differences we have observed could lie in a different activation of the DNA damage sensor kinases. For these reasons we treated at the same increasing concentrations of PDS the two cell lines and then checked the activation of different players of the DDR and TLS pathways by western blot.

In particular, the stabilisation of g-quadruplex sequences may slow the replication fork while encountering the obstacle, hence resulting in the activation of the checkpoint response. Thus we checked activation of the effector kinase, Chk1. As already mentioned, Chk1 is activated by phosphorylation on S345 and once active it can phosphorylate its downstream effectors. Using a specific antibody directed against S345 of Chk1, we detected a dose dependent phosphorylation of this residue (Fig. 33). More interestingly, the activation of the kinase was higher in XP30RO-eGFP polη cells compared to their parental cell line at the highest PDS dose (Fig. 33 compare lane 4 and lane 8). Together with the checkpoint, we analysed the activation of the TLS pathway by means of PCNA monoubiquitylation. A considerable higher signal of monoubiquitylation was detected with a specific antibody in polη proficient cells at the highest PDS dose (Fig. 33 compare lane 4 and lane 8). Interestingly increasing doses of PDS also caused an increase in DNA polη total quantity (Fig. 33 from lane 5 to 8). Whether this accumulation could be correlated to a higher production of the transcript or whether the polymerase may be stabilised in the presence of unresolved G4s will be an interesting point to be addressed. To summarise, the stabilization of G4s leads to a stronger activation of the kinase Chk1 and an increased ubiquitylation of PCNA in a manner dependent on the presence of DNA polη.

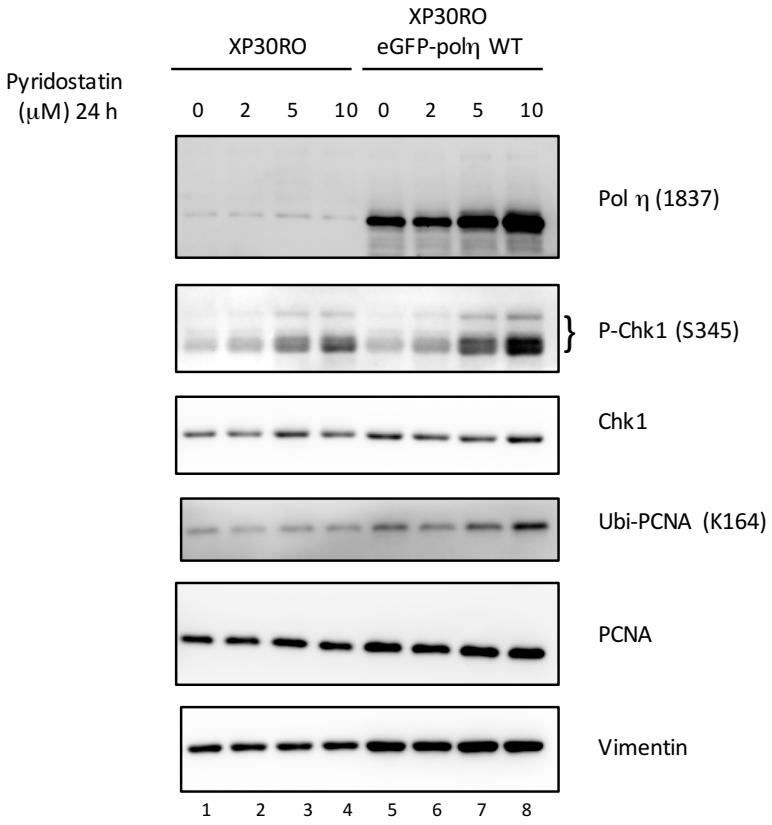


Fig. 33 Western blot analysis of S phase checkpoint activation and TLS after PDS treatment

Cells were treated with increasing doses of PDS and whole cell extracts were loaded on gel after 24 hours. Activation of Chk1 was analysed using a phospho-specific antibody against S345. The phosphorylated or hyperphosphorylated forms can be visualized as a shift during electrophoresis (}). Total Chk1 protein in the extracts was also controlled. Activation of the TLS pathway was assessed using an Ubi-PCNA antibody to detect its monoubiquitylation on K164. Total PCNA protein was also controlled in the extracts.

In these conditions, the slowing down of the replication fork after PDS treatment may lead to a higher accumulation of ssDNA, followed by RPA binding and its phosphorylation. This sequence of events recruits the E3 ubiquitin ligase Rad18 at the fork and thus permits PCNA monoubiquitylation. Given the crucial role of this axis for TLS activation, we tested whether a different activation of either RPA or Rad18 may be correlated to the previously observed differences in PCNA ubiquitylation. However, no differences in either Rad18 activation or RPA phosphorylation were visible between the two cell lines upon PDS treatment (Fig. 34). Thus, we can rule out the possibility that the increase of PCNA ubiquitylation may be related to a higher activity of the E3 ubiquitin ligase or indirectly by accumulation of ssDNA.

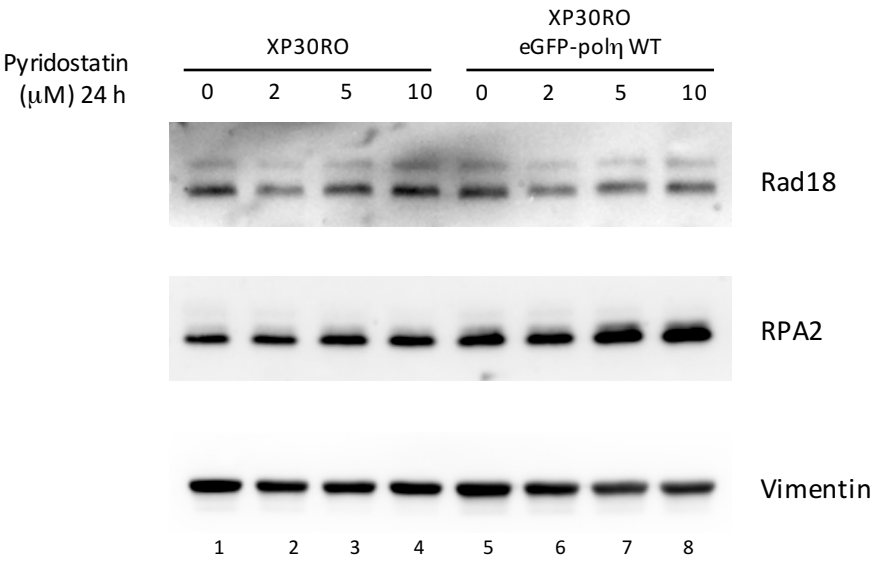


Fig. 34 Western blot analysis of Rad18 activation and RPA phosphorylation

Cells were treated with increasing doses of PDS and whole cell extracts were loaded on gel after 24 hours. Activation of Rad18 was analysed by checking the reduction of the ubiquitylated form visualised as a retarded band on gel. Phosphorylation of RPA2 was analysed controlling the gel shift of the RPA2 subunit.

3.3 Cells fractionation reveals dynamics of TLS in the presence of stable G4 structures

The majority of the dynamics of checkpoint and TLS activation occur on chromatin. To be able to discern subtler changes on protein dynamics on chromatin, we decided to perform cell fractionation after PDS treatment. Whole cell extracts in the same conditions were simultaneously obtained (Fig. 35A). Protein content can be subdivided in this case as the soluble fraction (Fig. 35B), containing both cytoplasmic and nuclear soluble factors (as indicated by the absence of histone H3, panel B) and the chromatin fraction (Fig. 35C), containing exclusively those proteins that were bound to DNA at

the time of extraction (as indicated by the absence of tubulin, panel C). By analysing whole cell extracts, a stronger monoubiquitylation of PCNA of about 2.5 fold was still detectable in XP30RO polη wt complemented cells at the highest PDS dose (Fig. 35A compare lane 4 and lane 5). We were able to confirm also the accumulation of eGFP polη as total protein content at increasing doses of PDS increasing at a maximum of almost 4 fold when compared to the untreated control (Fig. 35A from lane 5 to 8). No differences in the total amount of RPA2 were noticed comparing either the two cell lines or the treatment. Regarding the soluble fraction, no changes could be detected in all the proteins analysed (Fig. 35B). Interestingly chromatin protein fraction showed again a higher presence of DNA polη at the increase of PDS reaching an accumulation of about 10 fold when compared to the its matched control (Fig. 35C from lane 5 to lane 8). In light of these numbers, we can conclude that the maximum dose of PDS (10 μM) induces an accumulation of eGFPpolη at chromatin level that it cannot be simply explained by its increased total protein levels that are found in the cell upon PDS exposure. This result suggests that DNA polη may be required at the level of chromatin when G4 are stabilized and present an impediment to the progression of the replication. Interestingly, also PCNA monoubiquitylation was detectable on chromatin and doubled at the maximum PDS dose in the complemented cell lines while it did not change in XP30RO cells (Fig. 35C compare lane 4 and 8). Again, at the level of chromatin also RPA2 subunit did not change neither between the cell line nor upon PDS treatment. To conclude, differences between the two cell lines are clear and involve a different response along the Chk1 axis and a different activation of DNA damage tolerance pathway. However, the precise dynamics of G4 metabolism at stalled replication forks and the direct involvement of DNA polη will need further detailed studies.

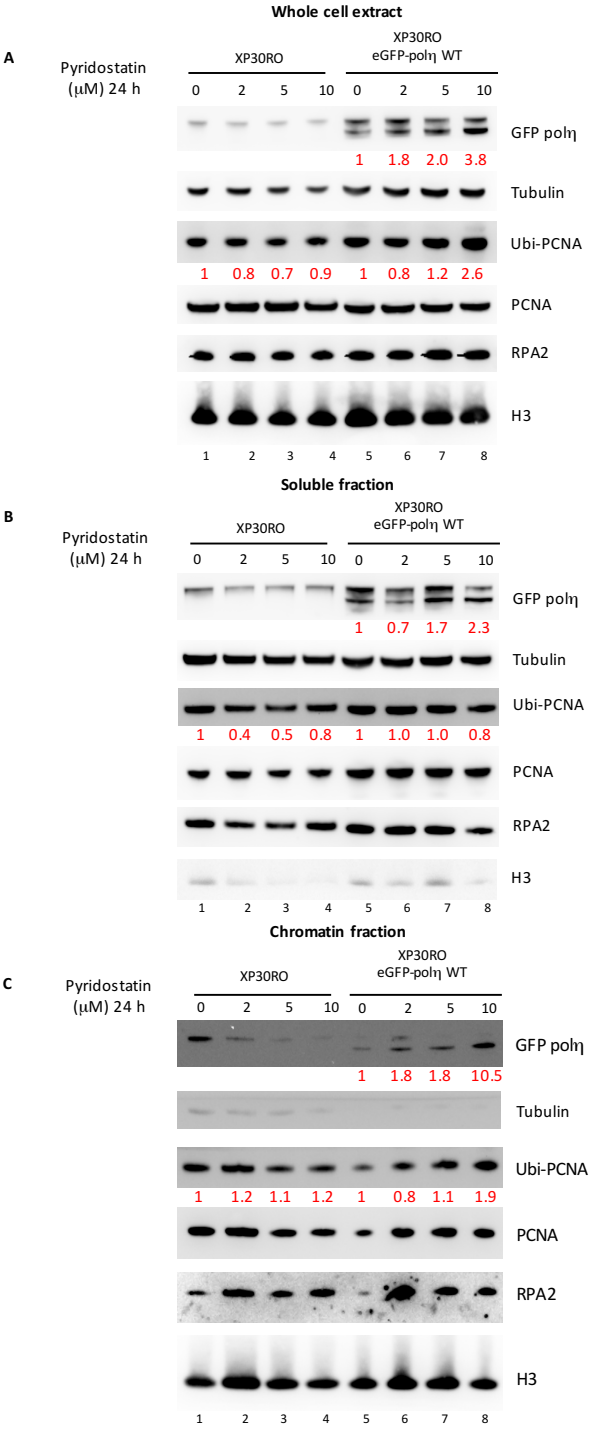

Whole cell extract
A
Pyridostatin (μM) 24 h
XP30RO
XP30RO eGFP-polη WT
0 2 5 10 0 2 5 10
GFP polη
1 1.8 2.0 3.8
Tubulin
Ubi-PCNA
1 0.8 0.7 0.9 1 0.8 1.2 2.6
PCNA
RPA2
H3
1 2 3 4 5 6 7 8
Soluble fraction
B
Pyridostatin (μM) 24 h
XP30RO
XP30RO eGFP-polη WT
0 2 5 10 0 2 5 10
GFP polη
1 0.7 1.7 2.3
Tubulin
Ubi-PCNA
1 0.4 0.5 0.8 1 1.0 1.0 0.8
PCNA
RPA2
H3
1 2 3 4 5 6 7 8
Chromatin fraction
C
Pyridostatin (μM) 24 h
XP30RO
XP30RO eGFP-polη WT
0 2 5 10 0 2 5 10
GFP polη
1 1.8 1.8 10.5
Tubulin
Ubi-PCNA
1 1.2 1.1 1.2 1 0.8 1.1 1.9
PCNA
RPA2
H3
1 2 3 4 5 6 7 8

Fig. 35 Western blot analysis of total, soluble and chromatin fractions

Cells were treated with increasing doses of PDS for 24 hours.
Fractionation of cells lysates was perfomed and provided whole cell extracts (A), soluble fraction (B) and chromating bound fraction (C). Fractionation was controlled by the absence of histone H3 in the soluble fraction and by tubulin in the chromatin fraction. Values were obtained by densitometric analysis and normalised on loading controls.

5. Discussion

DNA polη steric gate mutant incorporates ribonucleotides and it is a useful tool for DNA polymerase tracking

DNA polymerases have the most delicate task in genome replication, providing a correct copy and preserving its integrity. However, the genome is not homogeneous and presents regions that are inherently difficult to replicate eventually causing genomic instability. In addition to this, exogenous sources constantly threaten DNA requiring additional factors to preserve its integrity. DNA damage tolerance is one of the mechanisms that cells have evolved to counteract replication stress. Between the different players, DNA polymerase η and PCNA are among the main actors and remarkable efforts have been done during years to characterise their roles. Mounting evidences are redefining the role of DNA polymerase η outside of the TLS pathway. Its main advantage is given by the versatility of the polymerase catalytic site. This is a wider catalytic site with respect to replicative polymerases making it uniquely useful during replication of non canonical DNA structures or in the occurrence of replication stress. Seminal works have identified a role for this alternative polymerase in replicating past CFSs or across g-quadruplex DNA (Bergoglio V et al. 2013; Betous R et al. 2009). Thus, the emergence of DNA polη as a specialised polymerase in replicating non canonical DNA requires a more detailed investigation. Different techniques have been set up to follow polymerases activity. The most recent strategies have developed methods to force incorporation of ribonucleotides by the desired polymerase followed by sequencing of NTPs in the genome. This requires genetic engineering of the polymerase in its active site to change its sugar selectivity towards ribonucleotides. We started our project with the identification of the steric gate residue of polη by analysis of evolutionary sequence conservation. While performing in silico analysis, another group of authors confirmed our hypothesis on the identity of the steric gate residue for human DNA polη. In fact, Su Y. et al were able to define the mechanisms of NTPs incorporation and the main role of F18 by X-ray crystallography (Su Y et al 2016). More interestingly they also identified a secondary aminoacid, tyrosine 92 (Y92), as a critical residue to maintain the position of F18 and thus to support its steric gate function.

Future analysis may consider also this secondary residue to disrupt the function of the steric gate. *In vitro* analysis of the catalytic activity of the mutant polymerase excluded any major impairment of the active site due to the F18A mutation. Nevertheless, the mutant polymerase showed a lower processivity in all conditions tested. It must be taken into account that additional factors, such as PCNA, were not present in our reactions possibly explaining the loss of efficiency in DNA elongation. Moreover, as it was demonstrated by X-ray crystallography, incorporation of NTPs is a slower process compared to incorporation of dNTPs and further confirming the mechanisms we were observing *in vitro*. Overall, the mutation of the single F18 residue stimulated ribonucleotides incorporation while having little effects on the catalytic activity of the polymerase. This data made us confident about our predictions and we generated a mutant construct to proceed with the *in vivo* characterization. No deleterious effects were noticed in the behaviour of XP30RO cells complemented with the DNA polη F18A transgene. Mutation of F18 lies in a region that is far from the C-term regulatory motifs. Thus as expected the mutant polymerase retained its correct nuclear localisation and the capability to form discrete foci after UV irradiation. The only difference with the wt complemented counterpart was in the capability of UV damage bypass. Both long term clonogenic assay and short term cell cycle analysis highlighted a deficiency, although to different extent, in UV damage bypass for the F18A mutant cells. This phenotype was similar to what observed in yeast where the presence of the steric gate mutated polymerase decreased cells viability in the presence of UV (Donigan K et al. 2015). Loss of RNase H2 gene in yeast recovered cells survival thus correlating repair of genomic embedded ribonucleotides with the observed phenotypes. As we have demonstrated *in vitro*, UV lesions can be bypassed with ribonucleotides by the mutant polymerase. However, the presence of an active RER pathway would cause cleavage of NTPs by RNase H2. In this scenario, an attempt to bypass damage by mutant DNA polη with ribonucleotides, and its subsequent removal by RER, results in the generation of gaps in DNA that represent a more detrimental source of damage for the cells. The simultaneous presence of a polymerase inserting ribonucleotides and RNase H2 could create a futile bypass cycle in F18A cell lines and may account for reduced UV damage bypass capability.

Deeper analysis of this cycle may help confirm our hypothesis and may shed light on the bypass of DNA damage in a background of genomic DNA filled with ribonucleotides. Despite this phenotype, in the absence of exogenous sources of DNA damage, F18A cells behaved as their wt counterpart and thus represented an ideal substrate for DNA polη tracking in unchallenged conditions. Taking into account the reduced processivity and somehow decreased damage bypass, the cell line could be still very useful for tracking polη after genotoxic insults like UV irradiation and treatment with DNA crosslinking agents.

