The Role of Ubiquitination and SUMOylation in DNA Replication

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Abstract

DNA replication is a tightly regulated conserved process that ensures the faithful transmission of genetic material to define heritable phenotypic traits. Perturbations in this process result in genomic instability, mutagenesis, and diseases, including malignancy. Proteins involved in the initiation, progression, and termination of DNA replication are subject to a plethora of reversible post-translational modifications (PTMs) to provide a proper temporal and spatial control of replication. Among these, modifications involving the covalent attachment of the small protein ubiquitin or the small ubiquitin-like modifier (SUMO) to replication and replication-associated proteins are particularly important for the proper regulation of DNA replication as well as for optimal cellular responses to replication stress. In this article, we describe how the ubiquitination and SUMOylation processes impact DNA replication in eukaryotes and highlight the consequences of deregulated signals emanating from these two versatile regulatory pathways on cellular activities.

Regulation of eukaryotic DNA replication

Initiation of DNA replication

Eukaryotic DNA replication is tightly regulated such that cells replicate their entire genome once and only once in a given cell cycle (Machida *et al.*, 2005). For mammalian cells, this is no easy task since each proliferative somatic cell must efficiently replicate approximately 6 billion base pairs (in male cells) from roughly 250,000 replication origins scattered throughout the genome with each division cycle (Cadoret *et al.*, 2008; Sequeira-Mendes *et al.*, 2009; Karnani *et al.*, 2010). With roughly 600 million new blood cells born in the bone marrow of an adult human (Doulatov *et al.*, 2012), one cannot grasp the magnitude of the task the replication machinery has to accomplish. The core machinery of DNA replication is highly conserved in all living organisms, but eukaryotes diverge significantly in its regulation owing to the larger, more complex genomes (Kaguni, 2011). In bacteria (e.g. *Escherichia coli*), replication initiates at individual replication initiation sites or

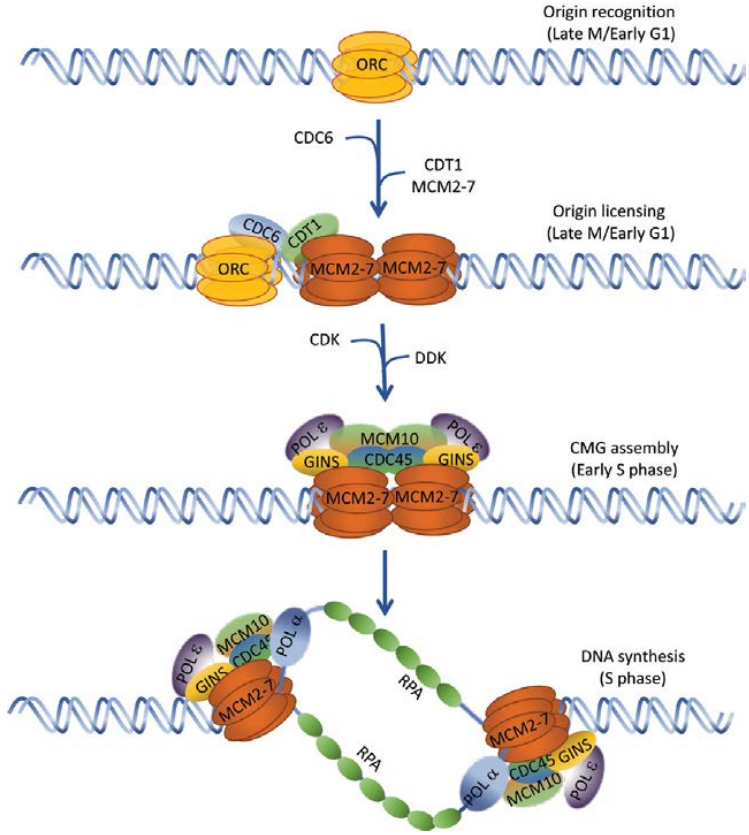


Figure 14.1 Regulation of replication initiation in eukaryotes. A model depicting the step-wise assembly of the pre-replication complex (Pre-RC) in late mitosis and during G1 phase of the cell cycle, followed by replisome assembly. The six-subunit ORC complex binds to origins of DNA replication in late M and early G1. This is followed by the recruitment of the replication licensing proteins CDC6 and CDT1, and the loading the of the MCM2–7 helicase (origin licensing). At the G1/S transition, the Dbf4-dependent kinase (DDK) and CDK enzymes promote the assembly of the replicative helicase, or the CMG complex, which is marked by the recruitment of the GINS complex (Sld5, Psf1, Psf2, Psf3), along with CDC45. MCM10 aids in this process by recruiting and stabilizing DNA polymerase α (POL α). Other proteins [e.g. Treslin (Sld3 in yeast), RecQL4 (Sld2 in yeast), and TopBP1] help in the replisome assembly (not shown). As DNA synthesis begins in S-phase, the unwound DNA is stabilized by the single-stranded DNA binding protein RPA, and DNA polymerases (POL ε and POL δ) initiate replication.

