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Regulation of translesion DNA synthesis: Posttranslational modification of lysine residues in key proteins

Justyna McIntyre[a,*], Roger Woodgate[b]

[a] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Pawinskiego 5a, 02-106 Warsaw, Poland
[b] Laboratory of Genomic Integrity, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-3371, USA

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ABSTRACT

Posttranslational modification of proteins often controls various aspects of their cellular function. Indeed, over the past decade or so, it has been discovered that posttranslational modification of lysine residues plays a major role in regulating translesion DNA synthesis (TLS) and perhaps the most appreciated lysine modification is that of ubiquitination. Much of the recent interest in ubiquitination stems from the fact that proliferating cell nuclear antigen (PCNA) was previously shown to be specifically ubiquitinated at K164 and that such ubiquitination plays a key role in regulating TLS. In addition, TLS polymerases themselves are now known to be ubiquitinated. In the case of human polymerase η, ubiquitination at four lysine residues in its C-terminus appears to regulate its ability to interact with PCNA and modulate TLS. Within the past few years, advances in global proteomic research have revealed that many proteins involved in TLS are, in fact, subject to a previously underappreciated number of lysine modifications. In this review, we will summarize the known lysine modifications of several key proteins involved in TLS; PCNA and Y-family polymerases η, ι, κ and Rev1 and we will discuss the potential regulatory effects of such modification in controlling TLS *in vivo*.

1. Introduction

Posttranslational modifications (PTMs) of proteins by attaching different functional groups to amino acids widens the target protein's range of function and provides additional mechanisms by which the modified protein can be regulated. For example, PTMs can control a protein's activity by influencing its ability to interact with protein-partners, alter its enzymatic activity, subcellular localization, and change the stability of the protein. Of all the experimentally identified PTMs in mammals, serine phosphorylation is the most frequent modification followed by lysine, which represents over 15% of all experimentally identified amino acid modifications (calculation based on data from [1]). Lysine can be modified in a variety of ways including, but not limited to: ubiquitination, ubiquitin-like protein (UBL) modification *e.g.* SUMOylation, ISGylation, neddylation, FATylation and other lysine modifications such as acetylation, methylation, butyrylation, crotonylation, glycation, malonylation, phosphoglycerylation, propionylation, succinylation, myristoylation [1–4].

Eukaryotic cells have evolved a plethora of mechanisms in order to protect genome stability by removing DNA lesions, or preventing their conversion into permanent mutations [5]. Importantly, due to partially overlapping functions of some of these pathways, or time and conditional cellular requirements, their actions need to be precisely controlled. Recent studies in the DNA repair field have accumulated evidence of an ever expanding role of ubiquitination in regulating diverse DNA repair mechanisms and pathways involved in genomic stability maintenance (reviewed in [6]). Ubiquitin- and ubiquitin-like-dependent signaling processes have an important function in controlling cellular responses to DNA damage by navigating through the range of DNA damage repair, or tolerance mechanisms (reviewed in [6–10]). The majority of DNA lesions are repaired by one of the specialized DNA repair pathways; however the repair processes can be slow and incomplete and as a consequence a number of DNA lesions remain in the template DNA. This causes a severe problem, especially during the S-phase of the cell cycle, when DNA is replicated, because efficient and accurate classical DNA polymerases are blocked at DNA lesions. At this critical juncture, distinct mechanisms are required to temporarily

Abbreviations: TLS, translesion synthesis; PCNA, proliferating cell nuclear antigen; pol, polymerase; PIP, PCNA-interacting peptide; RIR, Rev1-interacting region; UBM, ubiquitin binding motif; UBZ, ubiquitin binding zinc motif; polι, DNA polymerase iota; polη, DNA polymerase eta; polκ, DNA polymerase kappa; UBL, ubiquitin-like protein; PTM, posttranslational modification; PRR, post replication DNA repair.

* Corresponding author. Tel.: +48 22 5921115.
E-mail address: justyna@ibb.waw.pl (J. McIntyre).

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tolerate cellular DNA damage, thereby avoiding the permanent block to the replication fork and the threat of cell cycle arrest. Lesion tolerance can be achieved in two different ways; one *via* a damage avoidance pathway using the information from the undamaged sister chromatid as a template for replication of the damaged DNA region, or *via* translesion synthesis (TLS), which employs specialized DNA polymerases to synthesize past the lesion.

Over the past dozen years, it has become evident that modification of lysine residues through the covalent linkage of ubiquitin, or ubiquitin-like proteins, plays a central role in controlling both DNA damage avoidance mechanisms and TLS. This review will attempt to summarize the known sites and cellular effects of ubiquitination of several key proteins involved in TLS. We will recap the individually discovered and experimentally confirmed sites of ubiquitination and ubiquitin-like modifications of TLS proteins and combine them with recent data derived from multiple proteome-wide approaches that reveal a hitherto underappreciated extent of lysine ubiquitination of many of the TLS proteins.

2. Types of lysine modifications

2.1. Ubiquitination

In eukaryotic cells, ubiquitination is involved in the regulation of almost all cellular processes, including cell division, membrane transport, signal transduction, DNA repair, endocytosis, inflammatory signaling, apoptosis, *etc.* [11–14]. It has been estimated that roughly 10% of human genes encode for proteins involved in ubiquitin metabolism [15]. The malfunction of ubiquitination processes and ubiquitin-mediated proteolysis has been implicated in various pathologies, including neurodegenerative disorders, inflammatory diseases and cancers [16–19]. Due to their important cellular functions, ubiquitination pathways are significant targets for therapeutics [20,21].