Before proceeding with the polymerase tracking strategy, we have verified the accumulation of ribonucleotides *in vivo*. Insertion of ribonucleotides in DNA can occur either as a single insertion event or by primer elongation with NTPs that leads to longer stretches of DNA:RNA hybrids in genomic DNA. The possibility to detect DNA:RNA hybrids has relied on the affinity of the S9.6 antibody for such structures. It must be taken into account that use of this antibody precludes detection of single ribonucleotides scattered in DNA. In fact, the minimal sequence detected by the antibody is of about 6 consecutive ribonucleotides forming a hybrid. Increasing data about the affinity of the antibody for other RNA structures such as RNA:RNA hybrids or RNA secondary structures must be considered. Accordingly, from our immunofluorescence staining it is clear how the antibody may recognise structures different from genomic hybrids, since cytoplasmic stain is always detectable. However, the nuclear accumulation we detected was specifically increased in the presence of the F18A cell line. In addition, another evidence confirming the specificity of the signal came from the use of RNase HII as a control reaction. In fact, after pre treatment with RNase HII, staining with S9.6 was barely detectable. This was a clear indication that the signal we were detecting with previous staining was a substrate of RNase HII, thus it could be specifically attributed to the presence of nuclear DNA:RNA hybrids. As anticipated, insertion of ribonucleotides can be efficiently detected by the S9.6 staining while single NTPs cannot be precisely quantified. In this scenario we were still able to detect an increase in mutated cell lines but neglected the contribution of single insertion events. Single ribonucleotides can be isolated by treating genomic DNA with RNase HII. Nicks that are generated can be filled by using a labelled nucleotide analogue.

In order to obtain preliminary data we performed reactions in the presence of a radioactive precursor (α^{32}P-dCTP). By performing such an assay, we confirmed the previous results. We were able to show an accumulation of hybrids and we could detect an increase of ribonucleotides in genomic DNA from F18A cells. This provided a higher level of resolution for NTPs detection and allowed us to quantify more precisely the ribonucleotides load. While being extremely sensitive, radioactive precursors require a dedicated and more laborious handling of samples. To counteract these requirements, more recently a wide variety of labelled nucleotide precursors were made available. Taking advantage of such substrates, we decided to improve the assay with the use of a fluorescently tagged nucleotide precursor. Again in this case we detected an increase of signal from the mutated cell lines compared to wt complemented or XP30RO cells and thus we confirmed the previous data. Interestingly in some experiments we also observed an increase in ribonucleotides also in the XP30RO cells. In light of this, we speculate that in the absence of DNA polη, another polymerase with lower sugar discrimination capability may insert ribonucleotides in genomic DNA. Future studies on such mechanisms may reveal the activity of alternative polymerases in the absence of DNA polη. In this scenario abrogation of the RER pathway would be necessary to detect ribonucleotides that could be inserted at low frequency by other polymerases. Quantitation of ribonucleotides in genomic DNA is useful to validate cell lines as substrates for polymerase tracking experiments. The final aim would be to be able to isolate DNA fragments containing ribonucleotides. With this in mind, one possibility is to cleave genomic DNA with RNase HII at the level of ribonucleotides and then label nicked DNA with a different analogue that would allow DNA isolation. One of the most used techniques to label DNA is biotinylation. Therefore, we set up conditions to biotinylate samples treated with RNase HII and isolate DNA containing ribonucleotides. However, being able to isolate DNA was more challenging than predicted. At the moment we are still improving all conditions, from binding to elution, to be able to isolate sufficient amounts of biotinylated DNA for amplification of the desired targets. We are sure we could be able to perform the final amplification steps since we could amplify, even if at low amounts, the GAPDH locus from nicked genomic DNA.

In this case, DNA presented a relevant amount of nicks ending up with numerous biotinylated sites, thus facilitating affinity binding with streptavidin resin. In the case of RNase HII treatment, we are expecting to generate considerably lower amounts of nicks thus having lower amounts of biotinylated DNA. Moreover, it is inevitable to lose material given the number of reactions and steps required for the entire process. For these reasons, another point we are addressing is the increase of the input material to prevent loss of substrate for the final amplification steps. One of the main targets of DNA polη are common fragiles sites (CFSs) (Bergoglio V et al. 2013). Presently, we failed at isolating these sites in our assay. Besides technical improvements, it must be taken into account that in previous works detection of DNA polη at these sites was successful only after induction of replication stress (Bergoglio V et al. 2013). Thus, we may consider to reproduce such conditions in order to be able to detect the activity of the polymerase at these sites. In addition to this, in all of the previously described cases, the amount of genomic ribonucleotides is reduced by the presence of RER pathway in the cells. Thus, in all of our experiments we must consider that we are underestimating the actual insertion of NTPs by the mutant polymerase. Similar works in yeast have always taken advantage of RNase H2 deleted strains (Donigan K et al. 2015). Given the relevance of this active removal of ribonucleotides we also decided to work in a background with inactive RNase H2. At first we used a lentiviral system against the catalytic subunit, RNase H2A. Although being a promising strategy, we did not obtain a strong reduction of the protein levels in our cell lines. Surprisingly, the reduction obtained was sufficient to have an appreciable increase of ribonucleotides as detected by treatment of genomic with RNase H2. These data confirmed our hypothesis that we might be underestimating genomic ribonucleotides in the previous analysis. Given these results, we are planning to perform future experiments in the RNase H2 knockdown cellular background taking advantage of the siRNA technique. Indeed, using such strategy, we obtained a higher reduction of the subunit at protein level. Hence, unexpectedly siRNAs demonstrated to be more powerful in reducing RNase H2A subunit with the advantage of a safer and easier technique. Taken together our data, we obtained and characterised a cellular system that will be suitable to trace DNA polη.

Previous attempts to track the activity of the polymerase relied on the chromatin immune precipitation technique (ChIP) (Bergoglio V et al. 2013). Since DNA polη has a high mobility (Sabbioneda S et al. 2008), its immunoprecipitation required the use of a catalytic dead mutant that immobilized the polymerase at the chromatin level. In spite of being advantageous for immunoprecipitating the protein, cells carrying the catalytic dead form of the polymerase are less viable and present a chronic activation of the S phase checkpoint. Thus, using a viable and active form of the polymerase would make results more comparable to physiological conditions, preventing effects on cell's viability. Moreover, ChIP relies on the physical isolation of the protein from DNA, this leads to an underestimation of the loci that are replicated by the polymerase. In fact, only DNA regions where the polymerase is bound at the moment of the crosslinking will be finally isolated. The use of a tracking strategy permits isolation of all the sites replicated by the polymerase thus providing a higher resolution. The possibility to track the specialised polymerase for the first time in human cells would shed light on the role of this specialised polymerase both with its role in damage bypass but also outside its TLS activity.

PCNA K164R mutants have a different cell cycle progression after replication fork block or slow down

In the context of TLS, PCNA has a major role acting as a molecular platform for highly dynamic protein-protein interactions. K164 is a key residue which modifications regulate the DNA damage tolerance pathway. Given the importance of this residue many groups have made efforts to elucidate its role. However, in diverse model organisms, different results were obtained. For example, these contrasting evidences did not clarify the interaction between ubiquitylated PCNA and DNA polη during genome replication. K164R mutation in chicken cells led to the hypothesis of two temporally distinct TLS pathways (Edmunds C et al. 2008). For instance, Rev1 may be employed to assist the fork in damage bypass during replication and would not require PCNA ubiquitylation. On the contrary, PCNA ubiquitylation would be necessary to recruit DNA polη in a post replicative process.

Opposing to these findings, in mice cells the introduction of K164R homozygous mutations on PCNA, resulted in a reduction of DNA polη recruitment as detected by the lower number of replicative foci (Langerak P et al. 2009). Finally, in human cell lines, the reduction of Ubi-PCNA by chemical depletion of total ubiquitin with epoxomycin, did not alter DNA polη recruitment after UV damage (Sabbioneda S et al. 2008). These findings have clearly stressed the need of a human cell line to study the role of K164. In recent years, the power of the CRISPR/Cas9 technique has been employed to introduce precise mutations at desired genomic loci (Doudna J et al. 2014). Although being recognised as a very efficient method to generate knockout models, the use of such system for the introduction of single allelic changes at target genes remains at considerable low efficiency (Mali P et al. 2013). Lately, PCNA K164R mammary gland tumor cell lines were generated by CRISPR/Cas9 (Buoninfante O et al. 2018). In this case however, a different strategy was employed. In fact, at first target cells were transduced with viral vectors carrying either PCNA wt or K164R cDNA. This led to random integration of the construct. After integration of the transgene, CRISPR/Cas9 was used to knock-out the endogenous PCNA gene. Although being useful in their study, this system does not target precisely the endogenous gene. Moreover random integration of the construct cannot be compared to the precise targeting of the endogenous locus. In contrast we decided to target directly the endogenous locus of PCNA and thus to performed a knock-in strategy. For this purpose, the concomitant use of either ssDNA or dsDNA oligonucleotides carrying homology with the target sequence are useful to generate single nucleotide variations (Yang H et al. 2013). At first we took advantage of this strategy to target K164 in the genomic locus for PCNA. Screening for Ubi-PCNA by western blot allowed us to immediately verify the final outcome of our editing. Efficiency of homologous recombination is generally attested to be below 10% of repair events. This percentage is further reduced considering the final aim of obtaining a precise targeting event with the correct insertion of the desired mutation on both alleles. Being PCNA a fundamental gene we were confident that undesired recombination events would lead to cell death thus reducing the number of non precisely targeted cells. Screening by western blot resulted in only one clone with reduced Ubi-PCNA, so we decided to sequence the K164 locus in these cells.

Sequencing revealed the presence of a wt allele, in contrast with what expected from western blot analysis. We did not verify in more details the causes of such phenotype. We speculate that unscheduled recombination events may have occurred but remained undetected in the genomic region that we amplified. In any case, in the end we had not obtained a targeted K164R clone and thus we decided to improve our strategy. To begin, we wanted to be able to screen a lower number of clones while increasing the possibility to detect a successful targeting event. This is possible by introducing selectable markers with the desired mutation. Introduction of a selective marker poses two main problems. First, introduction of a marker should not affect the coding sequence. Second the presence of a marker requires additional sequences that can be carried only on a dsDNA sequence such as plasmid DNA. With this purpose, changed the substrates for HR mediated repair taking advantage of a plasmid system carrying the puromycin resistance marker. Given the necessity to insert a selectable marker, we decided to change our gRNAs to target the intronic region of the PCNA locus between exon 5 and exon 6, instead of targeting directly the K164 locus on exon 5. In addition, to have a more precise and faster screening strategy we moved to detection of puromycin insertion by PCR. By sequencing puromycin resistant clones from MRC5 cells, we found a wt and a K164R heterozygous clone. The targeted wt clone was unexpected since those cells were transfected with the K164R mutant donor plasmid. We hypothesise that a partial recombination may have brought the puromycin resistance in the PCNA locus by using the homology arms but stopped before inserting the K164 coding sequence at 990 bp from the Cas9 cut and at 120 bp from the begin of the left homology region in the pDonor-D09 plasmid. Further genomic analysis would clarify the events occurred in the locus. In any case, we managed to isolate another correctly targeted wt clone that was a suitable control cell line for the subsequent experiments. Unfortunately, we did not obtain any homozygous clone for K164R. We started speculating that the cell line may carry multiple copies of the genomic locus, hence explaining the low efficiency of homozygous targeting in our genome editing strategy. Results of karyotypic analysis of the PCNA locus revealed that our MRC5 cell lines carried on average three copies of the locus. This partially explained the unsuccessful homozygous targeting, since simultaneous targeting of more than one locus is a low probability event.

Copy number variation is not infrequent in transformed cell lines, and thus targeting of multiple loci is one of the obstacles in such a cellular background. We decided to face this issue in future experiments by using another donor plasmid that carries a different selection marker. This will allow to target once more the K164R puromycin resistant clones and stimulate homologous recombination at a secondary locus by the use of a different selective agent. Even though we had not obtained an homozygous clone, we decided to test the effects of the partial loss of PCNA ubiquitylation in the heterozygous cells. Notably, ubiquitylation of PCNA was reduced in the K164R cells in unperturbed conditions. This reduction did not have a phenotypic effects in cells not perturbed by exogenous replication stress. This effect rules out the possibility of an hypomorphic mutation of the sliding clamp that would have affected its normal replication activity. This hypothesis was also demonstrated by analysis of cell cycle progression where all cells showed a similar distribution in untreated conditions. On the other hand, either treatment with hydroxyurea or UV irradiation revealed a different behaviour of K164R mutated cells, while parental cells and WT targeted cells had a comparable behaviour. In more details, in the presence of hydroxyurea, the lower levels of Ubi-PCNA caused an accumulation of mutated cells in early steps of S phase, while after UV irradiation these cells had a faster progression through S phase. Although both treatments should impair the fork progression it must be taken into account that they act with different mechanisms. Inhibiting the ribonucleotide reductase, HU depletes cells of nucleotide precursors, causing a slower progression of the replication fork. UV irradiation instead acts more severely blocking physically the forks at the level of lesions on template DNA. For the latter case, rescue of fork progression requires the activation of the TLS pathway or more complex fork remodelling processes. Given the two distinct phenotypes between wt and K164R cells, a decrease in Ubi-PCNA subunits seems to impair DNA replication when the forks are slowed down. A slower progression through cell cycle in K164R cells was expected accordingly to models in which Ubi-PCNA controls responses to replication stress. In contrast, our evidence show that a reduction of Ubi-PCNA would speed S phase progression UV irradiation. This is in contrast with the established polymerase switch model in which ubiquitylation of PCNA is thought to be a necessary step for DNA damage bypass by specialised polymerases.

A faster progression through S phase may be explained by the presence of an alternative bypass of the damage. We may speculate that other more processive polymerases such as polζ, may be recruited at the fork explaining the faster cell cycle progression. It would be interesting to test the presence of alternative TLS polymerases and their requirement for Ubi-PCNA as a binding factor. Another possible mechanism acting could be re-priming by the newly characterized PrimPol (Helleday T et al. 2013). Fork repriming leaves a gap at the level of the lesion and priming by PrimPol allows a fast resumption of fork replication. The gap is left unresolved until post replicative mechanisms such as template switch or TLS repair the gap. Such a mechanism would provide damaged cells a faster escape from DNA lesions in the absence of other bypass pathways. Thus we may depict a mechanism in response to UV damage, where in the absence of K164 ubiquitylation TLS polymerases may be able to assist the fork via different interactions. Perhaps, reciprocal interactions in the pathway have been further complicated by findings that both Rad18 and DNA polη may cooperate to recruit and activate each other to stimulate TLS activity (Watanabe K et al. 2004; Durando M et al. 2013). A faster damage bypass may also involve recombinogenic mechanisms. This would not explain why such mechanisms are not employed in the absence of DNA polη, such as in XP30RO cells, to improve cells' sensitivity to UV irradiation. However, it should be reminded that K164 is also SUMOylated and this modification is thought to prevent unscheduled recombination events during S phase (Pfander B et al. 2005). Although this modification is less prominent, is still present in human cells (Moldovan G et al. 2012). This mechanism should however be distinct from template switch since this pathway also relies on polyubiquitylation of K164. Thus, loss of K164 may allow recombination to occur, resulting in damage bypass and a faster progression of the S phase. Future analysis of UV damage bypass may be interesting and possibly reveal alternative players in DNA damage bypass. The establishment of a homozygous cell line will help at defining such mechanisms.

Presence of DNA polη correlates with different G4 stabilisation effects at molecular and cellular level.

Interest on g-quadruplex DNA is rising mainly due to the correlation of such DNA structures with expression of oncogenes such as c-myc and k-ras (Balasubramanian S et al. 2011). Moreover, small G4 ligands are being tested to treat sensitive cancer cells causing DNA damage and hopefully their death (Zimmer J et al. 2016; Moruno-Manchon J et al. 2017). Given this interest it is of fundamental importance to characterise their dynamics in terms of stabilisation and resolution. While a considerable amount of data has been produced on the role of diverse families of helicases in their resolution, only recently there has been a focus on the interactions between replication and g-quadruplex DNA to prevent genome instability (Rodriguez R et al. 2012). In particular, the role of TLS in the dynamics of G4 DNA has come to attention. Our interest focused on activation of the TLS pathway in the presence of stabilised G4 structures. The availability of cells deficient for DNA polη and the complemented DNA polη wt cells gave us the possibility to test TLS activity in g-quadruplex resolution. Pyridostatin (PDS) has been used to induce G4 stabilisation both *in vitro* and *in vivo* and thus we tested this compound on our cell lines. In all cases, we tested increasing micro molar doses of PDS and we obtained differences in our cell lines only at the maximum dose of 10 μM. At first we tested cell's viability by treating the cells with PDS. At the maximum dose, we obtained a significant reduction of cell's viability for XP30RO cells that are deficient in polη compared to XP30RO cells complemented with wild type polη. Hence, we hypothesised that the absence of DNA polη may affect viability in the presence of stabilized G4, thus linking the activity of the polymerase with g-quadruplex DNA resolution. Further analysis on the type of cell death in XP30RO cells after PDS treatment will be of future interest. This remarkable difference in cell's viability convinced us about the possible involvement of TLS in G4 dynamics. In particular, we hypothesise that S phase checkpoint activation may link g-quadruplex stabilisation to the TLS pathway. Our initial hypothesis predicted that if polη was important for G4 bypass, its absence would result in a stronger activation of the DDR, due to the block of the replication forks. Interestingly, we had an opposite result with complemented cells showing a stronger Chk1 activation.