origin of chromosome replication (*OriC*) where two replication forks assemble and move in opposite direction at a rate of 1 Kb/sec/fork to replicate the entire 4.4 Mb circular chromosome within 30 minutes (Katayama, 2017). The AAA+ ATPase replication initiator protein DnaA, which is conserved in virtually all bacteria, recognizes and binds with high specificity to high density GATC repeat sequences (DnaA box) within these replicons, and both DNA binding and ATP hydrolysing activities of DnaA are essential for replication initiation (Hansen and Atlung, 2018). Initiation of DNA replication in eukaryotes (Fig. 14.1) is similarly dependent on the binding of a DnaA-like six-subunit origin recognition complex (ORC) to replication origins in an ATP-dependent manner (Bell and Stillman, 1992; Bell and Dutta, 2002). ORCs from various eukaryotes exhibit a wide range of sequence-recognition specificities. For example, whereas ORC from budding yeast

specifically recognizes 11-bp or 17-bp conserved sequences within the ≈400 autonomously replicating sequences (ARS) (Dhar *et al.*, 2012), the fission yeast ORC recognizes AT stretches (but without sequence consensus) through the AT-hook motif present on the Orc4 subunit (Chuang and Kelly, 1999; Segurado *et al.*, 2003; Dai *et al.*, 2005; Hayashi *et al.*, 2007). The six-subunit ORC complex from high eukaryotes binds DNA without sequences specificity (Vashee *et al.*, 2003; Schaarschmidt *et al.*, 2004), although replication initiates from genomic loci that are enriched for AT-rich sequences, dinucleotide repeats, asymmetrical purine-pyrimidine sequences, and matrix attachment region (MAR) sequences (Li and Stillman, 2012; Kumar and Remus, 2016). Additional epigenomic features, such as the DNA topology, transcription factors and regulatory elements, local chromatin environment as well as the replication initiation proteins CDT1 and CDC6 play a role for the selectivity of ORC to stably bind replication origins (Masai *et al.*, 2010; Li and Stillman, 2012; Kumar and Remus, 2016). Replication initiation in high eukaryotes is also dependent on histone methylation. For example, recent studies demonstrate a critical role for histone H4 methylation at Lys-20 (H4K20) at replication origins in the nucleation of DNA replication (Tardat *et al.*, 2010; Beck *et al.*, 2012a). Mono-methylation of H4K20 (H4K20me1) is catalysed by the histone methyltransferase (HMT) SET8 (also known as PR-SET7), which deposits a single methyl group on Lys-20 of nucleosomal histone H4 (Nishioka *et al.*, 2002; Xiao *et al.*, 2005). When tethered to specific genomic loci, catalytically active, but not inactive, SET8 recruits pre-RC proteins on chromatin and replication initiates from these sites (Tardat *et al.*, 2010). Mono-methylated Lys-20 of H4 is subject for further methylation [di- and tri-methylation (H4K20me2 and H4K20me3, respectively)] by the SUV4-20H1/H2 HMTs (Schotta *et al.*, 2008). The conversion of H4K20me1 to H4K20me2/3 by SUV4-20H1/H2 likely plays an important role for SET8-dependent replication initiation, as the recruitment of ORC1 as well as the ORC-associated protein (ORCA) protein (both capable of binding H4K20me *in vitro*) to chromatin requires SUV4–20H1/H2 (Beck *et al.*, 2012a).