Protein ubiquitination is a dynamic and reversible process where a three-step enzymatic cascade conjugates a small, regulatory protein, ubiquitin, to a specific lysine residue in a target protein [22]. Initially, one of the ubiquitin-activating enzymes (E1s) forms an ATP-dependent thioester bond with ubiquitin. The activated ubiquitin is then transferred from the E1 enzyme to one of multiple ubiquitin-conjugating enzymes (E2s). E2 then transfers the activated ubiquitin to a protein substrate, either by itself, or with the help of one of the many ubiquitin ligases (E3s). Ubiquitin is linked through its C-terminal glycine residue to a specific internal lysine residue of the target protein. It has been also shown that in some proteins, ubiquitin can be attached to the N-terminus of the protein and in rare cases to a serine, threonine or cysteine residue [23–25]. Monoubiquitinated substrates can undergo further ubiquitination [26–28]. There are seven lysine residues in ubiquitin; K6, K11, K27, K29, K33, K48 and K63; all of them can be involved in polyubiquitin chain assembly. Additionally linear N-terminal polyubiquitin chains can also be formed [29]. Depending on the type of ubiquitin-chain linkage, polyubiquitinated proteins might be destined for degradation by the 26S proteasome in an ATP-dependent manner or alternatively, polyubiquitination might provide a signal for distinct cellular processes such as the inflammatory response or DNA repair [10]. Chains that are linked through K48 are the principal signal for degradation by the proteasome [30,31]. Recent studies, based on mass spectrometry have shown that homogeneous chains consisting of K29, K11, K27 and K6-linkages, heterogeneous chains with mixed lysine linkages, as well as multiple nearby monoubiquitination and, in cases of substrates up to 150 amino acids, even monoubiquitination can promote proteasomal degradation [32,33]. Chain elongation of ubiquitinated substrates is mediated *via* another class of ubiquitin ligases, E4s [34,35]. Ubiquitination can be reversed through the activity of de-ubiquitinating enzymes (DUBs), which primarily disassemble polyubiquitin chains before protein degradation, but will also cleave off a single ubiquitin moiety, or a polyubiquitin chain to regulate protein functionality [36].

2.2. Ubiquitin-like posttranslational modification

Besides ubiquitin, at least 10 different ubiquitin-like proteins (UBLs) exist in mammals (reviewed in [37,38]) with SUMO, NEDD8 and ISG15 being the best known. UBL modifiers, similar to ubiquitin, form an isopeptide bond between their C-terminal glycine and lysine residues of the substrate [38]. UBLs often have low sequence homology, but share a similar three-dimensional structure [38]. Posttranslational modification with UBL proteins can alter cellular function, stability, interactions with protein partners, or subcellular localization of the target protein [37,39]. Protein modification by UBLs follows the same three-step cascade similar to ubiquitination in that it is catalyzed by sets of analogous activation (E1), conjugation (E2s) and ligation (E3s) enzymes and can be reversed by deconjugating enzymes [40].

SUMO (Small Ubiquitin-like MOdifier) is the most studied UBL modifier and is expressed in all eukaryotes, mainly as a single variant. However in human cells there are four different paralogs (SUMO1–4), representing various homology, expression levels and substrate preferences. Many proteins interacting with a SUMOlyated substrate possess specific SIM domains (from SUMO-interaction motif) [41]. SUMOylation of a target protein can influence the protein degradation, signal transduction, localization, transcription activation, cell cycle, chromatin organization, DNA repair and other functions (reviewed in [42]). Dysfunction of SUMOylation can lead to neurodegenerative diseases, heart defects, diabetes or cancer [42–45].

One ubiquitin-like molecule, ISG15 (the interferon-stimulated gene 15), has a primary sequence that consists of two domains with significant similarity to ubiquitin [46]. Interestingly, ISGylation shares some of the E2 and E3 enzymes used in ubiquitination and ISGylated proteins can also be targeted for degradation by the 20S proteasome [47,48]. ISG15 is only found in vertebrates. Type I interferon, viral infection, lipopolysaccharides and some types of genotoxic stress can rapidly induce ISG15 conjugation [49,50] and it has been shown that enhanced ISGylation correlates with carcinogenesis [51].

Another example, NEDD8 (neural precursor cell-expressed developmentally downregulated-8), shares 60% identity and 80% homology with ubiquitin [52], and as a consequence, it can be incorporated into polyubiquitin chains by the E2 and E3 ubiquitin-conjugating enzymes [53]. The best characterized substrates known to be neddylated are cullins, scaffold proteins of SCF ubiquitin ligases (Skip-1, cullin, F-box) which regulate ubiquitination and proteasomal degradation of proteins involved in cell cycle control, transcriptional regulation, signal transduction [37,54]. Other, non-cullin neddylation substrates include proteins involved in RNA splicing, DNA replication and repair and proteasomal degradation [55].

3. Identifying ubiquitination and UBL modification sites

The identification of lysine residue(s) to which ubiquitin, or UBL proteins are conjugated, is important for understanding its biological significance. Locating ubiquitination, or UBL sites, can be performed experimentally, using conventional approaches, such as site-directed mutagenesis of a potentially modified residue [56,57], or by using antibodies against ubiquitin, or UBL proteins [58,59]. Recently, however, high-throughput methods and

mass-spectrometry have also been frequently employed [60–67]. A collection of experimentally determined proteins which can be ubiquitinated and/or UBL-modified proteins in which the modified residues have been verified have been assembled into several searchable databases, such as UbiProt (http://ubiprot.org.ru/) [68], SCUD (http://scud.kaist.ac.kr) [69], for ubiquitin modification and SysPTM (http://www.sysbio.ac.cn/SysPTM) [70] and CPLM (http://cplm.biocuckoo.org/index.php) [71] for general PTMs.

3.1. Prediction of ubiquitination and UBL sites

The experimental detection of the potential sites of modification is time- and labor-intensive, which is why multiple efforts have been undertaken to computationally predict protein ubiquitination and UBL sites [72–78]. Generally, they all are based on analysing features of experimentally verified ubiquitination sites. The datasets are used for training *via* various algorithms and tested for their prediction ability. Catic and co-workers discovered that ubiquitination sites are preferably exposed at the molecular surface and reside in loop regions [72]. Kim et al. suggest the ubiquitination sites localize within a net negative charge [62]. The analysis of Radivojac et al. calculated 586 sequence attributes for each lysine of the positive and negative datasets and demonstrated that the ubiquitination sites are often located in intrinsically disordered regions [74]. This is in contrast to predictions made based on the correlation of ubiquitination with protein structure performed by Walsh et al. [78] and might be a result of different data sources (*Saccharomyces cerevisiae vs. Homo sapiens*). These findings generally indicate that ubiquitination site preferences seem to be poorly conserved across different species [61,76,77], therefore organism-specific predictors should be used for optimal results.

Several ubiquitin site predictors, such as UbiPred [73], UbPred [74] and CKSAAP_UbSite [76], trained on yeast datasets, have been developed. However, quite recently, some predictors have become available exclusively for mammalian sites, such as hCKSAAP_UbSite [77], UbiProber [79] and RUBI [78].

For SUMOylation, a consensus motif has been determined as ψKxD/E [80] (where ψ is a hydrophobic residue I, L, M, P, F, V or W and X is any residue). However, this motif is not very precise; about 40% of known SUMOylation sites do not match the consensus, and no detectible SUMOylation was found in some proteins having the ψKxD/E motif [81]. In the last decade, several methods have been used to generate SUMOylation site predictors, which include GPS, MotifX, SUMOsp, SUMOsp 2.0; seeSUMO, SUMOplot, SUMO-hydro and SUMmOn among others [81–87]. SUMOylation frequently appears to be site-specific, thus prediction programs recognize SUMO-modified sites primary on amino acid sequence information.