This activation of the S phase kinase correlated with an increased activation of the TLS pathway as detected by higher amounts of Ubi-PCNA in polη complemented cells. Therefore, it could be possible that this efficient activation of the replication stress pathway may be fundamental for g-quadruplex DNA resolution preventing genome instability. Accordingly, this response to G4 stabilisation would explain the advantage of DNA polη complemented cells in terms of cell viability as previously detected. Interestingly, we also noticed a dose dependent increase of the TLS polymerase. Further analysis will be useful to discern whether this increment can be attributed to a higher level of transcript for the polymerase or to an increased stability of the protein. From the evidence we collected, polη seems to facilitate checkpoint activation and thus sensitise cells to G4 stabilisation by PDS. How polη may stimulate checkpoint activation will be of interest for future experiments. G-quadruplex stabilisation also led to a higher ubiquitylation of PCNA in the presence of polη. Ubiquitylation of PCNA is normally controlled by Rad18 that is in turn stimulated by the presence of ssDNA bound by phosphorylated RPA. However, no differences in RPA2 protein was detected between the two cell lines. We failed to detect the presence of Rad18 on chromatin and thus it would interesting to analyse its dynamics to correlate its activation with PCNA ubiquitylation. More interestingly polη itself was shown to stimulate PCNA ubiquitylation (Durando M et al. 2013; Masuda Y et al. 2015). The presence of polη on chromatin stimulates PCNA ubiquitylation in a feedback loop. This mechanism may explain what we are observing after PDS treatment. In fact, the increase of DNA polη that we observe at chromatin level may stimulate PCNA ubiquitylation thus linking the two phenomenons. Intriguingly, the increase of DNA polη at chromatin level cannot be merely explained by a general increase of the total protein quantity. Thus, we speculate that after PDS treatment there must be a system that recruits the specialised polymerase on the chromatin. In the end, we can hypothesise from our results that the presence of DNA polη guarantees the activation of an efficient response to g-quadruplex stabilisation. The presence of an active checkpoint and an efficient activation of the TLS pathway may facilitate G4 resolution. This would ultimately result in a higher viability of cells in the presence of stable g-quadruplex DNA.

6. Conclusions and perspectives

A considerable amount of literature has identified players that run to the rescue of the fork and TLS has a major role in damage tolerance and in bypassing various types of DNA damage. In addition, DNA polη seems to be required also to replicate genomic regions that represent a challenge for the replicative fork during normal replication in unchallenged conditions. Common fragile sites require the activity of DNA polη in circumstances where replicative polymerases are slowed down such as the treatment with aphidicoline (Bergoglio V et al. 2013). Under-replication of these sites may lead to their breakage and result in gross chromosomal rearrangements, a mechanism that has been identified in cancers (Glover T et al. 2017). The importance of this specialised polymerase in replicating DNA either following DNA damage or in unchallenged conditions, underlines the necessity to analyse its activity. To study the role of DNA polη it is necessary to track its activity along the genome, thus defining its contribution in the replication of DNA. We established a mutated cell line that permits identification of DNA polη activity following the incorporation of ribonucleotides. At present, this is the most high resolution technique to follow polymerases along the genome (Koh K et al. 2015; Clausen A et al. 2015; Daigaku Y et al. 2015). This would be the first attempt to perform a polymerase tracking experiment in human cells and will represent a major achievement to define TLS role in genome replication. We have characterised the cell line, although we encountered difficulties in isolating specific genomic loci that were predicted to be DNA polη substrates. However, we should take into account that such loci, such as CFSs were identified only in conditions that slow the replication fork. It could be possible to hypothesise that only when the replication slows down, then specialised polymerases may gain access to the fork and bypass the obstacle. In light of this, in the future we are planning to perform our experiments in the presence of aphidicoline, a well known inhibitor that targets replicative polymerases. The final aim would be to construct libraries from mutated cell lines that will be used for next generation sequencing (NGS) to identify genomic embedded ribonucleotides as a DNA polη specific signature.

Once the methodology has been established, results may be translated to the context of cancer therapeutics by identifying DNA polη activity in cancer cells and after treatment with chemo therapeutics. In fact, overexpression of the polymerase has been correlated with development of cancer chemoresistance (Srivastava A et al. 2015; Zhou W et al. 2013). After treatment with cis-platin derivates, bulky adducts are generated in cells. These should impede DNA replication in cancer cells leading to death. Tumor cells take advantage of the capability of DNA polη to bypass such structures and thus overexpress it to develop chemoresistance. Apart from acquired chemoresistance, DNA polymerase η also contributes substantially to the mutagenic load of tumour cells (Supek F and Lehner B 2017). Identification of hotspots of DNA polη substrate sequences may unravel new players in chemoresistance or in tumor progression and thus give the possibility to improve present treatments.

DNA polη activity is also controlled by PCNA and in particular its post translational modifications. Ubiquitylation of lysine 164 (K164) has a pivotal role in the TLS branch of the DNA damage tolerance pathway, facilitating recruitment of specialised polymerases such as DNA polη (Lehmann A et al. 2007). Branching of K63-linked ubiquitin chains from the same residue, controls the error free branch of the pathway that takes advantage of homologous recombination to bypass DNA lesions. Finally, SUMOylation also occurs on K164 and finely regulating the template switch. This complex picture of events has led to the identification of the K164 residue as fundamental in contributing at different extents to genome stability. The role of the residue in humans is yet unclear and confounded by conflicting studies in different model organisms. Our effort is the first to clearly define the role of K164 in humans by generation of a CRISPR/Cas9 mutated human cell line. While our first attempt of genome editing did not result in a successfully targeted cell line, improvement of our strategy that facilitated screening, resulted in the generation of a heterozygous clone. Quite surprisingly, we have observed a clear difference in cell cycle progression between the wild type and K164R cells. Thus, this is already a promising tool to study dynamics of genomic replication in the absence of PCNA ubiquitylation. Even more surprisingly, we observed a different progression according to either replication stalling, as in the case of UV irradiation, or replication slow

down as in the case of HU treatment. These phenotypes will be analysed more in depth in future experiments aiming to identify different players in the two conditions. To do this, the generation of a homozygous clone will be of fundamental importance to finally elucidate the role of K164 in human cells. With this aim we have already targeted the heterozygous clone with a novel construct as homology donor, carrying a different selective marker. The use of a different marker would push towards selection of cells that carry a different allelic hit, thus increasing the probability of finding a homozygous clone. Once obtained different levels of experiments will be carried on in the K164R cell line. At first the identification of TLS polymerases dynamics by multiple techniques such as immunofluorescence or live cell imaging. This would finally define the role of PCNA ubiquitylation in the recruitment of the specialised polymerases. Biochemical techniques such immunoprecipitation would also strengthen findings regarding the interaction of the modified clamp and TLS polymerases. Many other players and interactors may be analysed in the same mutated background. Another important level of analysis will be devoted to the replication fork dynamics by studying replication fork progression by DNA fiber analysis. This would allow to study fork progression at high resolution in the absence of PCNA modifications combined with different treatments.

Another challenge for the replicative fork is the presence of structured non B-DNA as in the case of g-quadruplexes. Also in this case replication by specialised polymerases is one of the mechanisms identified that prevent genome instability (Eddy S et al. 2014; Barnes R et al. 2017). We have tested the possible role of DNA polη by stabilising *in vivo* G4 structures with pyridostatin. We obtained different responses to the treatment influenced by the presence of DNA polη. At first activation of the checkpoint was effective only in the presence of the specialised polymerase, with a milder activation in XP30RO cells. How the presence of the polymerase may be linked to checkpoint activation in this scenario, is still to be defined. Moreover, it would be of interest to assess whether the activation of the checkpoint may be advantageous for g-quadruplex resolution or whether this activation may become chronic and finally detrimental to the cells. We plan to tackle these questions by performing longer timepoints experiments and analysing other players in the DNA damage response pathway.

A more defined picture of the dynamics occurring after g-quadruplex stabilisation has been allowed by fractionation of cells. Separation of the soluble proteins, that comprise cytoplasmic and nucleoplasmic portion, from chromatin-bound proteins allowed us to monitor phenomenons occurring at chromatin level. One of the most interesting findings was the accumulation of DNA polη on chromatin after G4 stabilisation. It will be of interest to understand whether this is an active accumulation mediated by other factors or by post translational modifications of the polymerase. Interestingly also Ubi-PCNA, that works as a landing platform for the specialised polymerase, increases upon G4 stabilisation. This clearly involves TLS in the resolution of non B-DNA structures. Whether the activity of DNA polη is actually required in resolving the structures will be addressed in future experiments by testing the capability of the polymerase to resolve structured DNA *in vivo*.

Overall we have established important new cellular tools to study dynamics of TLS players *in vivo*. Related investigations have usually taken advantage of simpler eukaryotic systems, such as yeast, and our will be the first attempt to obtain evidences from human cells. Our strategies are very promising to study genome replication and instability in a wider context. For instance, TLS players have been correlated with different steps of cancer evolution. As a consequence, such studies may be useful to analyse cancer development, progression and finally chemoresistance.

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The Regulation of DNA Damage Tolerance by Ubiquitin and Ubiquitin-Like Modifiers

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DNA replication is an extremely complex process that needs to be executed in a highly accurate manner in order to propagate the genome. This task requires the coordination of a number of enzymatic activities and it is fragile and prone to arrest after DNA damage. DNA damage tolerance provides a last line of defense that allows completion of DNA replication in the presence of an unrepaired template. One of such mechanisms is called post-replication repair (PRR) and it is used by the cells to bypass highly distorted templates caused by damaged bases. PRR is extremely important for the cellular life and performs the bypass of the damage both in an error-free and in an error-prone manner. In light of these two possible outcomes, PRR needs to be tightly controlled in order to prevent the accumulation of mutations leading ultimately to genome instability. Post-translational modifications of PRR proteins provide the framework for this regulation with ubiquitylation and SUMOylation playing a pivotal role in choosing which pathway to activate, thus controlling the different outcomes of damage bypass. The proliferating cell nuclear antigen (PCNA), the DNA clamp for replicative polymerases, plays a central role in the regulation of damage tolerance and its modification by ubiquitin, and SUMO controls both the error-free and error-prone branches of PRR. Furthermore, a significant number of polymerases are involved in the bypass of DNA damage possess domains that can bind post-translational modifications and they are themselves target for ubiquitylation. In this review, we will focus on how ubiquitin and ubiquitin-like modifications can regulate the DNA damage tolerance systems and how they control the recruitment of different proteins to the replication fork.

Keywords: DNA damage tolerance, translesion synthesis, ubiquitylation, SUMOylation, ISGylation, PCNA

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INTRODUCTION

DNA damage poses a constant threat to the genetic material. It can arise from products either of the cellular metabolism or by exposure to exogenous sources (physical or chemical). Regardless of its origin, DNA damage is addressed swiftly by the multitude of repair mechanisms that protect the integrity of the genome (Hoeijmakers, 2001). The DNA damage response provides an overall control network for the repair mechanisms and it allows the coordination of the complex biochemical reactions that lead to the elimination of DNA damage (Ciccia and Elledge, 2011). Unfortunately, in certain conditions, the cells are exposed to an amount of damage that the repair systems cannot handle completely. This could be caused either by an extreme insult, able to saturate one or multiple repair systems, or by damage that is repaired slowly. The result of both conditions

is the permanence of lesions in the template DNA. Nevertheless, the damaged template then must be replicated during S phase. Replicative DNA polymerases are extremely efficient and processive but are unable to cope with a distorted template caused by DNA damage. To solve this impasse, cells possess damage tolerance pathways that are tasked with the bypass of the damage, which eventually will be repaired at a later stage (Sale et al., 2012). Failure to bypass the damage is believed to be one of the main causes of replication fork blocks, cell cycle arrest and eventually cell death.

During S phase, the damaged template can be replicated by either a special class of DNA polymerases, in a process called DNA translesion synthesis (TLS), or by a damage avoidance pathway that uses the sister chromatid as a template, in a mechanism called template switch. TLS utilizes specialized low-fidelity DNA polymerases (η, ι, κ, ζ, and Rev1), mostly belonging to the Y-family, to bypass the damaged template, while template switch is proposed to use a recombination-like mechanism. A crucial difference between the two pathways is that the former is potentially error-prone, while the latter is thought to be error-free (Branzei and Foiani, 2007; Sale et al., 2012). Given this background, the choice of pathway is extremely important in order to bypass the damage with the lowest possible chance of introducing mutations. Post-translational modifications play a central role in controlling damage tolerance and, in the last few years, emerging evidence has shown that ubiquitylation and SUMOylation sit at a crucial crossroad that influences its outcomes (Huang and D'Andrea, 2006; Bergink and Jentsch, 2009; Bekker-Jensen and Mailand, 2011; Mailand et al., 2013; Pinder et al., 2013).

Ubiquitylation is a process that involves the addition of ubiquitin to a target protein. This process is conserved in all eukaryotes and it controls a variety of cellular functions, ranging from protein degradation to cell cycle progression. Ubiquitylation is reversible and utilizes three classes of enzymes to target ubiquitin to a desired protein (Hershko and Ciechanover, 1998). In the initial step, an ubiquitin activating enzyme (E1) forms a thioester bond with ubiquitin. Afterward, ubiquitin conjugating enzymes (E2) transfer the ubiquitin from the E1 to the target protein, either directly or with the help of an E3 ubiquitin ligase that confers specificity to its E2 partner. Ubiquitin is normally attached via its C-terminus to lysines on the target proteins. Once ubiquitin has been linked to its target, it can be further modified by the addition of additional ubiquitin moieties on one of the lysines that can be found on ubiquitin itself: K6, K11, K27, K29, K33, K48, and K63 (Ikeda and Dikic, 2008; Kulathu and Komander, 2012). The linkage to the different lysines confers diverse structural properties to the polyubiquitin chains, creating a different binding platform for a variety of processes. For example, K48-linked chains have a compact structure (closed chain) and they direct proteins to degradation by the proteasome (Varadan et al., 2002). On the other hand, K63 chains are linear and flexible and they seem to have a more prominent role in mediating protein–protein interactions (Varadan et al., 2004). SUMOylation shares a similar activating pathway with ubiquitin but uses SUMO (Small Ubiquitin MOdifier) as a substrate (Muller et al., 2001; Hay, 2005). In most organisms, a single SUMO is present but human cells express 4 different variants (SUMO1–4, Hay, 2005). Remarkably, while in the human genome we can find between 10 to 35 ubiquitin E2s and hundreds of putativeE3 ubiquitin ligases have been predicted, this number is greatly reduced in the case of SUMO, up to the point where UBC9 encodes the only known SUMO E2 (Hay, 2005). The aim of this review is to highlight the crucial role of both ubiquitylation and SUMOylation in the regulation of the DNA damage tolerance pathways.

UBIQUITYLATION OF PCNA

A number of E2 and E3 enzymes has been known for a long time to be involved in the replication of damaged DNA, among these the proteins encoded by Rad6, Rad18, Ubc13, Mms2, and Rad5 in the yeast Saccharomyces cerevisiae (Jentsch et al., 1987; Bailly et al., 1994, 1997; Xiao et al., 2000). All of these proteins have been shown to ubiquitylate, in different ways, the PCNA, assigning to PCNA a central role in the regulation of damage bypass during replication (Hoege et al., 2002; Mailand et al., 2013).

Proliferating cell nuclear antigen is a homotrimeric protein that acts as the processivity factor for DNA polymerases, in a role similar to *E. coli* β-clamp (Kuriyan and O'Donnell, 1993; Krishna et al., 1994a,b). Each subunit consists of two different domains connected by an interdomain connecting loop (IDCL). The IDCL makes contacts and tethers the DNA polymerases to the DNA. The binding to the IDCL of PCNA is mediated by a PCNA interacting peptide (PIP) motif present in the interacting partner. PCNA plays also crucial roles as a loading platform for a variety of proteins involved in different repair systems (Freudenthal et al., 2010; Dieckman et al., 2012). In yeast, PCNA was originally discovered to be ubiquitylated after the treatment with methyl methanesulfonate (MMS) by the complex formed by the ubiquitin ligase Rad18 and the ubiquitin conjugating enzyme Rad6 (Hoege et al., 2002) (**Figure 1**). Ubiquitylation was shown to be attached to lysine 164 that is located on the back side of the trimer, on the opposite side where the replicating polymerases make contact (front side, Freudenthal et al., 2010).

Once monoubiquitylated, PCNA (Ubi-PCNA) can be further modified resulting in the formation of K63-linked polyubiquitin chains (Hoege et al., 2002). The two modifications were proposed to channel the bypass toward different branches of damage tolerance, with monoubiquitylation leading to TLS and polyubiquitylation of PCNA steering the system toward template switch (Branzei, 2011; Giannattasio et al., 2014).

Orthologs of all the proteins involved in the process originally described in *S. cerevisiae* have been identified in both invertebrates and vertebrates and, overall, the system appears to be conserved across different organisms, although subtle differences are present. For example, in *Xenopus laevis*, PCNA is monoubiquitylated during an unperturbed S phase and this modification is required for the efficient progression of the replication fork in egg extracts, while polyubiquitylation of the trimer appears specifically only after DNA damage (Leach and Michael, 2005).

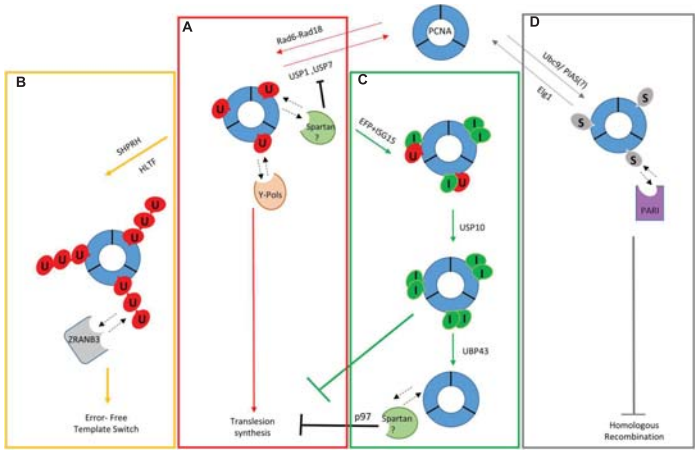

FIGURE 1 | Schematic model of ubiquitin and ubiquitin-like modifications in the DNA damage tolerance pathway. (A) Monoubiquitylation of PCNA leading to TLS. **(B)** Polyubiquitylation of PCNA leading to template switch. **(C)** ISGylation of PCNA and recovery from TLS. **(D)** SUMOylation of PCNA during unperturbed S phase and inhibition of Homologous Recombination. Dotted lines indicate interactions between regulators of the DDT and modified/unmodified PCNA.