Cell cycle regulation of replication initiation in eukaryotes

Initiation of eukaryotic DNA replication is cell cycle regulated, requires the ordered assembly of several proteins at replication origins, and occurs in two distinct steps that are temporally separated within the cell cycle (Fig. 14.1). The first step involves the establishment of pre-replicative complexes (pre-RCs) through the sequential assembly of ORC, CDC6, and CDT1, followed by the loading of the six-subunit helicase MCM2–7 (minichromosome maintenance proteins, subunits 2–7) at origins of replication in late mitosis (M) and early G1 (first Gap) phase of the cell cycle. Once the MCM2–7 complexes are loaded onto replication origins (origin licensing), the ORC-CDC6-CDT1 pre-RC components are no longer required to initiate replication. In the second step, licensed origins are activated in S phase (DNA synthesis phase) to generate active replication forks (origin firing), and this requires the conversion of the inactive double hexameric MCM2–7 helicase to an active replicative helicase, the CMG complex, which is composed of MCM2–7, its cofactor CDC45, and the GINS complex (Gambus *et al.*, 2006; Moyer *et al.*, 2006; Pacek *et al.*, 2006; Ilves *et al.*, 2010; Kang *et al.*, 2012). This conversion process, which is highly conserved from yeast to human, requires the activity of the Dbf4-dependent kinase (DDK) and the cyclin-dependent kinase (CDK). Both kinases are activated at G1/S transition, and their concerted activities promote the recruitment of several scaffolding proteins and DNA polymerase Polε to assemble the replisome. Studies in yeast have shown that while DDK phosphorylates multiple Mcm2–7 subunits to recruit the scaffolding protein Sld3 with its partners Sld7 and Cdc45, CDK phosphorylates the two other scaffolding subunits Sld2 and Sld3, thereby promoting their interaction with Dpb11 (TopBP1 in human) in cooperation with Polε and GINS (Gambus *et al.*, 2006; Moyer *et al.*, 2006; Pacek *et al.*, 2006; Ilves *et al.*, 2010; Muramatsu *et al.*, 2010; Kumagai *et al.*, 2010, 2011; Boos *et al.*, 2011; Kang *et al.*, 2012; Bruck and Kaplan, 2015, 2017; Fang *et al.*, 2016). Replisome assembly also requires the action of multiple protein complexes involved in monitoring replication fork progression, in coordinating DNA synthesis with chromatin assembly, and in responding to

genetic perturbations by generating checkpoint and damage signals (Leman and Noguchi, 2013).

Progression and termination of DNA replication

Origin firing in eukaryotes is temporally regulated with distinct early- and late-replicating genomic regions and exhibits significant flexibility that gives the cells control over situations that interfere with normal progression of replication forks (Renard-Guillet *et al.*, 2014). Activation of the CMG complex is tightly coupled to the activity of histone chaperones, nucleosome-remodelling complexes and chromatin-modifying enzymes (Groth, 2007, 2009; Jasencakova and Groth, 2010). These later factors facilitate nucleosomal disassembly ahead of the replication forks and reassembling nucleosomes with correct positioning following their passage. The DNA primase–POLα complex generates primers that will be extended by POLε (for continuous DNA synthesis of the leading strand) or POLδ (for the discontinuous replication of the lagging strand) (Bell and Dutta, 2002; Bell and Labib, 2016). Several other proteins are important for the maturation and ligation of the Okazaki fragments. In budding yeast, these include the flap endonuclease Rad27, the DNA helicase-nuclease Dna2, the Exo1 exonuclease and the DNA ligase Cdc9 (Bell and Labib, 2016). DNA topoisomerases relieve topological stresses created by the moving replication forks, and many proteins and protein complexes aid in removing other barriers to the progressing replication forks, such as tightly-bound non-histone proteins. Other proteins must be recruited to deal with difficult to replicate genomic sequences or with actively transcribing genomic templates. Progression of DNA replication is also tightly coordinated with the establishment of sister chromatid cohesion as well as with the activity of multiple proteins and protein complexes involved in the sensing and repair of DNA damage that may be encountered during DNA replication (Waters *et al.*, 2009; Villa-Hernandez and Bermejo, 2018). Termination of DNA replication occurs at converging replication forks from neighboring origins of replication, although in some cases, termination occurs at chromosomal termination regions (TERs) defined by replication pausing elements contained within these TERs (Labib and Hodgson, 2007; Fachinetti *et al.*, 2010). Genomic and mechanistic studies in budding yeast identified 71 such regions, and further demonstrated that these TERs can influence fork progression and merging (Fachinetti *et al.*, 2010). Replication across these TERs, which are characterized by the accumulation of X-shaped structures, can be facilitated by the Rrm3 DNA helicase, and the fusion of the converging forks at these sites is aided by DNA topoisomerase II (Topo II or Top2 in yeast), thus counteracting abnormal genomic transitions (Fachinetti *et al.*, 2010). Termination of DNA replication is marked by the completion of local DNA synthesis, the decatenation of the two daughter strands by DNA topoisomerases and the final disassembly of the replisome (Dewar and Walter, 2017; Gambus, 2017).