The high specificity and sensitivity desired in the computational determination of ubiquitination and UBL sites remains a challenge. In a recent paper from Schwartz, that assessed the different available predictors, it was pointed out that 9 out of 11 predictors perform no better than random, or unseen data [88], demonstrating that at the present time, bioinformatic analysis alone appears to be insufficient in confidently identifying *bona fide* sites of ubiquitination or SUMOylation. In short, ubiquitin and UBL sites still need to be determined experimentally.

3.2. Proteome-wide profiling of ubiquitin and UBL modification

The need for the large-scale detection of modified proteins has been recognized for a long time and within the past several years the scientific community has witnessed an explosion in the global identification of ubiquitinated proteins and sites and to a lesser extent, UBL modification [61–67,89–96]. Major progress has been made possible due to the development of new affinity purification tools and the improvement of mass spectrometers with increased speed, resolution and accuracy, together with a decrease in false-positive recognition [97–99].

The abundance of modified proteins is usually too low to be directly detected by mass spectrometry, therefore a variety of methods that enrich modified proteins has been employed. The strategies for enriching substrates for high-throughput ubiquitination site recognition include the usage of epitope-tagged ubiquitin expression system [61,91,94,100,101] and the employment of specific ubiquitin binding domains and antibodies against ubiquitin or peptides containing a K-ε-GG remnant that is created by tryptic digestion of a ubiquitinated protein [93,102–104]. All of the enrichment methods, however, carry some limitations that might lead to artifacts that one cannot exclude during analysis. For example, in a system using epitope-tagged ubiquitin, modified proteins can be purified under denaturing conditions to help reduce false positive identification. On the other hand, exogenously overexpressed tagged ubiquitin might hinder the kinetics of ubiquitination reactions, as well as hamper the formation of linear ubiquitin chains and consequently interfere with the cellular functions of modified proteins [105,106]. The employment of tandem ubiquitin binding domains (UBDs) and ubiquitin antibodies circumvents this problem, but there is an increased risk of co-purifying contaminant proteins or a bias toward a specifically modified substrate (linkage specific polyubiquitinated or monoubiquitinated proteins) [90,91,107,108]. Both problems are circumvented by using the K-ε-GG remnant antibodies developed by Xu et al. [102], as they do not isolate the entire substrate, but rather a short peptide with the glycine–glycine signature obtained after tryptic digestion of a ubiquitinated substrate. This method is highly efficient and has been widely used for ubiquitination site profiling [62–65,89,92,109]. It is noteworthy that less abundant ubiquitin-like modifications, such as neddylation and ISGylation, also generate the same di-GG signature after trypsinization and the di-GG antibodies do not discriminate them from ubiquitination [62,110]. The COFRADIC technology, recently developed by Stes et al. provides an alternative strategy for ubiquitination studies and does not require overexpression, epitope tags or specific antibodies [110].

To date, literally hundreds of mammalian proteins have been described as SUMOylation targets, however precise identification of SUMOylated sites remains challenging due to low abundance of modified proteins *in vivo* and the dynamic character of SUMOylation [95,111–113]. Several attempts toward large-scale identification of SUMOylation sites have been undertaken. Most methods employ recombinant SUMO in *in vitro* or *in vivo* SUMOylation assays followed by antibody- or epitope-based purification, digestion by proteases and mass-spectrometry analysis of the peptide [94–96,112,114–117]. The remnant of SUMO proteins left on the modified lysine residue after digestion with trypsin is relatively long (19–32 amino acids depending on the SUMO isoform) leading to a complex fragmentation pattern which obstructs precise identification of the SUMOylated site. To overcome this problem and generate shorter SUMO isopeptide fragments, convenient cleavage sites were introduced into the SUMO protein allowing for the generation of a specific di-GG signature after tryptic digestion [66]. 84 out of 103 identified SUMO sites were located in direct, or an inverted SUMO consensus sequences and out of the 16 proteins that were identified, a new SUMOylation motif HCSM (Hydrophobic Cluster SUMOylation Motif) was identified.

Additionally, Tammsalu et al. identified over 1000 SUMOylation sites within 539 human proteins involved in cell cycle regulation, transcription and DNA repair. By using a His-tagged SUMO2 with a T90K mutation, they obtained a di-GG remnant after endoproteinase cleavage and the SUMO-enriched peptides were subsequently analyzed by mass spectrometry [67]. Very recently, Hendriks et al. identified 4361 SUMOylation sites in 1606

proteins in human cells, both under normal growth conditions and in response to heat shock stress, as well as to SUMO protease and proteasome inhibition [96].

TAP-tagged and GST-tagged Nedd8 were respectively used to identify 75 and 496 neddylated proteins by Xirodimas et al. and Jones et al. [55,118]. Various cullin and non-cullin neddylation substrates were discovered including proteins involved in RNA splicing, DNA replication and repair and proteasomal degradation [55,118].

There are limited reports of large-scale identification of ISGylated proteins. By using high-throughput immunoblotting, for example, Malakhov et al. discovered 76 ISG15 substrates involved in translation, glycolysis, stress response and cell motility [59]. Similarly, by using affinity selection and mass spectrometry Zhao et al. identified 158 proteins ISGylated in response to interferon and functioning in diverse cellular processes [119].

In summary, the wide-assortment of multiple large-scale analyses has identified thousands of ubiquitination and UBL modification sites in numerous proteins. However, the large amount of data collected poses a huge challenge to validate the global proteomic studies. As discussed above, different strategies raise a variety of technical and analytical issues, such as contaminant protein recognition, pseudo modification, and false-positive assignments. Various algorithms may also have different levels of sensitivity and specificity that may bias the data obtained [105]. Therefore, individual examination and critical evaluation is essential to confirm global modification site profiling.