In vertebrates, the main modification of PCNA is monoubiquitylation. It is observed after treatments that block the progression of the replication fork (Kannouche and Lehmann, 2004; Kannouche et al., 2004; Watanabe et al., 2004). In such conditions, it is possible to detect an accumulation of single-stranded DNA (ssDNA), likely caused by the uncoupling of the activities of the blocked replication fork and the DNA helicase. At this point, RPA readily binds the free ssDNA creating the substrate for the recruitment of Rad18 and Rad6 that ubiquitylate PCNA on lysine 164 (Davies et al., 2008). Rad18 and replication protein A (RPA) interact directly and the recruitment of Rad6/Rad18 to RPA-coated ssDNA has been observed *in vitro* (Huttner and Ulrich, 2008). Monoubiquitylated PCNA has increased affinity for TLS polymerases, whose interactions are mediated by their PIP-boxes (PCNA-interacting peptide) and ubiquitin-binding motifs (Kannouche et al., 2004; Bienko et al., 2005; Dikic et al., 2009). Upon fork stalling, replicative polymerases slow down and dissociate from the replisome followed by the recruitment of TLS polymerases (polymerase switching; **Figure 1A**). In the last few years, there has been a progressive discovery of new factors that help Rad18 in promoting the efficient ubiquitylation of PCNA. One of these factors is a TLS polymerase itself. It is interesting to point out that originally the recruitment of TLS polymerases was proposed to be an event that followed the monoubiquitylation of PCNA. New experimental data seem to suggest that TLS polymerases can influence themselves the state of PCNA, and an increase in PCNA ubiquitylation has been observed, in some cell types, after polη overexpression (Durando et al., 2013; Masuda et al., 2015). In these conditions, polη is believed to enhance and stabilize Rad18 in the proximity of PCNA. Rad18 and polη have been purified as a stable complex and their interaction has been proposed to be dependent on the phosphorylation of Rad18. Rad18 is phosphorylated, at a basal level even in unperturbed conditions but this modification is enhanced after DNA damage by DDK (Dbf4/Drf1-dependent Cdc7 kinase) and JNK (c-Jun N-terminal kinase; Day et al., 2010; Barkley et al., 2012). This hyper-phosphorylation is believed to increase the affinity of Rad18 for polη and promote their mutual recruitment to the chromatin, leading to the ubiquitylation of PCNA. However, this model of action is still controversial since it would make the accumulation of Ubi-PCNA an event dependent on ATR and Chk1, in contrast with previous established experimental evidence that demonstrated that ubiquitylation of PCNA is independent from both ATM and ATR kinases and their respective DNA damage checkpoints (Chang et al., 2006; Davies et al., 2008; Gohler et al., 2008; Niimi et al., 2008; Yang et al., 2008). A cohort of new factors that have been found to interact with Rad18 to promote efficient PCNA ubiquitylation include NBS1 (Yanagihara et al., 2011), Claspin and Chk1 (Yang et al., 2008), RPA (Davies et al., 2008), Spartan (see later in this review, Centore et al., 2012; Davis et al., 2012; Juhasz et al., 2012; Mosbech et al., 2012) and SIVA1 (Han et al., 2014).

In human cells, Rad18 is the principal E3 ligase that monoubiquitylates PCNA, but avian DT40 cells lacking Rad18 ($Rad18^{-/-}$) still show detectable levels of Ubi-PCNA, indicating the existence of another E3 ligase (Arakawa et al., 2006; Simpson et al., 2006). In fact, other minor pathways leading to the

ubiquitylation of PCNA have been proposed also in *S. cerevisiae* and in human cells under specific conditions. In human cells, RNF8 and $CRL4^{Cdt2}$ were identified as ubiquitin E3 ligases of PCNA, although their contribution is rather minor when compared to Rad18 (Zhang et al., 2008; Terai et al., 2010).

Rad18 is itself ubiquitylated and its modification is believed to control its availability and cellular localization. Rad18 has been reported to form a homodimer where the ubiquitin moiety on each Rad18 interacts with the UBZ (ubiquitin-binding zinc finger domain) of the other subunit (Miyase et al., 2005; Notenboom et al., 2007). Once Rad18 is de-ubiquitylated, it becomes active. The Rad18 dimer, which is considered inactive, is believed to localize mainly in the cytoplasm, while the active Rad18 monomer is distributed in the nucleoplasm. Recently, Rev1 has been shown to bind ubiquitylated Rad18 causing the release of non-modified Rad18 from the dimer, that is then free to ubiquitylate PCNA on the chromatin (Wang et al., 2016). This is another example of the extensive crosstalk between TLS polymerases, Rad18 and PCNA, further strengthening the idea that the regulation of DNA damage tolerance is far from a simple linear pathway.

Once ubiquitylated, PCNA can be further modified via K63-linked polyubiquitylation. In yeast, the complex formed by Ubc13-Mms2 (E2) and Rad5 (E3) is responsible for this modification (Hoege et al., 2002; Parker and Ulrich, 2009). In human cells, polyUbi-PCNA is hardly observed in comparison to yeast (Chiu et al., 2006) although all the proteins involved are believed to be conserved. Two Rad5 orthologs have been identified: helicase-like transcription factor (HLTF) and SNF2 histone linker PHD RING helicase (SHPRH; **Figure 1B**). HLTF is characterized by ATPase and HIRAN domains that promote fork regression *in vitro*, a crucial step in the stabilization of the replication fork in the presence of DNA damage (MacKay et al., 2009; Blastyak et al., 2010; Achar et al., 2015). Both HLTF and SHRPH can catalyze the addition of ubiquitin chains to Ubi-PCNA *in vitro* and their silencing, mediated by siRNA, results in a decrease in polyUbi-PCNA in living cells (Motegi et al., 2006, 2008; Unk et al., 2008, 2010). Recent evidence suggests that the loss of HLTF and SHPRH increases mutagenesis induced by UV and MMS treatment, respectively (Lin et al., 2011). HLTF has been shown to have also a role in the mono-ubiquitylation of PCNA and in the recruitment of polη (Lin et al., 2011). Surprisingly, mouse embyonic fibroblast (MEF) cells lacking both SHPRH and HLTF are still competent for PCNA polyubiquitylation and the double mutant is not hypersensitive to DNA-damaging agents (Krijger et al., 2011a). This seems to suggest the existence of yet another E3 ligase involved in PCNA ubiquitylation, at least in mouse. In light of all of this evidence, it is clear that further investigation will be required in order to understand the role of the Rad5 orthologs in higher eukaryotes.

GOING BACK: THE DE-UBIQUITYLATING ENZYMES

Ubi-PCNA plays a central role in the bypass of damaged DNA by facilitating the access of TLS polymerases to the replication fork. However, unscheduled recruitment of low-fidelity TLS polymerases would result in replication errors and mutagenesis on undamaged DNA, thus the level of Ubi-PCNA must be strictly controlled. Ubi-PCNA in human cells is negatively regulated by the ubiquitin-specific protease 1 (USP1; Huang et al., 2006) (**Figure 1A**). USP1 interacts with the activating protein partner UAF1 (USP1-associated factor 1) and de-ubiquitylate Ubi-PCNA in the absence of DNA damage (Cohn et al., 2007). USP1 is subjected to an auto-cleavage reaction, which regulates its cellular concentration (Cohn et al., 2007). Furthermore, high doses of UV-C light result in the down-regulation of the USP1 transcript, thus ensuring its down-regulation when the ubiquitylation of PCNA needs to be promoted (Huang et al., 2006). Indeed, Ubi-PCNA levels correlate nicely with the reduced expression levels of USP1 after UV treatment (Niimi et al., 2008). Differently from UV, USP1 is still present after hydroxyurea or MMS treatment, two genotoxic agents that induce a strong ubiquitylation of PCNA (Niimi et al., 2008). This observation suggests the possible presence of other negative regulators.

USP1 has been shown to protect the cells from genomic instability, as monitored by the formation of micronuclei, caused by the erroneous recruitment of polκ and the following decrease in fork progression (Jones et al., 2012). USP1 was the first and most prominent DUB involved in the negative regulation of PCNA ubiquitylation; however, recent data seem to suggest the involvement of more DUBs in the control of PCNA. Some of these DUBs either act directly on PCNA or can regulate other proteins that control its ubiquitylation. Among these, USP7, also called HAUSP, is the DUB that controls the stability of p53 by counteracting the activity of Mdm2, the E3 ligase responsible for its degradation (Li et al., 2002; Cummins and Vogelstein, 2004; Sheng et al., 2006). Recently USP7 has been shown to regulate indirectly the ubiquitylation of PCNA via the stabilization of either Rad18 or polη (Qian et al., 2015; Zlatanou et al., 2016). Other work has shown that USP7 can de-ubiquitylate Ubi-PCNA *in vitro* and it suppresses UV- and oxidative-stress-induced PCNA monoubiquitylation *in vivo* (Kashiwaba et al., 2015). PCNA ubiquitylation after DNA damage is normally very stable and can be detected days after the original genotoxic treatment (Niimi et al., 2008). Another DUB involved in the de-ubiquitylation of PCNA is USP10. USP10 can interact directly with PCNA via its PIP box and its silencing results in increased Ubi-PCNA 24 h after UV irradiation (Park et al., 2014). The activity of USP10 is remarkably deferred compared with USP1 as no difference could be appreciated in the levels Ubi-PCNA at 0 and 12 h after UV irradiation (Park et al., 2014), whereas silencing of USP1 results in the accumulation of Ubi-PCNA even in the absence of DNA damage (Huang et al., 2006). This seems to suggest that USP10 may control the de-ubiquitylation of Ubi-PCNA during the recovery from UV irradiation (see ISGylation, later on). An USP1 ortholog has not been identified in yeast. Recently, ubiquitin protease 10 (UBP10) was reported to de-ubiquitylate Ubi-PCNA in *S. cerevisiae* (Gallego-Sanchez et al., 2012). Cells lacking UBP10 accumulate Ubi-PCNA in response to DNA damage resulting in an increased interaction between PCNA and Rev1. UBP10 appears to de-ubiquitylate Ubi-PCNA during S phase and its protein levels remain constant

after UV treatment suggesting that UBP10 in yeast and USP1 in human regulate the de-ubiquitylation of PCNA by different mechanisms (Gallego-Sanchez et al., 2012).

NEW READERS OF UBIQUITYLATED PCNA

Once PCNA is ubiquitylated, it provides a loading platform for a variety of proteins involved in the replication of damaged DNA. As already mentioned, Ubi-PCNA can recruit a plethora of TLS polymerases allowing damage bypass and the restart of a stalled replication fork (Sale et al., 2012). Recently at least two new proteins have been described to be able to read the state of ubiquitylated PCNA and to help in maintaining the stability of the fork: Spartan, also called DVC1, and ZRANB3 (Centore et al., 2012; Davis et al., 2012; Mosbech et al., 2012) (**Figures 1A–C**).

Spartan is a substrate of the anaphase promoting complex and localizes to replication factories in a manner dependent on both its PIP and UBZ domains (Davis et al., 2012; Mosbech et al., 2012). In its absence, cells become hypersensitive to DNA damage agents and they are deficient in the DNA damage tolerance (DDT) response. Spartan can bind to p97 via its SHP domain (Davis et al., 2012; Mosbech et al., 2012). p97 encodes for a chaperone protein that can remodel ubiquitylated proteins in an ATP-dependent manner (Meyer et al., 2012).

As mentioned, Spartan PIP box and UBZ domain are needed for its accrual in replication factories and DNA damage foci. While all the data in the literature consistently report that PCNA is required for Spartan recruitment, the role of Ubi-PCNA as the target of Spartan's UBZ is still controversial. Spartan can bind Ubi-PCNA *in vitro* (Centore et al., 2012) but there are discording evidences that this may occur *in vivo*. Two groups reported that Spartan could relocalize to replication factories when Rad18 is depleted by siRNA, a condition that results in the absence of Ubi-PCNA (Davis et al., 2012; Mosbech et al., 2012). Spartan itself is ubiquitylated and this modification prevents further binding to ubiquitin targets and decreases its accumulation in focal structures (Centore et al., 2012).

Given all the conflicting evidence, the role of Spartan is still under scrutiny, with at least two proposed models of actions. In the first Spartan is thought to bind to Ubi-PCNA and to promote both Rad18 and polη recruitment to the chromatin. Its binding would shield Ubi-PCNA from being de-ubiquitylated by USP1 or by another DUB, and in its absence PCNA ubiquitylation appears to be reduced (Centore et al., 2012) (**Figure 1A**). At the opposite side of the spectrum, an alternative mechanism proposes Spartan acting as a negative regulator of TLS. In this scenario, Spartan is thought to recruit p97, which in turn will remove polη from the replication fork in order to resume processive replication (**Figure 1C**). This model is substantiated by increased focal retention of polη and increased mutagenesis when Spartan is silenced (Davis et al., 2012; Mosbech et al., 2012). Recently, three patients showing early onset hepatocellular carcinomas and progeroid syndrome have been found to carry a mutation in SPRTN (Lessel et al., 2014). When Spartan was mutated or depleted, the cells showed signs of genomic instability, defects in replication fork progression and cell proliferation. Interestingly, depletion of polη in a background mutated in SPRTN did not rescue the replication phenotypes, indicating that polη is potentially not the main target of Spartan activity (Lessel et al., 2014). The discovery of this new progeroid syndrome further stresses the importance of SPRTN, but additional investigation is needed to clarify the mechanism of action of this protein essential for the DDT.

Proliferating cell nuclear antigen polyubiquitylation is proposed to channel the DDT to an error-free damage avoidance branch named template switch (Hoege et al., 2002; Branzei and Foiani, 2007; Branzei, 2011). The molecular mechanism of this pathway is still not completely understood and, until recently, we did not know the role of K63-linked chains attached to PCNA. In the last couple of years the protein ZRANB3/AH2, has been proposed to be able to recognize specifically polyubiquitylated PCNA and to promote template switch by stimulating fork regression (Ciccia et al., 2012; Weston et al., 2012; Yuan et al., 2012). ZRANB3 encodes for an annealing helicase/translocase and it can interact with polyUbi-PCNA via multiple domains. A canonical PIP motif and an APIM (C-terminal AlkB2 PCNA-interaction motif) domain mediate the direct interaction with the PCNA trimer while an NPL4 zinc finger (NZF), a variant of ubiquitin-binding domain, recognizes K63-linked ubiquitin chains specifically (Ciccia et al., 2012). This domain is able to bind to polyUbi-PCNA *in vitro* and it is needed for the localization of ZRANB3 to damage sites. All these structural motifs are required for restarting the fork after DNA damage (**Figure 1B**).

Experimental observations suggest that ZRANB3 may play three different roles at the stalled replication fork: (1) it can stimulate fork regression in order to stabilize the fork and minimize the amount of ssDNA that is generated (Ciccia et al., 2012). (2) ZRANB3 can disrupt D-loop formation *in vitro* and this in turn could result in the prevention of inappropriate homologous recombination (HR) (Ciccia et al., 2012); (3) it can act as a strand-specific endonuclease pointing to a role not only in damage bypass but also in damage repair (Weston et al., 2012).

ZRANB3 may act in parallel or in conjunction with HLFT that also has a helicase activity and can stimulate fork regression *in vitro* (Blastyak et al., 2010; Achar et al., 2015). Further work will be needed in the future to completely elucidate ZRANB3 role in damage tolerance and repair.

PCNA SUMOylation AND ISGylation

Another prominent post-translational modification of PCNA is its SUMOylation. It was originally identified in yeast and only recently it was observed in human cells.

In yeast, PCNA is SUMOylated (S-PCNA) on Lys164 (major) and Lys127 (minor) by the combined action of Ubc9 (E2) and Siz2 (E3) or by Ubc9 alone, respectively (Hoege et al., 2002) (**Figure 1D**). SUMOylation occurs during normal S phase and/or in response to high doses of DNA damage (Juhasz et al., 2012). SUMOylated PCNA interacts with Srs2 helicase, which has been shown to prevent HR by disrupting Rad51 filaments (Papouli

et al., 2005; Pfander et al., 2005). Srs2 has a non-canonical PIP-box with limited affinity for PCNA and it binds stably only when the clamp is SUMOylated. A SUMO interacting motif that is located in tandem after the PIP in the protein carboxyl terminus of Srs2 mediates this interaction (Kim S.O. et al., 2012).

Given the catalytic activity of Srs2 and the timing of this modification, it is believed that SUMOylation of PCNA acts as a negative regulator of unscheduled HR during S phase, where this kind of pathway could be detrimental to the cell. In yeast, one of the replication factor C (RFC)-like complexes, Elg1-RFC also has a role in regulating S-PCNA. RFC is a complex consisting of Rfc1-5 and it works as clamp loader/unloader. All eukaryotic cells contain a series of three alternative RFCs, containing Elg1, Ctf18, or Rad24 in place of Rfc1(Kim and MacNeill, 2003). Elg1-RFC is required for the efficient unloading of SUMOylated PCNA from the chromatin during S phase. In cells lacking Elg1, PCNA accumulates on the chromatin and it is possible to detect an increase in SUMOylated PCNA (Parnas et al., 2010; Kubota et al., 2013b).

In *X. laevis* S-PCNA is present during unperturbed replication in cell extracts, but it is not required for the replication of either ssDNA or sperm chromatin (Leach and Michael, 2005).

In human cells, S-PCNA had eluded detection for a number of years and it has been detected only recently after overexpression of SUMO1, although to a much less extent than the levels detected in yeast (Moldovan et al., 2012). PCNA was found to be SUMOylated on both Lys164 and Lys254 under specific conditions (Gali et al., 2012). As in yeast, mammalian UBC9 acts as the E2 enzyme but surprisingly, at least *in vitro*, the SUMOylation of PCNA does not require the Siz1 orthologs (PIAS1-4) in either lysine residues (Gali et al., 2012).

A PCNA-SUMO fusion protein not only prevents HR, but also DNA double-strand break formation, as monitored by a marked reduction of γH2AX foci (Gali et al., 2012). Two putative functional homologs of Srs2 have been identified in human cells: PCNA-associated recombination inhibitor (PARI; Moldovan et al., 2012) and F-box DNA helicase (FBH1; Fugger et al., 2009; Bacquin et al., 2013). Both PARI and FBH1 have been reported to interact with PCNA and to have PCNA-dependent anti-recombinogenic activity, but only PARI seems to specifically interact with SUMOylated PCNA, at least *in vitro* (Moldovan et al., 2012). On the other hand, FBH1 needs to be degraded, via $CRL4^{Cdt2}$ pathway in order to allow efficient recruitment of polη to replication factories (Bacquin et al., 2013).

In human cells, ATAD5, the ortholog of yeast Elg1 appears to have a somehow different role from its yeast counterpart as it interacts, at stalled replication forks, with the USP1/UAF1 complex and facilitates USP1-mediated PCNA de-ubiquitylation (Lee et al., 2010; Kubota et al., 2013a).