Ubiquitin-dependent regulation of DNA replication

Overview of the ubiquitin-proteasome system

ATP-dependent and ubiquitin-mediated proteasomal degradation through the ubiquitin-proteasome system (UPS) provides an efficient mean to regulate protein abundance and maintain homeostatic regulation of cellular physiology, and is involved in almost all cellular activities (Kornitzer and Ciechanover, 2000; Amir *et al.*, 2001; Ciechanover and Schwartz, 2002; Glickman and Ciechanover, 2002; Hershko, 2005; Schwartz and Ciechanover, 2009). The process ensures the timely down-regulation of cellular proteins via the 26S proteasome, where roughly 80% of all intracellular proteins are digested into small peptides (Skaar *et al.*, 2014). Proteasomal degradation is preceded by the covalent attachment of multiple copies of the highly conserved 76 amino-acid ubiquitin protein [linked together through Lys-48 (Lys-48 linkage) or Lys-11 (Lys-11 linkage)] to substrate proteins (Fig. 14.2). This occurs in a series of enzymatic reactions involving the activity of an E1 ubiquitin-activating enzyme, the transfer of the activated ubiquitin to an E2 ubiquitin-conjugating enzyme (UBC), and the selective transfer of ubiquitin to the substrate through the activity of an E3 ubiquitin ligase (Glickman and Ciechanover, 2002; Groll and Huber, 2003; Kornitzer and Ciechanover, 2000; Teixeira and Reed, 2013). Whereas Lys-48 and Lys-11- polyubiquitination signal proteolytic