4. Post replication DNA repair (PRR) pathway

DNA damage tolerance pathways allow for temporal acceptance of the presence of DNA lesions in the genome when there is a risk of cell death. The most frequent and deleterious are DNA lesions that arise during the DNA replication. Very efficient and extremely faithful polymerases guarantee fast and accurate DNA duplication during S-phase. However, any distortion in the DNA structure can hinder this proficient process and cause the replication fork to stall. If stalled for too long, the replication fork can collapse, generating DNA double strand breaks that lead to genome instability. It is therefore critically important to resume replication even in the face of persistent DNA damage [120,121]. Based on genetic studies, PRR utilizes two major mechanisms that allow blocked replication to resume. The first is indirect bypass using DNA damage avoidance mechanism and the second is DNA translesion synthesis (TLS). The mechanism of DNA damage avoidance is not clear, however it is thought to involve template switching where the undamaged sister chromatid is used as a temporary replication template or homologous recombination [122,123]. In contrast, during TLS, the highly precise and efficient DNA replicases that are blocked by a DNA lesion are replaced by specialized, low-processivity TLS polymerases that are able to carry out DNA synthesis past the damaged site. These TLS DNA polymerases can either bypass the lesion unassisted, or with the help of another TLS polymerase in a two-step process [124].

4.1. PCNA modifications

It should be noted that in budding yeast, the decision as to which DNA damage tolerance pathway will be undertaken to rescue a stalled replication fork depends on the type of posttranslational modification to proliferating cell nuclear antigen (PCNA), which is a central player in the PRR pathway.

PCNA, a replication processivity factor, is a ring-shaped homotrimeric complex which encircles double stranded DNA and slides along the DNA [125]. The monomers, each comprising two structurally similar domains, are linked in head-to-tail mode. PCNA monomers interact with DNA through their DNA binding motifs (61–80 residues) located on an internal surface. On the outer surface, the N- and C-terminal halves of PCNA are linked by the interdomain-connecting loop (IDCL) positioned above a hydrophobic pocket that provides a docking site for the PCNA-interacting peptide (PIP) motif of proteins that interact with PCNA [126]. PCNA interacts with multiple proteins involved in replication, cell cycle regulation and DNA repair and coordinates their access to replication forks (reviewed in [127,128]).

In response to replication fork stalling, PCNA undergoes monoubiquitination at K164 by Rad6 and Rad18 (E2-ubiquitin conjugation and E3-ubiquitin ligase enzyme, respectively) [129,130]. Monoubiquitinated PCNA interacts with TLS polymerases *via* their ubiquitin binding domains (UBDs), thereby activating the TLS pathway. Rad6-Rad18 is the main source of PCNA monoubiquitination, though some residual, conditional ubiquitination can be observed in yeast and chicken cells lacking Rad6 or Rad18 [131–133]. There are also reports that human PCNA can be monoubiquitinated by $CRL4^{Cdt2}$ or Rnf8 ubiquitin ligases [134,135]. PCNA ubiquitination is reversible and modified PCNA can be deubiquitinated *via* USP1 or BPLF1 (only in human cells) [136–139].

PCNA that is monoubiquitinated at K164 can undergo further ubiquitination. In budding yeast, K63-linked polyubiquitin chains, catalyzed by the Ubc13-Mms2/Rad5 E2-E3 enzymes, promote template switching [130]. In human cells, there are two Rad5 homologues, HLTF and SHPRH [140–143] serving as the E3 ligases for K63-chain formation. Like yeast Rad5, they both interact with RAD6/RAD18 and MMS2/UBC13 complexes [140,141], however, their role is not fully understood and their function in both damage avoidance and TLS sub-pathways of PRR have been suggested [144]. Furthermore, Krijger et al. suggested the existence of yet another E3 ligase, as PCNA polyubiquitination was observed in HLTF/SHPRH double mutant mice [145]. An additional difference in PCNA polyubiquitination between lower and higher eukaryotes regards a requirement for MMS2, as the protein seems to be dispensable for this process in mammalian cells [146]. PCNA polyubiquitination, similar to PCNA monoubiquitination, is negatively regulated by USP1 [138,141].

Human PCNA is a stable protein with an estimated half-life of over 20 h. The stability can be attributed to proteins such as MUTH2, ERK8 and NRAGE, which protect PCNA from polyubiquitination by blocking proteasome degradation signaling through K48- or K11-linked chains [147–149].

Yeast PCNA can also be ubiquitinated at K107 in response to replication stress caused by the presence of unprocessed Okazaki fragments in ligase-deficient cells. K107 monoubiquitination signals checkpoint activation and both mono- and K29-linked polyubiquitination on K107 involves Mms2, Ubc4 and Rad5 [150]. Human PCNA is also ubiquitinated in ligase I depleted cells, but the modified residue has not yet been identified [150].

In addition to ubiquitination, PCNA can be modified by SUMO. Initially, this modification was identified in yeast, followed by a handful of other species including *Xenopus* and chicken DT40 cells [129,131,151]. Recently, a low level of SUMOylation of human PCNA has also been reported [152,153]. SUMOylation is a reversible process and Ulp1 hydrolase removes SUMO from PCNA [154].

In budding yeast, PCNA SUMOylation occurs mostly during S-phase progression and, by influencing PCNA interactions with various partners, controls DNA replication and repair. PCNA SUMOylation promotes the binding of the Srs2 helicase, an inhibitor of recombination and thereby prevents unwanted recombination events at the replication fork [155]. Kim et al. showed that the non-canonical Srs2 PIP box has relatively low affinity for unmodified PCNA but it enhances significantly upon PCNA SUMOylation [156]. Another interaction involving Elg1, the large subunit of an

alternative clamp loading complex, implies the involvement of SUMOylation of PCNA in its unloading from DNA in yeast cells [157,158]. Conversely, ATAD5, a human homologue of yeast Elg1, does not seem to have a preference for unloading SUMOlyated PCNA despite possessing a SUMO interacting motif [159,160]. On the other hand, it has been shown to be involved in deubiquitinating PCNA by recruiting the USP1 complex to ubiquitinated PCNA [155]. PCNA SUMOylation in yeast cells has also been shown to inhibit Eco1-PCNA-dependent sister chromatid cohesion [161].

Yeast PCNA can be SUMOylated on K164 and to a lesser extent on K127, with the involvement of SUMO conjugating and ligating E2-E3 enzymes, Ubc9 and Siz1 (K164), or just Ubc9 itself (K127) [129]. Gali et al. showed that hPCNA can be SUMOylated on K164 and K254 which prevent DSB formation and inappropriate recombination in response to replication fork arrest by DNA lesions [152]. In another study, the human analog of Srs2, PARI, was shown to promote the interaction with SUMOylated PCNA, correspondingly obstructing homologous recombination [153].

Monoubiquitination of PCNA on K164 strengthens the interaction with TLS polymerases, while yet another lysine modification, ISGylation, promotes release of polymerase η (polη) from PCNA [162]. Upon UV irradiation, either K164 or K168 is assumed to undergo ISG15 modification that induces PCNA de-ubiquitination and polη discharge followed by PCNA de-ISGylation and resumption of normal replication.