Last year ISGyaltion, another ubiquitin-like modification, was discovered to affect PCNA.

ISG15 (interferon-stimulated gene 15) was the first identified ubiquitin-like protein and it is strongly stimulated by type I interferon (Haas et al., 1987; Loeb and Haas, 1992). As ubiquitin and SUMO this post-translational modification relies on a chain of three classes of enzymes to be linked to its substrates: UBE1L is the activating E1 enzyme, followed by UBCH8 (E2) and finally by EFP and HERC5 (E3s; Yuan and Krug, 2001; Kim et al., 2004; Zhao et al., 2004; Dastur et al., 2006; Zou and Zhang, 2006). PCNA was reported to be bi-ISGylated 24 h after UV irradiation by EFP on both K164 and K168 (Park et al., 2014). Mutations of either residues resulted in the complete disappearance of ISGylated PCNA indicating that ISGylation at one site influences the state of the other. The late response to UV irradiation suggested that ISG15 had a role in the recovery from DNA damage and post-replication repair (PRR). The E3 ligase EFP interacts with Ubi-PCNA and this interaction is propaedeutic to PCNA ISGylation (Park et al., 2014). This modification in turn recruits USP10 that de-ubiquitylates PCNA in order to block TLS and resume normal replication. Eventually, UBP43 removes ISG15 from PCNA (**Figure 1C**). ISGylation-deficient mutants of PCNA show increased recruitment of polη to the chromatin many hours after UV irradiation (Park et al., 2014).

UBIQUITYLATION OF TLS POLYMERASES

As mentioned before, PCNA is not the only player that is modified in order to control PRR. All the members of the Y-family of DNA polymerase (η, ι, κ, and Rev1) involved in DNA TLS have been identified to be modified by ubiquitin or ubiquitin-like modifiers (Sale et al., 2012). Furthermore, all four of them contain ubiquitin-binding domains (UBM or UBZ; (Bienko et al., 2005; Guo et al., 2006, 2008; Plosky et al., 2006).

Probably, the best characterized of the group is polη, the major TLS polymerase involved in the error-free bypass of cyclobutane pyrimidine dimers (CPDs), the main adduct created by UV irradiation. CPDs are repaired slowly by the nucleotide excision repair (NER) and have a higher probability to persist in the genome until DNA replication. The importance of the bypass performed by polη is exemplified by the fact that individuals carrying an inactivating mutation are affected by *Xeroderma pigmentosum* Variant (XPV; Masutani et al., 1999). Regardless of the importance of its function, polη shares a common characteristic with other Y-family polymerases, a wide catalytic site. This structural feature, while beneficial for damage bypass, makes the polymerase intrinsically error-prone compared to replicating polymerases when using undamaged DNA as a template. For this reason, its recruitment to the replication fork needs to be tightly regulated. Polη is recruited to replication factories in a manner dependent on its PIP-box and UBZ, a specialized ubiquitin-binding zinc finger (Kannouche et al., 2001, 2002; Bienko et al., 2005, 2010; Sabbioneda et al., 2009). The presence of both domains stabilizes the interaction between the polymerase and Ubi-PCNA after DNA damage (Kannouche et al., 2004; Bienko et al., 2010). Mutants in either the PIP-box or the UBZ are required for focal accumulation of the polymerase but they retain a partial bypass activity, indicating that they work in parallel to ensure efficient binding with PCNA (Bienko et al., 2010). Ubiquitylation of PCNA provides a positive regulation by increasing the affinity between polη and the clamp when the replication fork is blocked (Kannouche et al., 2004).

Conversely, ubiquitylation of the polymerase works as a negative regulator by preventing its recruitment on the chromatin (Bienko et al., 2010). *In vivo*, a small amount of polη is monoubiquitylated, in the absence of damage, in its nuclear localization signal directly adjacent the PIP-box. The modification occurs primarily on K682 but in its absence, also K686, K694 and K709 have been found to be ubiquitylated (Bienko et al., 2010; Jung et al., 2011). Ubiquitylation is strictly dependent on the UBZ of polη. Recently, PirH2 was discovered to be the E3 ligase responsible for this monoubiquitylation (Jung et al., 2011). Ubiquitylation of polη is believed to cause a conformational change in its C-terminus with the attached ubiquitin binding intra-molecularly to polη's UBZ. In this closed confirmation, neither the UBZ, blocked by the binding to the ubiquitin attached to polη, nor the PIP-box, that is located between the UBZ and K682, are available to stabilize its interaction with PCNA (Bienko et al., 2010). Ubi-polη is indeed excluded from the chromatin and replication foci. After DNA damage, ubiquitylated polη gradually disappears. The polymerase can be then recruited to the chromatin and it becomes proficient for TLS. The de-ubiquitylation of the polymerase is believed to be carried out by the DUB USP7 (Qian et al., 2015). It is important to note that only 10% of polη is ubiquitylated in the absence of damage at any given time, indicating that some other forms of regulation are keeping polη under negative control. In some cellular background, polη gradually disappears in the hours following UV irradiation. This process is believed to be mediated by Mdm2 that polyubiquitylate the polymerase and marks it for proteasomal degradation (Jung et al., 2012). A similar system, mediated by $CRL4^{Cdt2}$ has also been observed in *Caenorhabditis elegans*. Interestingly in this system, the degradation of polη is prevented by its SUMOylation by the SUMO E3 ligase GEI-17 (Kim and Michael, 2008). It is still unclear whether polη is SUMOylated in human cells.

Similarly, to polη also its paralog polι is ubiquitylated (Bienko et al., 2005; McIntyre et al., 2015). This polymerase is thought to bypass lesions when polη is not present (Wang et al., 2007; Vidal and Woodgate, 2009). *In vitro*, polι can bypass different typologies of DNA adducts with different degrees of fidelity (Washington et al., 2004a,b; Frank and Woodgate, 2007).

Polι is characterized by two UBMs that are needed for its modification and correct localization in replication foci (Bienko et al., 2005; Bomar et al., 2010). It is speculated that the ubiquitylation of polι might be important for its interaction with polη (McIntyre et al., 2013).

The deoxycytidyl transferase Rev1 possesses two UBMs (Bomar et al., 2010) and gets ubiquitylated *in vivo* (Guo et al., 2006; Kim H. et al., 2012). The UBMs are needed for the efficient interaction with Ubi-PCNA (Guo et al., 2006; Wood et al., 2007). In yeast, deletion of UBM2 severely affects UV-induced mutagenesis, a pathway that is strictly dependent on TLS (Wood et al., 2007; Terai et al., 2010). Mutations in Rev1's UBMs make the cells hypersensitive to UV in the DT40 system (Guo et al., 2006). In chicken cells, Rev1 and its UBMs have been shown to have a role in replication fork progression in the presence of UV in a process that is independent from Ubi-PCNA (Edmunds et al., 2008). Finally, Rev1 appears to be able to bind to the Fanconi core complex via FAAP20 and this interaction is believed to promote Rev1 recruitment to replication foci and ultimately Rev1-dependent mutagenesis (Mirchandani et al., 2008; Kim H. et al., 2012).

The last TLS polymerase that has been reported to be ubiquitylated is polκ (Guo et al., 2008). Polκ is characterized by two UBZ domains in its c-terminus (Bienko et al., 2005) that have been reported to be important for the interaction with PCNA and the localization in foci after UV irradiation (Guo et al., 2008). Polκ has also been shown to be important for NER, and its repair function depends on its UBZs (Ogi et al., 2010).

The role of polκ ubiquitylation is currently not clear but it is likely to promote protein–protein interaction similarly to the other members of the Y-family of DNA polymerases.

DNA DAMAGE TOLERANCE AND CANCER

Post-replication repair and the damage tolerance systems provide an essential safety mechanism that allows the completion of DNA replication and it is an important pathway to preserve genome stability. At the same time, it can act as a double-edged sword since a number of its components, such as TLS polymerases, are intrinsically error prone and can be a source of mutations if they are not correctly regulated. Mutations are one of the major driving forces that lead to cell transformation and tumorigenesis, therefore it is important to define the contribution of PRR in the context of cancer. The dichotomy of protection versus increased risk is emblematic in the case of polη. As already mentioned in this review, a deficiency in polη is the cause of XPV (Broughton et al., 2002). Like other XP groups that are mutated in NER, XPV patients are sensitive to sun light and are extremely prone to both melanoma and non-melanoma skin cancers (Fassihi et al., 2016). Polη is the main polymerase that is able to bypass CPDs in an error-free manner and it possible to envisage that when missing, its role is carried out by other TLS polymerases with different degrees of fidelity.

In these cases, the ultimate and less than desirable outcome would be the introduction of mutations that are responsible for the transformation of the skin cells. It is important to note that polη-deficient patients are the most prone to skin cancers among the *X. pigmentosum* groups (Fassihi et al., 2016). XPV patients tend to have milder skin phenotypes and are normally diagnosed much later in their life, when they have already accumulated a number of UV-induced mutations. This higher mutation load correlates with the possibility of developing more skin tumors in their adult life (Fassihi et al., 2016). In this context, it is clear that polη protects the cells from cancer. On the other hand, the survival capability conferred by this polymerase can be hijacked to make tumors more resilient. *In vitro*, cells lacking polη are more sensitive to cisplatin, one of the most used first line drug in chemotherapy (Albertella et al., 2005a). Increased expression of polη associates with worse prognosis and survival in a cohort of patients suffering from non-small cell lung cancer patients previously treated with platinum (Ceppi et al., 2009). Polη seems also to be involved in the cellular response after treatment with

nucleoside analogs, which are commonly used in the clinic as cancer drugs (Chen et al., 2006). Interestingly, mutations in polη are hardly found in patients with sporadic skin carcinomas (Glick et al., 2006; Flanagan et al., 2007; Lange et al., 2011) but its overexpression has been reported (Albertella et al., 2005b). Polη ortholog, polι, has been found to be elevated in breast cancer cells and in these cell lines a reduced mutation frequency was recorded when the polymerase was depleted *in vitro* (Yang et al., 2004). Furthermore, mutation in polι have been linked to an increased predisposition of developing lung cancer in both human (Sakiyama et al., 2005) and mouse (Wang et al., 2004; Lee and Matsushita, 2005).

Two of TLS polymerases extensively characterized for their role in mutagenesis and cancer are polζ and Rev1. Polζ is thought to be the major player involved in error-prone replication of damaged templates *in vivo*. In mice, conditional Rev3 knockout results in increased genome instability and tumorigenesis in a p53-null background (Wittschieben et al., 2006, 2010; Lange et al., 2013). Similarly to polη, there is experimental evidence indicating that the presence of both Rev1 and polζ can confer drug resistance both *in vitro* and *in vivo* (Xie et al., 2010). Conversely, Rev3 inhibition makes lymphoma and lung cancer cells more sensitive to platinum-derived drugs (Doles et al., 2010), once again underlying the dichotomy of TLS regarding cancer and genome protection. All of these evidences point to the idea that transient inhibition of TLS could be synthetically lethal to tumor cells that rely on the TLS mutator activity for survival. TLS polymerases are not the only proteins involved in damage tolerance that have been linked to cancer development. The expression of the E3 ligase HLTF has been found to be altered in transformed cells and in numerous tumors. A reduced expression of HLTF, due to hyper-methylation of its promoter, has been found in colon and colorectal cancer, esophageal squamous cell and gastric carcinomas (Debauve et al., 2008). Interestingly HLTF is overexpressed in transformed cells, indicating that a differential modulation of its expression could be needed at different stages of tumorigenesis (Debauve et al., 2008). Given the role of HLTF in the control of the error-free branch of damage tolerance, it is tempting to speculate that it could be beneficial for tumor cells to inactivate HLTF in order to channel the PRR pathway toward the more mutagenic TLS bypass, thus allowing the malignant cells to accumulate more mutations. As mentioned before a SPRTN deficiency has been linked with a new progeroid syndrome with propensity to develop early onset hepatocellular carcinomas, but it is still not clear whether this phenotype is directly linked with its proposed control of polη (Lessel et al., 2014). In conclusion, a tight regulation of TLS and the DNA damage tolerance pathway in general is required to preserve the delicate balance between protecting the genome stability and inducing cellular transformation.

THE UNANSWERED QUESTIONS

In the last decade, mounting evidence has pointed out the crucial role of ubiquitin, and other ubiquitin-like modifications, in the control of PCNA and TLS. Nevertheless, we still do not know whether PCNA ubiquitylation is strictly required for TLS. A series of experimental hints suggest that there is more to the story and we still have only a partial picture of the regulation of the damage tolerance pathway. For instance, MEF cells carrying the PCNA K164R mutation can be further sensitized by the deletion of other TLS genes, indicating that some steps of the pathway could be independent from Ubi-PCNA (Hendel et al., 2011). Furthermore, PCNA ubiquitylation is not required for polη-mediated somatic hyper-mutation in mouse B cells (Krijger et al., 2011b).

In human cells the phosphorylation of polη, that occurs on the chromatin, is dependent on its UBZ, indicating that the binding to ubiquitin is needed for this regulatory modification (Gohler et al., 2011). However, this phosphorylation does not require Ubi-PCNA and can occur in its absence (Gohler et al., 2011). Dynamic studies on polη show that Ubi-PCNA helps in stabilizing the polymerase in replication foci but do not exclude the possibility that other ubiquitylated proteins may play a role in its initial recruitment (Sabbioneda et al., 2008). Consistent with this hypothesis polη is still recruited to replication factories after chemical depletion of Ubi-PCNA caused by prolonged treatment with the proteasome inhibitors MG132 or epoxomicin (Sabbioneda et al., 2008). It must be noted that mouse cells carrying a homozygous K164R mutation appear to be deficient for polη recruitment (Krijger et al., 2011b), and so far no explanation has been found for these conflicting evidences.

CONCLUSION

We are now starting to grasp the complexities of the regulation of PRR and TLS, the continuous dance between protein partners and the intricacies that lie behind such an important tolerance pathway. Meanwhile, behind the scenes, the hunt for the next big ubiquitylated/SUMOylated target still rages on.

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Phosphorylation regulates human polη stability and damage bypass throughout the cell cycle

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ABSTRACT

DNA translesion synthesis (TLS) is a crucial damage tolerance pathway that oversees the completion of DNA replication in the presence of DNA damage. TLS polymerases are capable of bypassing a distorted template but they are generally considered inaccurate and they need to be tightly regulated. We have previously shown that polη is phosphorylated on Serine 601 after DNA damage and we have demonstrated that this modification is important for efficient damage bypass. Here we report that polη is also phosphorylated by CDK2, in the absence of damage, in a cell cycle-dependent manner and we identify serine 687 as an important residue targeted by the kinase. We discover that phosphorylation on serine 687 regulates the stability of the polymerase during the cell cycle, allowing it to accumulate in late S and G2 when productive TLS is critical for cell survival. Furthermore, we show that alongside the phosphorylation of S601, the phosphorylation of S687 and S510, S512 and/or S514 are important for damage bypass and cell survival after UV irradiation. Taken together our results provide new insights into how cells can, at different times, modulate DNA TLS for improved cell survival.

INTRODUCTION

DNA replication is crucial for the perpetuation of genetic information. To ensure its completion, cells possess damage tolerance mechanisms that are capable of replicating a template even when DNA damage is present. One of such tolerance systems is DNA translesion synthesis (TLS) and it takes advantage of specialized DNA polymerases to allow the progression of the replication fork in the presence of a distorted template (1). The majority of TLS polymerases belong to the Y family and include polη, polι, polκ and Rev1 along with the B-family member polζ (1). The Y family polymerases can bypass the damage by virtue of a more open catalytic site that can accommodate DNA lesions, such as cyclobutane pyrimidine dimers (CPDs), created by exposure to UV light (2). As a trade-off, this wider catalytic site is prone to errors during nucleotide incorporation, so TLS polymerases need to be tightly controlled. This regulation is achieved in multiple ways in different model organisms: while in *Saccharomyces cerevisae* the concentration of TLS polymerases seems to be regulated (3), in mammalian cells TLS polymerases are believed to be regulated mainly by modulating their localization and recruitment to DNA (3). Post-translational modifications (PTMs) play a fundamental role in such control, and numerous lines of evidence now support the notion that ubiquitylation, SUMOylation and phosphorylation are crucial in activating and blocking TLS in different contexts (4–9). Polη is one of the main TLS polymerases and is the only one that can bypass CPDs in an error-free manner. The genetic disease, xeroderma pigmentosum variant, which is caused by inactivating mutations in the *POLH* gene, exemplifies its importance (10). Polη can be considered the archetypal TLS polymerase on the subject of PTMs as it has been shown to be ubiquitylated, phosphorylated and more recently SUMOylated (4–5,7,9,11–12). Polη is monoubiquitylated on one of four lysines present on its PCNA (proliferating cell nuclear antigen) interacting region (PIR) (4). This modification occurs in the absence of DNA damage and prevents the interaction of the polymerase with ubiquitylated targets that is mediated by its UBZ (ubiquitin binding zinc finger) motif. Once ubiquitylated, polη transitions to an inactive state via an intramolecular conformational change. This structural change allows the UBZ to make contact with the ubiquitin attached to the PIR, thus blocking it from binding to other ubiquitylated proteins. Monoubiquitylation has been shown to prevent polη from

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interacting with PCNA, the polymerase clamp, which plays a crucial role in controlling damage tolerance. The E3 ligases PirH2 and Mdm2 have been suggested to be responsible for this regulatory monoubiquitylation of polη and previously it has been shown to be linked to the proteasomal degradation of the polymerase (13–15).

While the ubiquitylation of polη appears to have an inhibitory effect on TLS, the phosphorylation of the polymerase leads to its full activation. After DNA damage polη is phosphorylated by the PI3 kinase Ataxia Telangiectasia and Rad3 related (ATR) on serine 601, and this modification is required for efficient damage bypass (5). Phosphorylation of S601 occurs only on chromatin and it requires polη UBZ and depends on Rad18 but not on PCNA ubiquitylation. In recent years it has been suggested that other polη residues might be phosphorylated but direct evidence of their modification is still missing (12).

Here we report that polη exists in multiple phosphorylation states even in the absence of DNA damage and its phosphorylation changes in a cell cycle-dependent manner. We identify novel phosphorylated sites and show that one of them (S687) affects polη protein stability.

MATERIALS AND METHODS

Cell culture and treatments

All the experiments in this study were carried out with SV40-transformed cells cultured in Dulbecco's modified Eagle's medium containing 10% Fetal Bovine Serum. XP30RO (XP-V, also designated GM3617) carries a homozygous deletion in the *POLH* gene, resulting in a truncated protein of 42 amino acids. The SV40-transformed cell line was originally established by Cleaver *et al*. (16). The absence of the polη protein was routinely monitored by immunoblotting and by UV sensitivity assays. MRC5V1 cells (also called MRC5) are SV40-transformed normal lung fibroblasts.

eGFP-tagged mutants of polη (S687A and SSSAAA) cloned in pEGFP-C3 were created by Quickchange site directed mutagenesis (Stratagene) using the following primers and verified by sequencing:

S687A: primer forward 5′-GCAAAAGAAATCCCAAG GCCCCTTTGGCCTGCAC-3′, primer reverse 5′-GTG CAGGCCAAAGGGGCCTTGGGATTTCTTTTGC-3′.

SSSAAA: primer forward 5′-CTCAGGCTCCCATGG CCAATGCCCCAGCCAAGCCCTCATTACC-3′, primer reverse 5′-GGTAATGAGGGCTTGGCTGGGGCATTG GCCATGGGAGCCTGAG-3′.

Once obtained, eGFPpolη and its mutants were subcloned in pCDNA3.1(+) (Thermo Scientific) by digestion with NheI and BamHI to obtain plasmids suitable for the TNT Quick coupled Transcription/Translation system (Promega).

XP30RO stably expressing S687A or SSSAAA were created using previously described procedures (5,17). Briefly 4×10^5 cells were seeded on a 6 cm dish and transfected (see below) with the relevant construct. After 24 h, the cells were diluted in four 10-cm dishes and selected in 1 μg/ml G418. Single colonies were collected after 14 days in culture with cloning discs (Sigma–Aldrich) and expanded. Finally the clones were screened for the presence of eGFPpolη before enrichment with a cell sorter.

MG132 (Sigma–Aldrich) was used at 10 μM for 1, 3 or 6 h. Roscovitine (Selleckchem) was used at the final concentration of 20 μM for 1, 3 or 6 h. Dinaciclib (Selleckchem) was used at the final concentration of 100 nM for 2 or 8 h. VE-821 (Sellekchem) was used at 10 μM for 1 hour.

Cell synchronization was performed with a double thymidine (Sigma–Aldrich) block and a release in nocodazole (Sigma–Aldrich) if not otherwise stated. Cells were treated for 16 h with 2.5-mM thymidine, released in medium without thymidine and then incubated again for 16 h in thymidine. Finally, the cells were released into 50 ng/ml nocodazole for up to 16 h and were harvested at different time points. Transfection was carried out with Viafect (Promega) or Genejuice (Novagen) according to the manufacturers' instructions.

2D gel electrophoresis

All reagents and solutions for 2D-PAGE (polyacrylamide gel electrophoresis) were obtained from Bio-Rad except as noted. Cell pellets were resuspended in 100 μl of rehydration buffer with protease and phosphatase inhibitors and sonicated for 3 cycles/30 s with a Diagenode Bioruptor. Benzonase (50U) (Merk Millipore) was added and the extracts were incubated end over end for 30 min at 4°C. Samples were then centrifuged at 13 000 rpm for 30 min at 4°C and quantified by Bradford assay. A total of 120 μg protein extract were diluted with rehydration buffer to a final volume of 200 μl.

A total of 200 μl of each sample were loaded on a 7 cm IPG strip (pH3–10) by passive rehydration for 30 min followed by an active rehydration in a Bio-Rad Protean i12 IEF cell (14 h 50V 20°C).

The first dimension was performed at 50 mA on a voltage gradient. Following focusing, each strip was incubated for 20 min twice in equilibration buffer with 20 mg/ml dithiothreitol (DTT) or 25 mg/ml iodoacetamide (Sigma–Aldrich) respectively. Strips, embedded in agarose, were then loaded on 10% polyacrylamide gels.

Electrophoresis was performed at 100 mA for 10 min and then at a constant current of 200 mA for 60 min.

SDS-PAGE and western blotting

Proteins separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred onto nitrocellulose membranes. After blocking in 5% skim milk powder in Tris-buffered saline, pH 7.5, 0.1% Tween-20 for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4°C. The membranes were then incubated with secondary antibody and visualized using an enhanced chemiluminescence detection kit (Millipore) on a LAS500 system (GE Healthcare). Western blots were analyzed with Imagequant (GE Healthcare).

Antibodies

Antibodies used in this study included: anti-mouse-BrdU (Becton Dickinson, 1:200); Vimentin (Sigma–Aldrich,

1:1000); Vinculin (Abcam, 1:1000); Phospho-CDK Substrate Motif [(K/H)pSP] (Cell Signaling 1:1000); Polη (Abcam, 1:1000, used only for detection of eGFPpolη); Polη (custom made 1:500, detects endogenous polη); Polη P-687 (custom made, 1:200); PCNA (Santa Cruz, 1:1000).

RNA extraction, RT-PCR and real-time PCR

Total RNA was extracted using a Qiagen RNA Kit according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis with a M-MulV Reverse Transcriptase Kit (Roche). Amplification conditions were as follows: 60 min at 37°C followed by 10 min at 70°C. Real-time polymerase chain reaction (PCR) was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad) in a LightCycler 480 (Roche). Reactions were run in triplicate in three independent experiments. The housekeeping gene used was GAPDH.
The primers used are:
GAPDH: primer forward 5′-ACCACAGTCCATGCCATCAC-3′ primer reverse 5′-TCCACCACCCTGTTGCTGTA-3′.
Polη: primer forward 5′-AGTTCGTGAGTCCCGTGGG-3′ primer reverse 5′-GCTTGGCAACAAGTCTGCC-3′.

Clonogenic assay

Cell lines expressing different eGFP constructs were seeded and UV irradiated the following day at the indicated doses. After 13 days, the number of colonies was assessed after 70% EtOH fixation and methylene blue staining (0.1% Page-Blue; 50% MetOH; 7.5% Acid Acetic).

Post-replication repair (PRR) assay

Cells were washed in phosphate-buffered saline (PBS) and irradiated with 8 Jm^{-2} as previously described (4). Briefly, cells were incubated in 2 ml of medium containing 0.3 mg/ml caffeine for 30 min before the addition of 1.85 MBq of ^{3}H-thymidine. Cells were then released in fresh caffeine-containing medium (0.3 mg/ml) supplemented with 10 μM thymidine and 10 μM deoxycytidine for 150 min. At the end of the chase period, cells were collected in PBS containing ethylenediaminetetraacetic acid (EDTA) (0.2 g/l). Cells were then lysed in 0.2 ml buffer (1% SDS, 0.75M NaOH, 0.75M NaCl, 20 mM EDTA) layered on top of 5 ml of a 5–20% sucrose gradient (in 0.1 M NaOH, 0.1 M NaCl). After exposure to visible light for 1 h, the gradients were spun at 38 500 rpm for 70 min in a Sw55 rotor. At the end of the run, 25 fractions were spotted on Whatman grade 17 paper, precipitated by trichloracetic acid and washed twice in ethanol before being counted on a Perkin Elmer scintillation counter. The weight-average molecular weights of the distributions were calculated as (18), omitting the top and bottom three fractions.

Flow Cytometry analysis

Cells were harvested after incubation with 30 μM bromodeoxyuridine (BrdU; Sigma–Aldrich) for 30 min, fixed in 1 ml of cold 70% ethanol and stored at 4°C. DNA was denatured with 2N HCl and 0.5% Triton X-100, then neutralized with 1 ml of 0.1M sodium borate pH8.5. The cell pellet was resuspended in PBS, 0.5% Tween 20, 1% bovine serum albumin (BSA) with a mouse anti-BrdU antibody (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated at room temperature for 1 h, followed by 1-h room temperature incubation with anti-mouse-488 (Alexa anti-mouse 488, Molecular Probes/Invitrogen). Samples were finally incubated with 10 μg/ml Propidium Iodide and analyzed on a S3 flow cytometer (Biorad). Data were analyzed using the program ProSort (Biorad) or FCS Express (DeNovo). When Propidium Iodide only staining was performed, cells were fixed in 1 ml of cold ethanol and resuspended in PBS, 0.1% Tween 20, 50 μg/ml Propidium Iodide, 5 μg/ml RNase A. After incubation at 37°C for 15 min, cells were analyzed by flow cytometry.

In vitro CDK assay

Assays for polη phosphorylation were performed with CDK1 and CDK2. The reactions were performed with the same units of enzymes (10U) or with the same quantity of enzymes (40 ng). Purified polη was incubated either with CDK1/cyclin B (Millipore) or CDK2/cyclin A (Millipore) in reaction buffer (8 mM MOPS/NaOH pH7.0, 2.5 mM MgAc, 0.05 mM ATP, 0.02 mM EDTA) for 10 min at 30°C. The reactions were stopped by addition of Laemmli buffer and boiled at 95°C for 5 min. Activity of the CDKs was checked by incubating the kinases with Histone H1 (Sigma–Aldrich).

Immunoprecipitation

Harvested cells were lysed with a lysis buffer (0.5% NP40, 40 mM NaCl, 50 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$, 1 mM N-Ethylmaleimide (NEM), protease inhibitors cocktail (Roche), phosphatase inhibitors (Roche) and 1 μl/ml Benzonase (Novagen)) and incubated on a rotary wheel at 4°C for 30 min. After incubation the mixture was centrifuged at 13 000 rpm for 15 min at 4°C and the supernatants were collected and quantified by Bradford assay. Samples were diluted in four volumes of immunoprecipitation (IP) buffer (125 mM NaCl, 50 mM Tris–HCl pH 7.5, protease inhibitors cocktail (Roche)). Protein extracts were pre-cleared with Protein A/G magnetic beads (100 μl/20 mg of protein, Thermo Scientific), previously equilibrated with three volumes of IP buffer, before IP with anti-GFP antibody (Thermo Scientific) bound to protein A/G magnetic beads.

Protein purification and MS analysis

Polη–6His, was purified from *Escherichia coli* as previously described (19). GST-PCNA was bought from Thermo Fisher Scientific. Flag-HA Polη was purified and analyzed by liquid chromatography-tandem mass spectrometry (MS) as described in (20).

Phosphatase treatment

A total of 120 μg protein extracts were incubated in phosphatase buffer (New England Biolabs) with or without

1600U of λ phosphatase (New England Biolabs) for 30 min at 30°C. After incubation the reaction was diluted with rehydration buffer up to a volume of 200 μl and processed as previously described for 2D-PAGE. Alternatively, immunoprecipitated proteins were incubated with 800U of λ phosphatase for 1 h in phosphatase buffer at 30°C before resuspension in Laemmli buffer.

Primer extension assay

Plasmids encoding eGFPpolη (WT, S687A and SSSAAA) or polη-6His (WT and S687A) were used for *in vitro* coupled transcription and translation reactions in rabbit reticulocyte lysate according to manufacturer's instructions (Promega). Polymerases were quantified by western blot analysis and equimolar amounts of proteins were used for the primer extension assays. A 5′ FAM-labeled 13mer (5′ GGGTGGAGGTGAC 3′) was annealed with a template 33mer primer (5′ TCACACTCTATCACACTC[cis-syn-TT]GTCACCTCCACCC 3′) containing a thymidine dimer (TT) at position +14 (TriLink Biotechnologies). Primer extension assays were performed in 10 μl of 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). Reactions were incubated at 37°C for 15 min and stopped with 10 μl of 2× loading buffer, (98% deionized formamide, 10 mM EDTA pH 8, 0.025% xylene cyanol and 0.025% bromophenol blue). Samples were boiled at 95°C for 5 min and rapidly chilled on ice. Reaction products were resolved on a 15% acrylamide-7M urea gel and then imaged on a Typhoon imager (GE HealthCare).

RESULTS

Polη presents multiple phosphorylation states

We previously identified and characterized S601 of polη and discovered how its phosphorylation was crucial for efficient damage bypass (5). The visualization of the phosphorylated state of the polymerase was extremely challenging and we had to develop a phospho-specific antibody in order to characterize the function of serine 601. In order to be able to assess the general phosphorylation state of the polymerase we adapted a new approach based on 2D SDS-PAGE. This technique relies on a first dimension that separates the proteins according to their isoelectric point followed by second dimension under denaturing conditions. This technique allowed us to amplify the small mobility shift caused by the polymerase phosphorylation as observed by canonical SDS-PAGE. We were surprised to note that polη separated in a number of different isoforms (Figure 1A) indicating that the polymerase could be modified by multiple phosphorylation events. To confirm that the different isoforms we were visualizing were indeed due to phosphorylation, we treated the cell extracts with λ−phosphatase. In such conditions all the negatively charged forms of polη disappeared indicating that our experimental approach had revealed multiple phosphorylated forms of the polymerase (Figure 1A). This finding was unexpected, as polη was previously shown to be phosphorylated only after DNA damage in order to trigger damage bypass (5). The damage-dependent phosphorylation of polη was carried out by the ATR kinase. By exploiting our experimental set-up we confirmed that while polη is significantly phosphorylated in the absence of DNA damage, after UV exposure its phosphorylation profile changes toward a more acidic state (Figure 1B and C) and we confirmed that this hyper-phosphorylation was dependent on ATR as it could be abrogated by the ATR-specific inhibitor VE821 (Figure 1B and C).

Polη is phosphorylated during an unperturbed cell cycle

We wondered if the presence of multiple phosphorylation states in the absence of DNA damage could have functional significance in the regulation of the activity of the polymerase. In order to assess if the phosphorylation changed during the cell cycle, we synchronized the cells in early S phase via a double thymidine block and then released the cells. This allowed us to sample the global phosphorylation status of the polymerase at different stages of the cell cycle, notably during S phase, during anaphase transition and finally in the G1 of the next cell cycle phase. As can be seen in Figure 2A and B the phosphorylation of polη increases toward the end of the S phase before reaching a maximum when the cells are arrested at the -metaphase-anaphase transition because of the treatment with nocodazole. Once the cells reach the G1 phase of the following cell cycle, the phosphorylation profile reverts to a more basic state, indicating that the phosphorylation of polη is cell cycle regulated and is low in G1 before increasing in late S-G2. A BrdU incorporation assay confirmed that the cells progressed synchronously (Figure 2C). The cell cycle-dependent phosphorylation of polη occurred also when the cells were released in the absence of nocodazole, indicating that the progression through the cell cycle was responsible for the modification and not the treatment with the drug (Supplementary Figure S1A).

CDKs are responsible for the cell cycle phosphorylation of polη

Given the peculiar distribution of polη phosphorylation through the cell cycle, we investigated if the polymerase could be the target of CDKs. To address this hypothesis we synchronized the cells in mitosis with nocodazole. In this condition, the phosphorylation of polη is maximal, with an evident shift toward an acidic isoelectric point (compare Figure 3A/B first and second blot). After treatment with nocodazole, the cells were further treated with two CDK inhibitors, specifically roscovitine and Dinaciclib. Both are known to be capable of repressing the activity of various CDKs (21,22). In both cases we detected a shift in the mobility of polη, consistent with a reduction in phosphorylated forms (compare third and fourth blot with the second in Figure 3A/B). Overall polη was unphosphorylated in roscovitine and Dinaciclib-treated cells, indicating that CDKs played a major role in regulating the cell cycle-dependent phosphorylation of the polymerase. The change in polη phosphorylation after CDK inhibition was also detectable in cycling cells without nocodazole arrest (Supplementary Figure S1B), indicating that the observed phenomenon was not due to the synchronization protocol. Although the phosphorylation was significantly reduced by

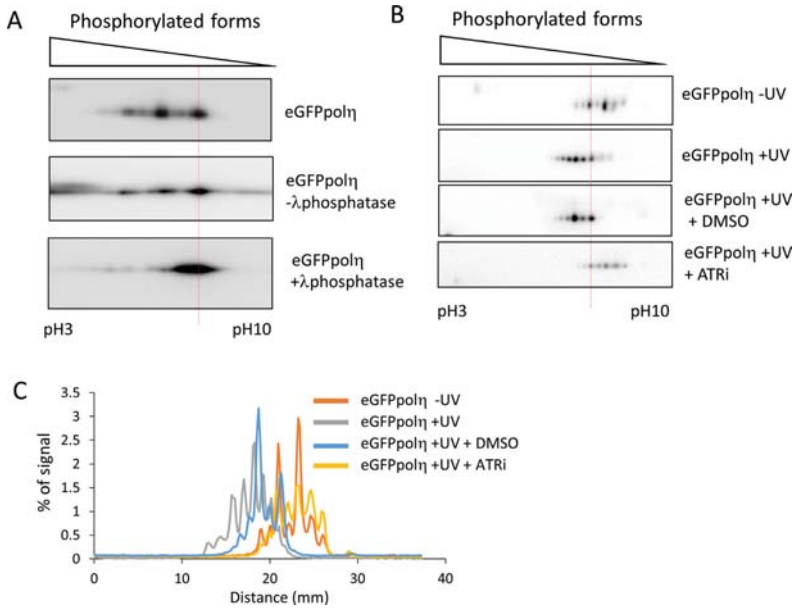

Figure 1. DNA polymerase η is phosphorylated even in the absence of DNA damage. (**A**) 2D-PAGE analysis of XP30RO cells expressing eGFPpolη shows the protein separating at different isoelectric points. Top blot, cell extract processed before incubation with phosphatase buffer. Middle blot, cell extract after incubation in phosphatase buffer at 30°C for 30 min. Bottom blot, cell extract after incubation with λ-phosphatase in phosphatase buffer at 30°C for 30 min. The different isoforms disappear after treatment with λ-phosphatase indicating that they represent phosphorylated forms of the polymerase. The 2D PAGE were aligned by using Vimentin and PCNA as triangulation markers (see Figure 2). The red dotted line is used as reference of the alignment. (**B**) DNA polymerase η becomes hyper-phosphorylated after damage in an ATR-dependent manner. (**C**) Densitometric analysis of the blots presented in B.

the treatments, some modified form of polη persisted, indicating that either redundant CDKs are involved or other kinases could phosphorylate polη.