There are 16 lysine residues in human PCNA and 13 of them have been reported to be ubiquitinated, either in individual experiments (K164), or by numerous large-scale methods (Fig. 1). Nine of the identified residues can also be UBL-modified, or acetylated, suggesting possible competition between different types of modification. Site-specific overlap between lysine ubiquitination and acetylation has been suggested for about 20% of identified protein ubiquitinations [61] and an interplay between ubiquitination and acetylation represents a common way to regulate protein stability. In most cases, lysine acetylation prevents ubiquitination and ubiquitin mediated proteolysis of modified protein, but there are some examples of acetylation-directed acceleration of protein degradation, by modulating protein–protein interactions (reviewed in [163]). Indeed, Yu et al. showed that UV-induced PCNA acetylation at K14 caused dissociation of PCNA from a complex with MTH2, and as a consequence, shortens its half-life, as PCNA is more easily degraded by the proteasome [147].

In PCNA, six of the lysines located in the N- and C-terminal domains were found to be targets of both ubiquitination and acetylation. Naryzhny and Lee [164] showed in Chinese hamster ovary (CHO) cells that the PCNA acetylation status plays an important role in regulating its function. Indeed, they suggested the existence of three PCNA isoforms differing in their acetylation status and subcellular localization. Additionally, they implied that acetylated PCNA is involved in DNA replication, while its deacetylated form with replication termination, as acetylated PCNA has higher affinity for polδ and polβ, compared to the deacetylated form.

UBL modifications were identified at four lysine residues in the C-terminal half of the protein. The most modified lysine residue is K164, as all of the global ubiquitin profiling studies detected ubiquitination at this site. Additionally, K164 can also be a subject of SUMOylation, ISGylation and acetylation. The structural studies of ubiquitinated PCNA suggest that the ubiquitin moieties attached to K164 of PCNA monomers extend away from the ring and do not block the interactions of PIP box containing proteins with PCNA. It seems, however, that the type of modification can control the interacting partner by strengthening the interaction between the ubiquitin, or UBL, on PCNA and UBD, or UBL binding motif on the protein [165].

Two of the identified lysine residues, K77 and K80, are placed within the DNA binding domain, implying possible obstruction of DNA–PCNA binding upon modifications at any of these sites. Two other lysines, K110 and K117, in which ubiquitination was detected in multiple studies, lie within a segment of 101–120 amino acids found to play an essential role for PCNA nuclear location [166]. Even though both K110A and K117A mutants are efficiently imported to the nucleus, K110A does not co-localize with replication foci suggesting that ubiquitination of this residue might influence PCNA foci formation.

4.2. Modifications of Y-family polymerases

While it is apparent that many of the recently characterized DNA polymerases have the ability to facilitate TLS of certain DNA lesions, the best characterized from an historical perspective are the four Y-family DNA polymerases polη, polι, polκ, and Rev1 and the B-family DNA polymerase, polζ [167,168]. Each of these polymerases presents a specific portfolio of DNA lesions they are able to bypass with differing efficiency and accuracy. The Y-family polymerases are multi-domain proteins. The catalytic domain generally occupies the N-terminus, while the C-terminus is engaged in protein-protein interactions and contains various protein-binding motifs. Polymerases η, ι and κ possess non-canonical PCNA-binding motifs (PIP box) and a Rev1 interacting region (RIR) [168]. Rev1 interacts with PCNA *via* a BRCT domain localized at its extreme N-terminus while the C-terminus of Rev1 interacts with polymerases η, ι and κ. All Y-family DNA polymerases have ubiquitin binding domains that bind non-covalently to ubiquitin, or ubiquitinated proteins. Polymerases ι and Rev1 possess two UBMs, while polymerases η and κ, have so called UBZs – UBDs that additionally bind a zinc atom (polη has one UBZ, whereas polκ has two UBZs) [168–170]. Besides possessing UBDs that facilitate the interaction with monoubiquitinated PCNA, human polη, polι, mouse polκ and Rev1 as well as yeast and nematode polη have been shown to be subject to ubiquitination themselves [169,171–177]. In general, most of the proteins that can non-covalently bind ubiquitin *via* different types of UBDs are themselves targets of monoubiquitination in a process called coupled monoubiquitination [178,179]. In this process, ubiquitin attached to an E2 ubiquitin conjugating enzyme, or E3 ubiquitin ligase, is recruited to the UBD containing substrate which becomes ubiquitinated in an E3-dependent, or independent mode [180].

4.2.1. DNA polymerase η modifications

Polη is possibly the best-characterized Y-family DNA polymerase and is mainly known for efficient replication past cyclobutane pyrimidine dimers, which are the main DNA lesions induced after UV-irradiation. As a consequence, a dysfunction in human polη results in the variant form of Xeroderma Pigmentosum, which is characterized by sunlight sensitivity and a high incidence of skin cancer [182,183].

It has previously been shown that human polη can be ubiquitinated *in vivo* in its nuclear localization signal (NLS) motif. K682 was identified as the main ubiquitination site, however when this residue is unavailable, three other close by lysines (K686, K694 or K709) can serve as a target [169,171]. Pirh2, an E3 ligase, was discovered to interact with human polη and monoubiquitinate it at one of the four lysine residues at the C-terminus [184,185]. Attaching a ubiquitin moiety to the C-terminus of polη prevents its interaction with PCNA and inhibits its ability to bypass UV-induced lesions and causes an increased sensitivity to UV radiation [171,185]. Therefore, monoubiquitinated polη needs to be actively de-ubiquitinated prior to interacting with PCNA and its recruitment to a stalled replication fork [171]. Additionally, polη is a subject of polyubiquitination by another E3 ligase, Mdm2, that targets polη for proteasomal degradation and controls its stability in response to UV-induced DNA damage [186]. Wallace et al. showed that human polη can also be polyubiquitinated