At this point, we could only conclude that CDKs were important in regulating the phosphorylation of polη, but we did not know if they directly phosphorylated polη or if the phosphorylation was mediated by one of the plethora of CDK substrates. To address this point we purified human polη and performed an *in vitro* kinase assay. CDK2 and CDK1 are the two major kinases involved in S phase and G2/M phase progression. For this reason we chose to test their kinase activity using polη as substrate. Equal units of CDK1/2 or equal amounts (ng) of active kinases (Supplementary Figure S2A) were incubated with polη before electrophoresis. In order to monitor the phosphorylation activity, we used an antibody able to recognize phosphorylated CDK targets at their consensus motif ((K/R)(S*)PX(K/R)). As can be seen in Figure 3C, polη was efficiently phosphorylated by CDK2 but hardly by CDK1. We therefore concluded that polη was phosphorylated directly by CDK2 in a cell cycle-dependent manner.

Identification of new polη phosphorylation sites

To try to identify the specific residues that were phosphorylated, Flag-HA-tagged polη (e-Polη) was stably expressed in HeLa cells. e-Polη was purified, excised from an SDS-PAGE gel (Figure 4A) and analyzed by MS/MS, leading to the identification of new putative phosphopeptides. The MS analysis confidently assigned a phosphorylation on S687 (Figure 4B, top panel), and another single phosphate on a peptide containing S510, S512, S514 (Figure 4B, bottom panel). In this case, it was not possible to pinpoint exactly which of the three closely clustered serines was modified. As can be seen in Figure 4C, the new phosphorylation sites lie toward the C-terminus of the polymerase with S510, S512, S514 located right after the catalytic domain while S687 resides in the Nuclear Localization Signal (NLS).

To verify that the phosphorylated peptides identified in the MS analysis were indeed phosphorylated, we established a series of XP30RO cell lines stably expressing alleles of the polymerases that were mutated at S687 and S510, S512, S514 (SSSAAA). All the alleles were tagged with eGFP, as we have previously shown that this tag did not

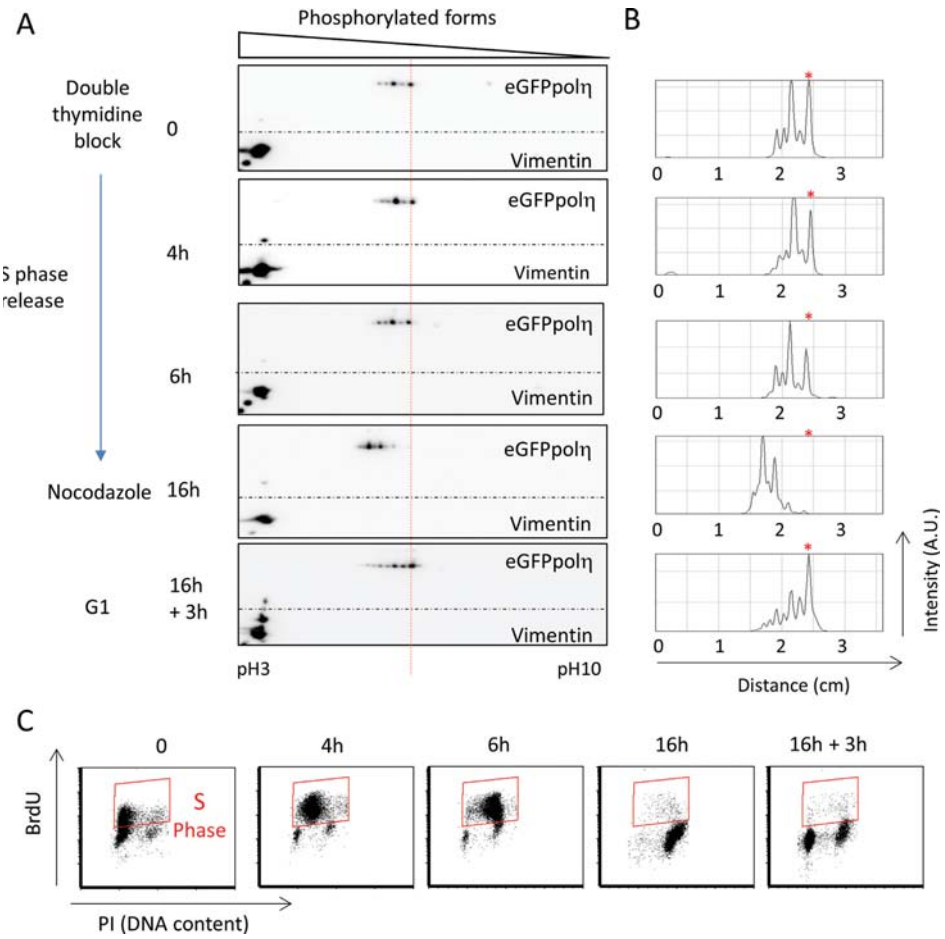

Figure 2. DNA polymerase η phosphorylation changes during an unperturbed cell cycle. XP30RO cells expressing eGFPpolη were synchronized by double thymidine block (0) before being released into S phase in the presence of nocodazole. After 16 h, the cells arrested in anaphase and they were subsequently released into the G1 phase of the next cell cycle (16 + 3). 2D-PAGE shows increasing amounts of phosphorylated polη during the transition from S to G2/M before a reduction in G1. The dotted line is used as reference of the alignment. (**B**) Densitometric analysis of the blots presented in A, the asterisk marks the reference peak as in A. (**C**) BrdU analysis of the experiment shown in panel A shows a synchronized progression through the cell cycle.

interfere with the protein catalytic activity (Supplementary Figure S3A), localization or function (4,17,23). We then performed a 2D-PAGE analysis to assess the phosphorylation pattern of the mutants. As can be seen in Figure 4D, both S687A and SSSAAA showed a marked reduction in the number of phosphorylated species, going from seven in the WT to four in each mutant.

Mutations in either of these residues did not completely abolish the phosphorylation of polη, indicating that S687 and S510, S512, S514 are phosphorylated independently and other phosphorylated forms of the polymerase are still present. Furthermore, the isoelectric point of the polymerase changed, with a new more basic spot appearing in the mutants. This could indicate that in the WT all of the polymerase is phosphorylated and the unphosphorylated form becomes evident only when some of the phospho-sites are mutated or the CDKs are inhibited (See Dinaciclib blot in Figure 3A and B).

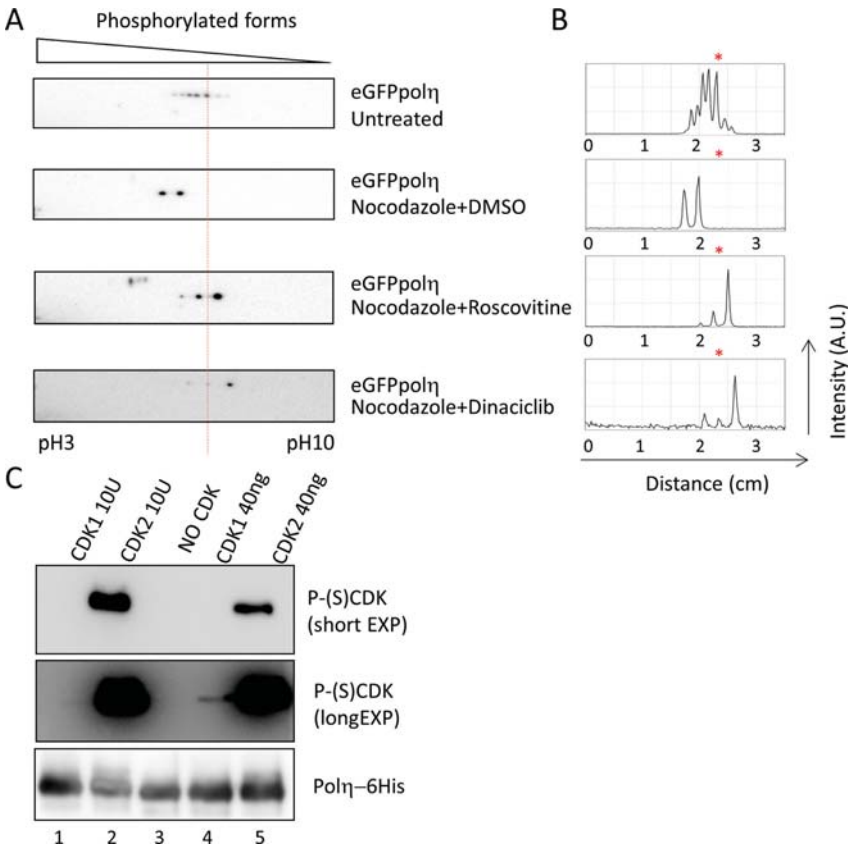

Figure 3. DNA polymerase η phosphorylation is dependent on CDKs. XP30RO cells expressing eGFPpolη were synchronized in anaphase by nocodazole before treatment with CDK inhibitors. (**A**) 2D-PAGE analysis of polη shows a reduction of phosphorylated forms after treatment with two pan CDK inhibitors (Dinaciclib and roscovitine). Gels were aligned by using Vimentin (shown) and PCNA (not shown) as first dimension migration markers. (**B**) Densitometric analysis of the blots presented in A, the asterisk marks the peak used as reference. (**C**) *In vitro* kinase assay with purified polη and purified CDK1 or CDK2.

Up to this point, we could successfully monitor the global phosphorylation status of polη, without being able to discern specifically the behavior of a specific residue. To overcome this limitation, we developed a phospho-specific antibody raised against P-S687. Unfortunately, we could not establish an antibody against the other residues due to the difficulty in pinpointing which serine was modified. The new antibody was able to detect phosphorylated polη specifically on S687 as can be seen in Figure 4E. We also verified that the signal was due to a phosphorylation modification as a signal was absent when polη was immunoprecipitated and treated with λ-phosphatase (Supplementary Figure S2, compare lanes 3 and 4 with 9 and 10). The development of this antibody gave us the opportunity to specifically study this modification *in vivo*.

S687 is a CDK target and is regulated during the cell cycle

As shown before, the phosphorylation of polη was affected by CDK inhibitors *in vivo* and was a direct target of CDK2 *in vitro*. The identification of new putative phosphorylated residues prompted us to examine if S687 and S510, S512, S514 were CDK targets *in vivo*. After IP, we were not able to detect a signal from the CDK phospho-specific antibodies when S687A was expressed, while it was unchanged in the SSSAAA mutant (Figure 5). This suggested that only S687 was a target of CDKs *in vivo*. It should also be noted

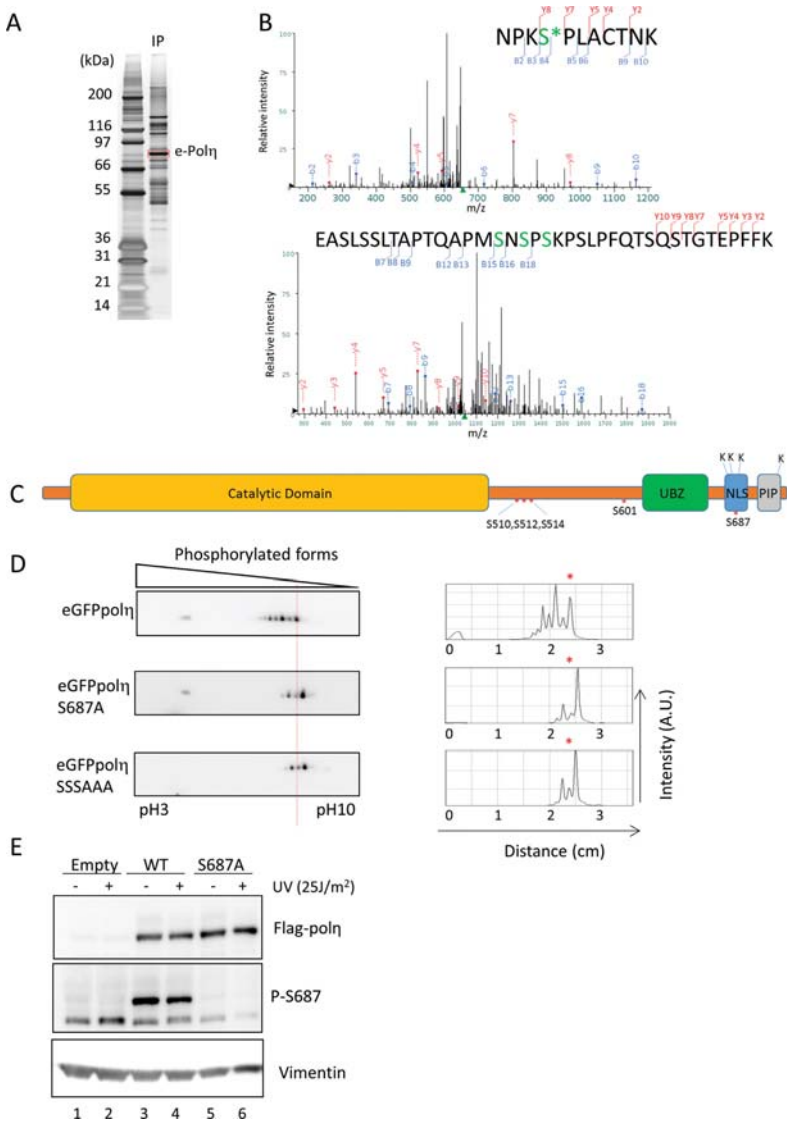

Figure 4. Identification of new phosphorylated residues in Polη. (**A**) e-Polη was purified and extracted from the gel for MS/MS analysis. (**B**) MS/MS spectra of the new phosphorylation sites of polη. B ions are marked in blue and Y ions are marked in red, both in the spectra and the peptide sequences. The top spectrum represents the peptide containing P-S687 (*site confidently assigned) while the bottom spectrum represents a peptide (aa 495–533) containing a single phosphate that could not be confidently assigned. Based on the fragmentation pattern the possible location of the phosphate was narrowed down to S510, S512 or S514. (**C**) Schematic representation of polη domains and alignment of newly identified phospho-residues. (**D**) 2D-PAGE analysis of XP30RO cells expressing eGFPpolη carrying S687A or S510A, S512A, S514A allele with respective densitometric analysis. The two mutant alleles show a reduced phosphorylation pattern. (**E**) Characterization of a new antibody against P-S687. Cells were transfected with Flag-polη either WT or S687A and probed with this antibody. Signal is present only in the case of the WT allele.

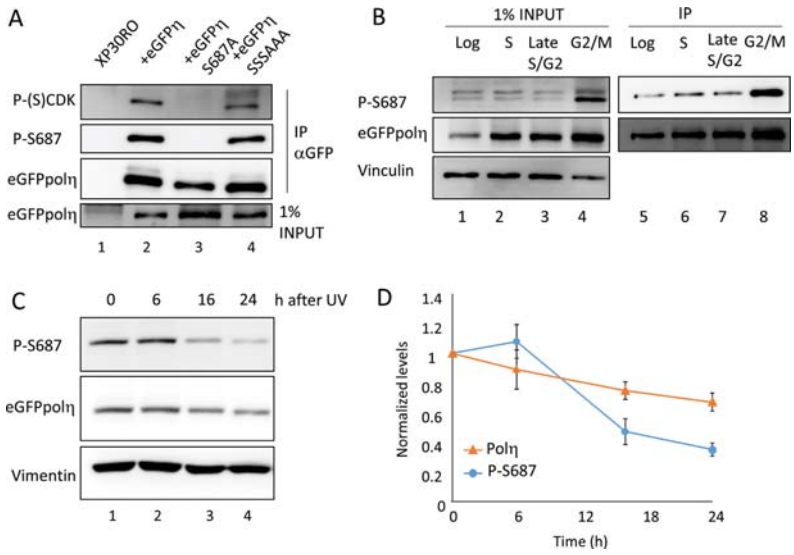

Figure 5. Serine 687 is phosphorylated by CDK and changes during the cell cycle and after UV irradiation. (**A**) eGFPpolη either WT, S687A or SSSAAA was immunoprecipitated from XP30RO derived cell lines and its phosphorylation status was analyzed with antibodies to P-(S)CDK and pS687. (**B**) XP30RO cells expressing eGFPpolη were synchronized by double thymidine block and released into the cell cycle in the presence of nocodazole, before IP of eGFPpolη. Polη and P-S687A increase in S phase and G2/M. (**C**) MRC5 cells were transfected with eGFPpolη and irradiated with 25 J/m^2 and followed for the indicated times. (**D**) Densitometric analysis of polη and P-S687 after UV irradiation. The signal of P-S687 was corrected for the levels of total polη. The plots represent the mean of three experiment ±S.E.M.

that the two phosphorylated regions are independently regulated as S687 is still phosphorylated in the SSSAAA mutant, indicating that the putative phosphorylation of S510, S512, S514 is not a prerequisite for the modification of S687 (Figure 5A, compare lane 2 with lane 4).

The phosphorylation of polη analyzed by 2D PAGE appeared to follow the progression of the cell cycle. To assess if S687 followed the same pattern and accounted for a part of the global cell cycle phosphorylation of polη we used the phospho-specific antibody to analyze polη in a single cell cycle after double thymidine synchronization. As can be seen in Figure 5B, phosphorylation of S687 peaks at the G2/M transition, with an 8-fold increase compared to the log phase sample, in a manner compatible with our previous observation (Figure 2A). Interestingly, we also detected a change in polη protein levels, with the polymerase being more abundant in S phase and G2/M than in the cells in log phase, where the G1 phase is more prominent.

As previously stated, polη is phosphorylated on S601 mainly after DNA damage. This holds true for both UV irradiation but also other DNA damaging treatments (5). In contrast, phosphorylation of S687 is detectable even in the absence of DNA damage and for this reason we speculated that it might be controlled in a different manner. To test the kinetics of phosphorylation of S687 after genotoxic insult we treated the cells with UV-C light and monitored the presence of P-S687 over time. In contrast to what was recently reported (11) while this paper was in preparation, P-S687 levels did not increase after damage but slowly diminished, being halved 24 h after irradiation (Figure 5C and D). This suggested a marked difference from P-S601. Interestingly the level of polη itself also changed slightly (Figure 5C and D) after UV damage in accordance with what was previously observed in *Caenorhabditis elegans* (8,24). It is important to mention that the signal of P-S687 shown in Figure 5D was corrected for the levels of total polη, so the decrease in phosphorylation could not be explained simply by the reduction in the total amount of the polymerase.