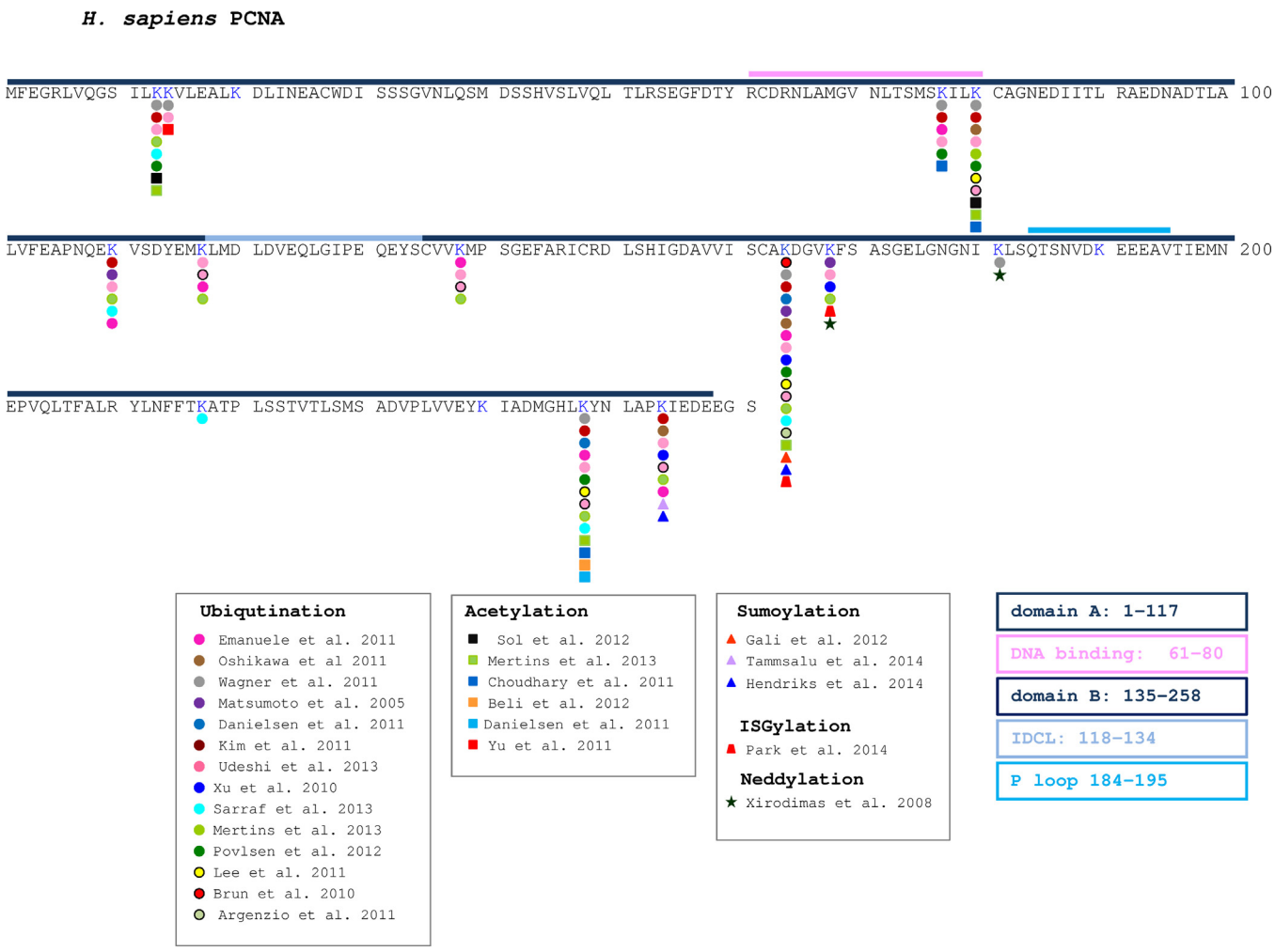

Fig. 1. Posttranslational modification of human PCNA. Lysines that have been indicated to be ubiquitinated are shown with a colored circle. Lysines that have been indicated to be acetylated are shown with a colored square. Lysines that have been indicated to be SUMOylated are shown by a colored triangle. The lysine residue that is ISGylated is shown by a red trapezium. The lysines that are known to be neddylated are shown by a green star. References are given in the appropriate associated box.

by a RING E3 ligase, TRIP (TRAF-interacting protein) (tumor necrosis factor receptor (TNFR)-associated factor) and TRIP promotes its localization in nuclear foci [187]. The TRIP homolog in *Drosophila melanogaster*, NOPO, enhances ubiquitination of polη during insect embryogenesis [187]. Most probably NOPO promotes non-proteolytic polyubiquitination, as its overexpression does not cause polη destabilization and additionally, NOPO interacts with Bendless (Ben), the *Drosophila* homolog of Ubc13, suggesting the formation of K63-linked polyubiquitin chains [188].

Recently, the deubiquitinating enzyme, USP7, has been reported to regulate the stability of human polη in two ways. On one hand, USP7 can directly deubiquitinate polη which stabilizes polη, and on the other hand, knockout of USP7 increases the steady-state level of polη by destabilizing Mdm2 [189].

S. cerevisiae polη was also found to be ubiquitinated [173,175,176]. However, the particular ubiquitination sites were not identified. Nevertheless, it has been shown that similar to human polη, the ubiquitination depends on a functional UBZ domain [169,175]. Ubiquitination of yeast polη is correlated with the cell cycle and increases during G1 and drops as cells enter S-phase, thereby allowing for the recruitment of polη to PCNA that is monoubiquitinated in response to a replication block [173]. There are contradicting reports about the stability of yeast polη [172,173] because it appears that the half-life of the yeast enzyme largely depends on the epitope tag used to identify the recombinant enzyme [190,this issue].

Interestingly, in response to DNA damaging agents (MMS and UV), nematode polη becomes SUMOylated by GEI-17 SUMO E3 ligase at K85 and K260 and protects it from degradation mediated by CRL4-CDT-2-dependent ubiquitination [174,181]. The SUMOylated lysine residues are conserved in human polη (K86 and K261 respectively), and very recently human polη has been reported to be SUMOylated at K163 (Patricia Kannouche and Emmanuelle Despras, personal communication).

In addition to ubiquitination and SUMOylation, human polη can also be phosphorylated by both ATR kinase and protein kinase C (PKC), in response to UV radiation. Potential phosphorylation sites were reported at S587, T617 and S601 by two independent groups [191,192]. The phosphorylation of polη seems to be required for cell survival after UV radiation and provides a link between DNA damage-induced checkpoint control and translesion synthesis.

There are 47 lysine residues in human polη. In 7 out of 13 large-scale studies, polη was found to be ubiquitinated (Fig. 2). In total, the ubiquitination of 14 lysine residues was reported, but 10 of them were identified in single analysis implying that they might represent non-specific modifications. All 7 global approaches that showed polη ubiquitination identified K682, thereby independently confirming the primary ubiquitination site originally found

Fig. 2. Posttranslational modification of human DNA polη. Lysines that have been identified to be ubiquitinated are shown with a colored circle. The serine residues that are thought to be phosphorylated, are shown by a colored star. References are given in the appropriate associated box.

by Bienko et al. [171]. Three neighboring lysine residues, K686, K694 and K709 were also identified as back-up sites of ubiquitination, again, as previously reported by Bienko et al. [171].

4.2.2. DNA polymerase ι modifications

DNA polι is a paralog of polη [193] and is thought to bypass a number of lesions *in vivo* especially when polη is absent (*e.g.* in XPV cells) and due to its reduced accuracy in synthesizing across photoproducts, polι-dependent TLS results in elevated mutagenesis [194–198]. Extensive biochemical studies performed *in vitro* with the highly purified enzyme suggest that polι is able to bypass a wide range of DNA lesions [199–202]. Interestingly, when copying an undamaged template, its accuracy varies 10,000-fold depending on the template base copied (reviewed in [203]). Its unusual preference of incorporating G opposite T (3–10-fold greater than the correct base, A) gives polι a distinctive signature [204,205] and is a result of the specific structure of its active site [206,207]. Another interesting feature of polι is that its N-terminus contains two partially overlapping catalytic domains; one with DNA polymerase activity and one with dRP lyase activity [208,209]. The C-terminus contains motifs characteristic of other Y-family polymerases and includes PCNA-interacting (PIP), Rev1-interacting (RIR) and ubiquitin-interacting (UBM1 and UBM2) motifs/domains.

To date, the only reported posttranslational modification in human polι is ubiquitination [169] and the cellular function of ubiquitinated polι is not fully understood. However, our previous studies implied that ubiquitination of either polη or polι is required for the two polymerases to physically interact [210].

There are 53 lysine residues in human polι. In 8 total large-scale studies, 24 lysine residues distributed along the entire length of the polymerase were found to be ubiquitinated (Fig. 3). Ubiquitination of half of these residues was detected just once, suggesting that the modifications might have appeared either accidentally, or represent rather rare conditional cases. The remaining 12 modified lysines were found to be ubiquitinated in 2–4 independent approaches, often using different experimental strategies. We believe that multiple autonomous detection of the same residue thereby increases the probability that the ubiquitination of any particular lysine has a functional meaning and possibly a broader cellular effect. Nonetheless, no single lysine has appeared in all of the analyzed studies.

Thirteen of the detected ubiquitination sites are located in the polymerase catalytic domain and the proposed dRP lyase domain, suggesting that ubiquitination of some of these lysines could possibly influence the enzymatic activities of polι. Ubiquitination of K309 was detected in four independent approaches and nearby K310 in two, giving these residues a higher possibility of *bona fide* modification. Ubiquitination targets were also reported in two adjacent lysines, K549 and K550, of the RIR motif suggesting control of the Rev1–polι interaction. The detection of ubiquitination of both lysines suggests that either one could serve as a modification target. Several other ubiquitination sites were detected at, or in close proximity to the UBM motifs, which could possibly affect the ability of polι to bind to ubiquitinated proteins (such as PCNA or polη).

4.2.3. DNA polymerase κ modifications

Polκ is able to bypass multiple types of DNA lesions including abasic sites and bulky adducts, but with rather low efficiency [211] and due to a constricted active site, cannot incorporate a base opposite a pyrimidine dimer [212]. When copying an undamaged template, polκ is quite accurate compared to other Y-family

Fig. 3. Posttranslational modification of human DNA polι. Lysines that have been indicated to be ubiquitinated are shown with a colored circle and the appropriate reference cited in the associated box.

polymerases, but can also extend mispaired primer termini [213]. Furthermore, after UV-irradiation the activity of polκ has been implicated in the gap-filling step of nucleotide excision repair [214].

Similar to other Y-family DNA polymerases, polκ possesses a catalytic domain at its N-terminus while the C-terminal half of the protein contains domains involved in protein–protein interactions including an RIR, two UBZ domains and at the extreme C-terminus, a PIP box [215,216]. The UBZ domains mediate the enhanced interaction with PCNA and some studies report that they are essential for UV-induced nuclear foci formation by polκ, but do not affect protein's half-life [177]. On the other hand, Okada et al. suggests that polκ can function independently of PCNA modification [216]. Recent studies of Wit et al. concluded that dependent on the type of DNA damage, PCNA ubiquitination may, or may not, be required for polκ activation [217].

To date, several reports show evidence for posttranslational modifications to polκ in eukaryotic cells. Guo et al. observed monoubiquitination of mouse polκ and confirmed that similar to other Y-family polymerases, monoubiquitination depends on UBDs [177]. Endogenously expressed murine polκ is a stable protein with a half-life estimated to be ~5.4 h [177]. The turnover of exogenously expressed polκ is somewhat faster (3.7–4.2 h), but does not change in polκ UBZ mutants. The suggestion that human polκ might also be ubiquitinated comes from the publication of Wallace et al. which reports an interaction between the C-terminus of polκ and the RING E3 ligase, TRIP [187]. On the other hand, the possibility of polκ E3-independent monoubiquitination has also been reported *in vitro* [179]. The biological function of polκ modification is not yet known. However, by influencing polκ interactions with other proteins, it may regulate its presence in replication factories.

Regarding other types polκ modification, Roerink et al. suggested that GEI-17, which is known to SUMOylate and consequently protect nematode polη from proteasomal degradation, most likely also acts on polκ [181], so it is entirely possible that polκ may also be SUMOylated *in vivo*.

Until now, the information about which lysine residues can be modified in polκ comes from large-scale proteomics analysis. There are 84 lysine residues in human polκ. Just four of the over 13 independent global studies have reported ubiquitination of polκ at 36 lysines (Fig. 4). It should be noted that most of these sites were detected in just one single analysis. Only K173, K461 and K541 were detected in two studies and K683 in three studies. Indeed, the majority of polκ expressed *in vivo* is not ubiquitinated [177], which probably explains the limited number of studies reporting ubiquitination of polκ. In a recent publication on proteome wide SUMOylation, Hendriks et al. [96] reported SUMO2 modifications at six polκ lysine residues; half of which, K55, K224 and K814 overlap with known ubiquitination sites.