The phosphorylation sites are important for damage bypass and cell survival

To test the effect of the different newly discovered phosphorylated residues on TLS, we used a series of XP30RO cells stably expressing different alleles of polη. The cell lines expressed eGFPpolη WT, S601A, S687A and SSSAAA. The cells were then sorted to perform the experiments with cells expressing polη at the same, near physiological, level. To assess for TLS proficiency, we monitored the cells in a clonogenic survival assay after increasing doses of UV. XP30RO cells are extremely sensitive to UV irradiation followed by incubation in the presence of caffeine and this has been

used as a diagnostic assay for a number of years. As can be seen in Figure 6A, expression of WT eGFPpolη rescued the UV sensitivity. Consistent with our previous report (5) the S601A mutant shows a lower survival than WT polη. Mutating S687 to alanine resulted in similar sensitivity and the survival rates of S601A and S687A are nearly identical. The triple S510512514A mutant appears to show a milder phenotype, although rescue of the sensitivity to the WT level was not complete. It is important to note that neither the S601A nor the SSS mutant affected the levels of phosphorylation at S687, as can be seen in Supplementary Figure S2C, and the signal disappeared only when serine 687 was mutated to alanine. Conversely, the phosphorylation of S601 was not affected in the S687A and SSS mutants suggesting that phosphorylation of S687 and P-S601 are indeed independent events.

To complement this analysis we examined also the post-replicative repair activity of the different mutants by following the size of nascent DNA in the presence of DNA damage. UV irradiated cells were pulsed with 3H-thymidine to label newly replicated DNA, before being chased further without the radioactive nucleoside. The size of newly synthesized DNA was then determined by running the DNA on an alkaline sucrose gradient. XP30RO cells cannot bypass the damage hence the DNA is replicated in shorter fragments (right side of the plot in Figure 6B). Complementation of XP30RO with WT polη rescues this deficiency and the cells are able to bypass the damage, resulting in DNA of larger size (left side of the plot in Figure 6B). All the mutants analyzed showed an intermediate phenotype, indicating that the damage bypass was impaired, ultimately resulting in UV sensitivity as shown by the clonogenic survival. Note that the catalytic activity of polη is unaffected in these mutants. It remained comparable between all our polη constructs, as monitored by *in vitro* primer extension assays (Supplementary Figure S3A).

Polη phosphorylation controls protein stability

While performing 2D-PAGE experiments we noticed that the treatment with roscovitine made the visualization of polη difficult. This required longer exposures for detection of the polymerase by western blot. Initially we linked this phenomenon to a decrease in protein expression after treatment with the CDK inhibitor. Roscovitine, by inhibiting the phosphorylation of RNApolII, is known to affect the rate of transcription of a number of genes (25). In agreement with this, we observed a drop in the levels of polη mRNA to around 30–50% of the original levels within 6 h of incubation with roscovitine, as monitored by qPCR (Figure 7A). The protein levels of polη appeared to be affected even more, as the roscovitine treatment resulted in a 70% reduction after 3 h and more than a 90% decrease after 6 h (lane 2 and 5 respectively of Figure 7B). The substantial decrease of polη at 6 h indicated that the reduction in protein levels could not be explained only by a block of transcription. To test if protein degradation was involved in this process we combined the treatment with roscovitine with the proteasome inhibitor MG132. The concurrent incubation of MG132 and roscovitine led to a rescue of this phenomenon (compare lanes 4 and 7 with lane 1 Figure 7B) and we could observe a stabilization of polη, resulting in a 7-fold increase at 6 h, even when its phosphorylation was blocked (compare lanes 5 with lane 7 Figure 7B). During these experiments we did not observe a significant difference in the cell cycle distribution, so we could exclude the possibility that the effects we detected resulted from the cells accumulating in different phases of the cell cycle upon drug treatments (Supplementary Figure S4A).

During our studies we also found that the stable cell line carrying the S687A allele of polη expressed the transgene reproducibly at a lower level (approximately at 30% of WT) than the matching control (Figure 7C, lane 1 and 5). This was a feature that we observed in multiple independent clones (data not shown) even after cell sorting, suggesting that it was not due to the chromosomal locus where the transgene was integrated. This observation was again consistent with a possible role of the phosphorylation of S687 in controlling the polymerase stability. We reasoned that if that were the case, blocking the proteasome with MG132 should restore the protein levels of the S687A mutant to the levels of the WT, possibly with a faster accumulation kinetic after addition of the drug. As can be seen in Figure 7C, exposure to MG132 for just 1 h resulted in doubling the levels of the S687A mutant (compare lane 5 and 6) while very little effect was detected for the WT control (lane 1 and 2). After 6 h of proteasome inhibition, the mutant protein reached the same protein level as its matched control (compare lane 4 and 8).

These two pieces of experimental evidence point to an important role for the phosphorylation in controlling polη stability. We also showed that the polη protein fluctuates during a cell cycle (Figure 5B), increasing toward late S and G2/M phases, matching the pattern of appearance of S687 phosphorylation. If the increase in polη stability was due to the establishment of phosphorylation, we predicted that the S687A protein level would not change during a cell cycle. To test this hypothesis we synchronized the cells in early S phase by double thymidine block before releasing them. Again, the WT showed an increase of polη in S and G2/M before dropping in the G1 of the next cell cycle. Consistent with our hypothesis the S687A mutant showed (i) lower overall protein levels; (ii) hardly any fluctuation in polη during the cell cycle (Figure 7D and E). We conclude that phosphorylation of S687 is important for the regulation of polη protein stability throughout the cell cycle.

DISCUSSION

DNA polymerase η protects the genome by allowing the completion of replication during stress conditions and can bypass CPDs in an error-free manner. Regardless of this characteristic, polη is potentially an error prone polymerase and, along with the other TLS polymerases, it has to be correctly regulated. In yeast, part of the control of TLS is achieved by transcriptional regulation and protein stability (26,27). In human cells, the current working model is that TLS polymerases are controlled by modulating their access to the replication fork by protein–protein interactions and by PTMs. In the case of polη, the protein levels have been reported to be constant throughout the cell cycle (28).

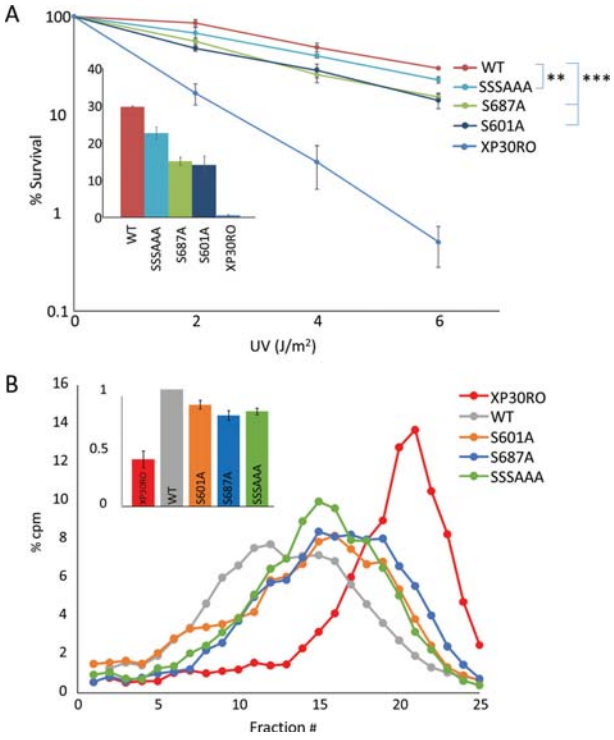

Figure 6. S687 is important for damage bypass and cell survival after UV. (**A**) Clonogenic survival of XP30RO cells expressing various mutant alleles of polη ($n > 3 \pm$ S.E.M). Statistical significance was assessed by *t*-test: $^{**}P = 0.0031$, $^{***}P < 0.0001$. The inset shows the survival of the cell lines at 6 J/m^2 on a linear scale. (**B**) Damage bypass assay using alkaline sucrose gradient fractionation of newly-synthesized DNA. The inset shows the normalized average molecular weight of DNA from the distributions ($n = 3 \pm$ S.E.M).

Here we show that polη is phosphorylated by CDK2 during an unperturbed cell cycle, with a peak in G2/M and this phosphorylation is important for regulating its stability and activity. We further identify the sites where this modification occurs and demonstrate that missense mutations at these sites affect TLS.

The identification of phosphorylation during an unperturbed cell cycle complements our previous observation that polη was phosphorylated on serine 601 after DNA damage (5). These lines of evidence suggests that phosphorylation plays an even more important role in the framework of TLS regulation. It is interesting to note that Polλ is phosphorylated in a similar manner and its phosphorylation on multiple sites by CDK2 protects it from ubiquitin mediated protein degradation in late S and G2 (29).

Increasing the levels of polη during S phase, with a peak after the bulk of DNA replication is completed provides an appealing form of control to avoid excessive recruitment of a potentially inaccurate polymerase during replication (Figure 8). On the other hand, once the bulk of DNA replication finishes and any remaining gaps in the DNA become a threat to cell survival, increasing the amount of polη would improve the cell's ability to bypass the damaged bases and complete DNA replication. In this situation the presence of polη provides a crucial viability advantage even if the polymerase is inaccurate and could generate mutations in the genome. This model of action is in agreement with observations in yeast and mammalian cells, which showed that TLS was able to function also in late G2 and was not limited to the replicating phase (28,30).

The demonstration that cell-cycle phosphorylation of polη peaks in G2/M suggests a molecular mechanism for this kind of regulation. Interestingly Rev1, another TLS polymerase, has also been found to accumulate in G2 in *S. cerevisiae* (26).

Recently Dai *et al*. identified the phosphorylation of Serine 687 of polη by immunoprecipitating Flag-tagged polη followed by MS (11). They detected a time-dependent in-

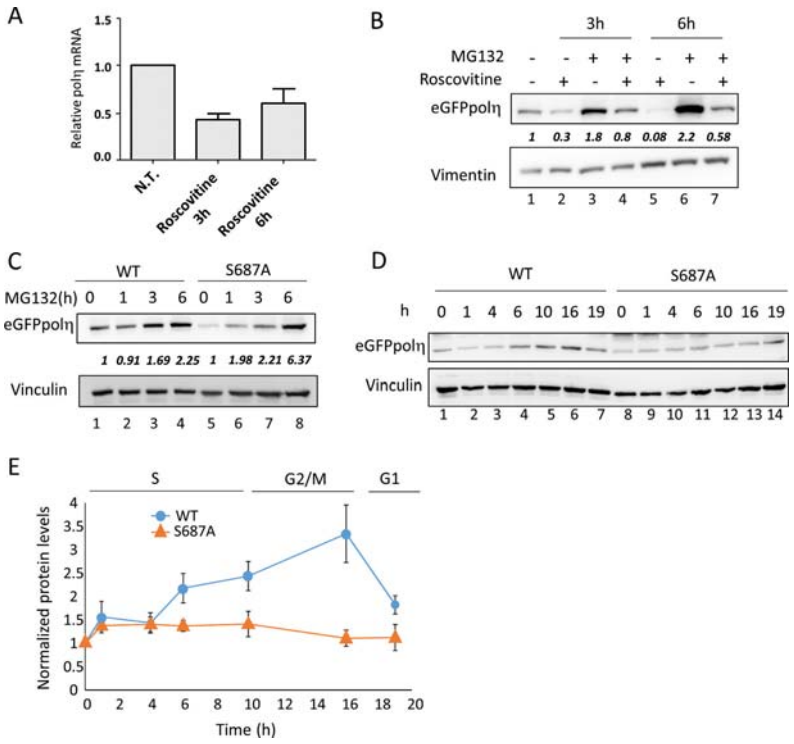

Figure 7. Phosphorylation of polη is important for its stability. (**A**) Transcript levels of polη after CDK inhibition. (**B**) Polη protein levels after roscovitine treatment, in the presence of MG132. Relative protein levels normalized to the untreated sample are displayed in red under the eGFPpolη blot. (**C**) XP30RO cells expressing eGFPpolη WT or S687A were incubated with MG132. Relative protein levels normalized to the 0 h sample of each allele are displayed in bold italic under the eGFPpolη blot. (**D**) XP30RO cells expressing eGFPpolη WT or S687A were synchronized by double thymidine block and followed in the cell cycle after release in nocodazole for 16 h, before final removal of the drug for 3 h. (**E**) Densitometric analysis of polη levels, either WT or S687A, during the cell cycle (mean of three experiments ± S.E.M).

crease in P-S687 after UV irradiation and they speculated that P-S687 mediated the release of the polymerase from PCNA after damage bypass was completed. This model originated from the observation that a C-terminal fragment of polη carrying a S687D mutation had lower affinity for PCNA *in vitro*. By using a phospho-specific antibody we have shown instead that P-S687 decreases over time after UV (Figure 5). One possible explanation for this discrepancy lies in the fact that Dai *et al*. used nocodazole in some of their experiments after UV irradiation, thus increasing the percentage of cells in G2/M. As we have shown, in G2/M the phosphorylation of S687 increases substantially in an unperturbed cell cycle so the compounded effect of UV and nocodazole treatments may have masked the decrease we have observed and resulted in a net apparent increase of P-S687. Furthermore, we failed to observe any difference in binding to PCNA *in vitro* between full length polη and polη phosphorylated on S687 (Supplementary Figure S3B). Both studies suggest that CDK2 is involved in polη phosphorylation and that S687 could be important for ending TLS activity after damage is bypassed.

The C-terminus of polη contains a number of important regulatory domains such as the NLS (aa 682–694), the UBZ (aa 633–654) and the PIP (aa 701–708). Furthermore, following DNA damage polη is phosphorylated on S601 by ATR. Serine 687 is located in the region that is also referred to as PIR that spans from the canonical PIP to the NLS and contains four lysines that can be ubiquitylated in the absence of DNA damage (4). We confirmed that the S687A mutation did not impair the correct localization of the polymerase (data not shown) and polη appears to be ubiquitylated normally (see Supplementary Figure S2C). Our analysis of P-S687 enables us also to propose that S687 is likely phosphorylated on the diffusing fraction of the polymerase,

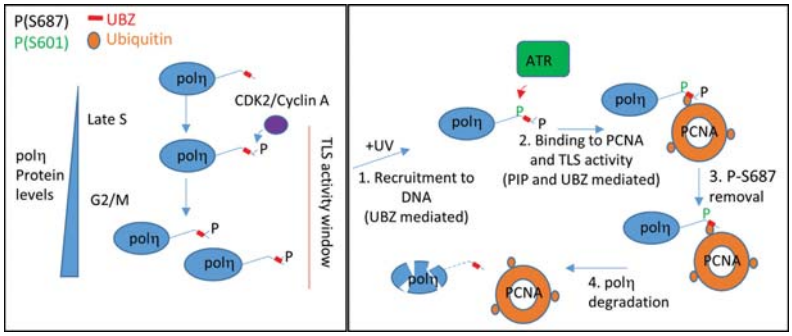

Figure 8. Proposed model of phosphorylation-controlled TLS. During an unperturbed cell cycle polη becomes phosphorylated by CDK2 on Serine 687 from late S to G2/M. This results in the accumulation of the polymerase, which is available for bypass activity (left panel). After damage, phosphorylated polη is recruited to the chromatin via its UBZ (1) and it is further phosphorylated on S601 by ATR. Once S601 has been phosphorylated, polη can interact with Ubiquitylated PCNA and perform TLS (2). Once the damage has been bypassed P-S687 is removed (3) leading to polη degradation (4).

for two reasons: (i) phosphorylation occurs in the absence of DNA damage when the majority of polη is not associated with DNA. (ii) The phosphorylation is not altered in the UBZ mutant (not shown) where polη recruitment to the chromatin is severely hindered. Both in *C. elegans* and mammalian cells, polη appears to be degraded after UV (8) in order to keep the TLS polymerase engaged at a minimum. In *C. elegans*, SUMOylation of polη protects it from degradation by CUL4/Cdt2 and it is removed after damage bypass, leading to removal of the polymerase. In human cells, SUMOylation of polη has been recently identified but with a completely different function (9). Our data suggest that phosphorylation could play a role similar to SUMOylation in *C. elegans*, as S687 defective alleles show enhanced degradation. Furthermore after UV, P-S687 is removed with kinetics similar to that of polη degradation. Serine 687 thus provides an appealing candidate to play the role of regulator of polη, making the polymerase available during the cell cycle and protecting it in the first few hours after DNA damage. In these first crucial moments, polη, stabilized by the phosphorylation of S687, is recruited to DNA via its UBZ. Once on the chromatin, ATR can phosphorylate S601 on polη and the polymerase can interact with ubiquitylated PCNA becoming proficient for damage bypass. Once TLS has been completed P-S687 is removed and this in turn leads to a decrease in polη that could favor resumption of normal replication (Figure 8). How the phosphorylation regulates degradation is an open question.

In recent years, several lines of evidence suggest that polη is important for the bypass of difficult to replicate templates, such as common fragile sites (CFS), even in the absence of damage (31,32). CFS are replicated in late S phase and a large percentage are still unreplicated as the cell enters G2 (33,34). The replication timing of CFS strikingly matches the accumulation of polη and it is tempting to speculate that the accumulation we observe of the polymerase in G2/M could be linked with its CFS bypass role.

It is also interesting to note that damage bypass is impaired *in vivo* in the phosphorylation mutants, although their catalytic activity is unaffected *in vitro*. As all the functional assays were performed after cell sorting, we can exclude the possibility that the phenotype is merely dependent on reduced protein levels, as the cells expressed the different alleles at similar levels right after sorting, comparable to endogenous polη in MRC5 cells (Supplementary Figure S4B).

Our results complement a recent finding that unveiled how the C-terminus of polη was important for protein stability, by analyzing a mutant allele derived from an XPV patient (10,35). One of the mutations present in XP872VI was a T692A transversion in addition to a STOP codon loss that resulted in a protein with eight extra aminoacids (35). Threonine 692 is located just outside the CDK2 consensus and still in the polη NLS. It is plausible that part of the enhanced degradation of the allele resulted from its defective phosphorylation, further supporting the physiological importance of serine 687 and phosphorylation in general in controlling TLS.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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