4.2.4. *Rev1 modifications*

Rev1 is a unique enzyme among Y-family polymerases, as it is only able to incorporate dCMP nucleotides opposite undamaged or damaged template G and some DNA lesions, including an abasic site [218–221]. Besides its distinctive enzymatic activity, Rev1

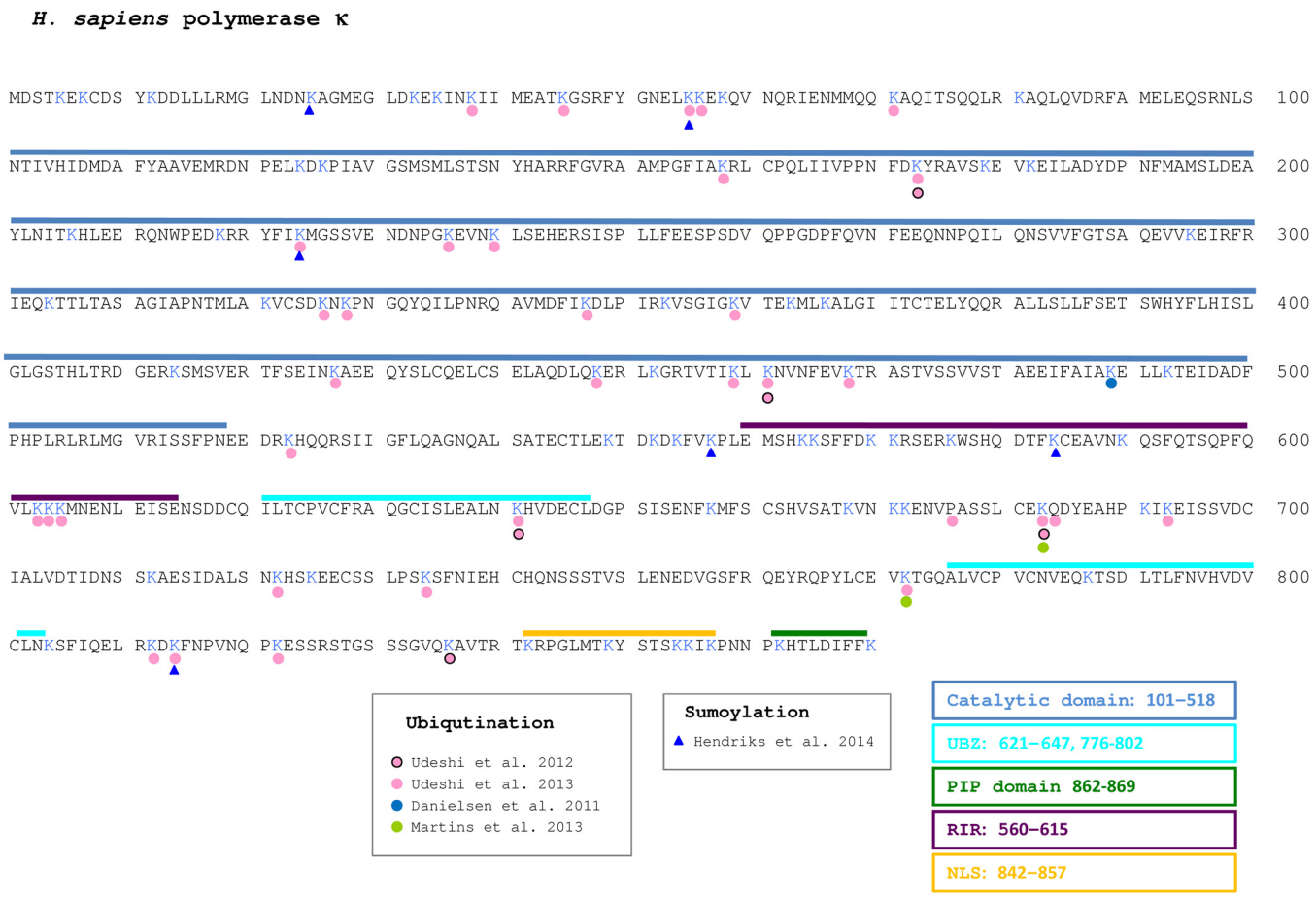

Fig. 4. Posttranslational modification of human DNA polκ. Lysines that have been indicated to be ubiquitinated are shown with a colored circle. Lysines that have been indicated to be SUMOylated are shown by a colored triangle. The appropriate references are cited in the associated box.

also plays a non-catalytic role in TLS as a scaffolding protein that coordinates the other TLS polymerases. The extreme C-terminus of Rev1 in higher eukaryotes is devoted to the interaction with pols η, ι, κ and ζ [222–227]. Additionally the two UBMs that are located close to the C-terminal domain permit the interaction with ubiquitin, ubiquitinated PCNA, or other ubiquitinated proteins [170,228]. Interestingly, Rev1 lacks a well-conserved PIP box, that is characteristic of the other Y-family polymerases and the interaction with PCNA is, instead, through the N-terminal BRCT domain of Rev1 [229,230].

In addition to non-covalent interacts with ubiquitin and ubiquitinated proteins, at least two studies have shown that mouse and human Rev1 can also be directly conjugated to ubiquitin [176,231]. However, the mechanism and sites of the ubiquitination remain unknown. Kim et al. reported that ubiquitinated human Rev1 can be recruited to nuclear foci by the *Fanconi Anemia* core complex, as it binds directly to the UBZ4 domain of FAAP20 protein [231]. Moreover, the level of *S. cerevisiae* Rev1 seems to be regulated in a cell cycle-dependent mechanism *via* ubiquitin-mediated proteasomal degradation, suggesting that it gets ubiquitinated before degradation [232].

There are 92 lysine residues in human Rev1. However, ubiquitination of Rev1 was detected in just one of the large-scale proteomic studies [64]. Out of 7 lysine modifications 5 localize to the N-terminus (K28, K41, K119, K134 and K140), which are located near, or within, the BRCT domain. The remaining ubiquitinated lysines were located in the middle of the protein (K678 and K770).

SUMO2 modification of human Rev1 at K99 has also been reported [96]. In another proteome-wide analysis, *Drosophila melanogaster* Rev1 was shown to be acetylated at K136 [233]. However, in human Rev1, this residue is replaced by arginine (R149). These results suggest that Rev1 modifications (most probably at the N-terminus), occurs either rarely, or under highly specific conditions not employed in the global proteomic studies.

5. Concluding remarks

Review of the published data reveals interesting differences in the extent of posttranslational modification of key TLS proteins. PCNA is the most highly modified protein and is subject to ubiquitination, acetylation, SUMOylation, ISGylation and neddylation. Human polη is a target of limited ubiquitination, primarily at one key residue, K682, and is also been reported to undergo phosphorylation and very recently SUMOylation. In contrast, nearly 50% of the lysines in polι can apparently be ubiquitinated. This is also in dramatic contrast to either the polκ or Rev1 proteins, which appear to undergo limited lysine (or any other) posttranslational modification. It will therefore be interesting to determine why polι is so highly ubiquitinated and the effects such ubiquitination have on the regulation and *in vivo* properties of the enigmatic polι enzyme.

Conflict of interest

The authors declare no conflict of interest.

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