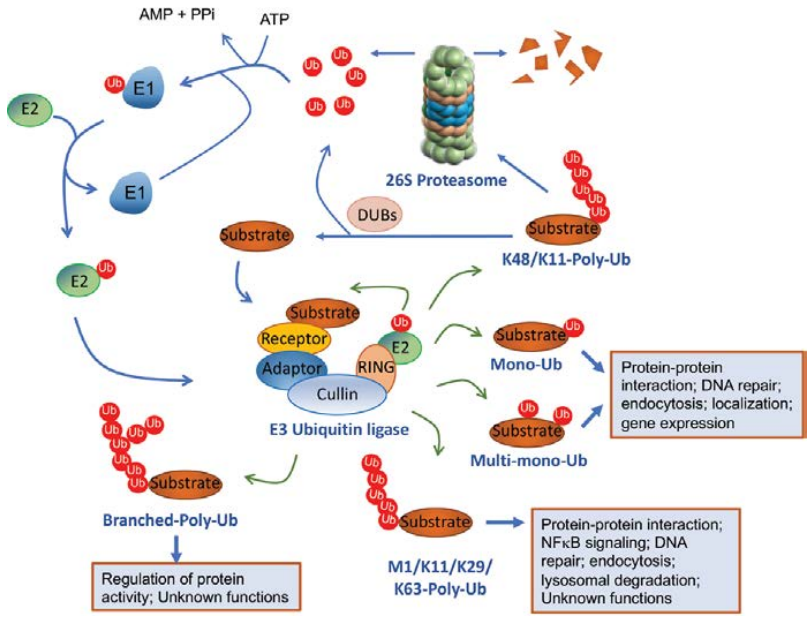


Figure 14.2 Regulation of protein ubiquitination. Protein ubiquitination involves the sequential activity of an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme and an E3 ubiquitin ligase (a cullin-based E3 ubiquitin ligase is shown as an example). The E3 ligase transfers the ubiquitin moiety (Ub) to the substrate through interaction with the E2-charged ubiquitin, forming a covalent isopeptide bond between the C-terminus of ubiquitin and a specific Lys residue on the substrate. Polyubiquitin chains (poly-Ub) can be formed by covalently conjugating the C-terminus of a ubiquitin moiety to one of seven Lys residues (e.g. Lys-48) or to the fist Met residue (M1) on another ubiquitin moiety. Polyubiquitination through Lys-48 (K48), and Lys-11 (K11) linkages directs the substrate to the 26S proteasome, where the substrate is proteolytically degraded into small peptides, with the ubiquitin moieties released and recycled. Other homotypic poly-ubiquitin chains [e.g. M1, Lys-63 (K63)], or the attachment of single ubiquitin moieties to individual (mono-ubiquitination) or multiple (multi-ubiquitination) Lys residues do not signal protein degradation and serves other distinct biological functions. A set of deubiquitinating enzymes (DUBs), which are highly specific cysteine proteases, can cleave the isopeptide bonds between the ubiquitin and ε-amino group of the substrate Lys or the Lys of the other ubiquitin moiety in a polyubiquitin chain. DUBs can also cleave the peptide bond between ubiquitin and the N-terminal methionine of another ubiquitin moiety.

degradation, other homotypic poly-ubiquitin chains involving ubiquitin conjugation through Lys-63 or Met-1, or the attachment of single ubiquitin moieties to individual (mono-ubiquitination) or multiple (multi-ubiquitination) Lys residues do not signal protein degradation, but play a role in various cellular process (Fig. 14.2). These include activities that impact protein–protein interaction, transcription factor activation, protein synthesis, and cellular response to DNA damage (Wang *et al.*, 2001; Tokunaga *et al.*, 2009; Yang *et al.*, 2010; Behrends and Harper, 2011; Dantuma and Pfeiffer, 2016; Schwertman *et al.*, 2016).

E3 ubiquitin ligases are critical for conferring specificity for the substrates to be ubiquitinated and, in some cases, for dictating the nature of substrate ubiquitination (Zheng and Shabek, 2017). Cullin-RING (*R*eally *I*nteresting *N*ew *G*ene) E3 ubiquitin Ligases (CRLs) represent the largest family of E3 ubiquitin ligases in mammals, promoting the polyubiquitin-mediated degradation of approximately 20% of total cellular proteins via the proteasome (Hotton and Callis, 2008; Deshaies and Joazeiro, 2009; Soucy *et al.*, 2009; Duda *et al.*, 2011; Hua and Vierstra, 2011; Lipkowitz and Weissman, 2011; Sarikas *et al.*, 2011; Lydeard *et al.*, 2013; Chen *et al.*, 2015). Other E3 ubiquitin ligases including the HECT (*H*omologous to the *E6*-AP *C*arboxyl *T*erminus) domain containing E3 ubiquitin ligases are described in more details in

recent excellent reviews (Li *et al.*, 2008; Deshaies and Joazeiro, 2009; Skaar *et al.*, 2014; Zheng and Shabek, 2017). CRLs are involved in many cellular processes, including DNA replication, cell cycle progression and cellular proliferation (Petroski and Deshaies, 2005; Bosu and Kipreos, 2008; Hotton and Callis, 2008). CRL family members include eight cullin proteins (cullin 1–3, 4A, 4B, 5, 7 and cullin 9) and a cullin-like protein ANAPC2 or APC2. The multi-subunit CRL1 E3 complex, better known as the SCF ligase (**S**KP1-**C**ullin1-**F**-Box protein), is the prototype of this family of E3 ligases and is best known for its role in controlling cell cycle progression, proliferation, and differentiation (Nakayama and Nakayama, 2005; Maser *et al.*, 2007; Welcker and Clurman, 2008; Huang *et al.*, 2010; Duan *et al.*, 2012; Lee and Diehl, 2014). The SCF ubiquitin ligase is built around the cullin 1 scaffold subunit, which binds the SKP1 (S-phase kinase-associated protein 1) adaptor protein through its N-terminal domain (Fig. 14.3). The SKP1 subunit bridges one of several substrate receptors with their cognate substrates to the cullin 1 subunit (Wang *et al.*, 2014). The cullin 1 C-terminal domain, on the other hand, is essential for substrate polyubiquitination through its interaction with a small RING domain protein (RBX1 or RBX2), which is essential for the recruitment of the E2 UBCs. The substrate specificity of the SCF ligase complex is dictated by a family of substrate receptors, which are collectively called F-box proteins owing to their interaction with the SPK1 protein through conserved F-box motif (Skaar *et al.*, 2014; Heo *et al.*, 2016). Mammalian cells express at least 69 F-box proteins, and thus assemble a large number of distinct SCF ligases. Each F-box protein is capable of recognizing a subset of ubiquitination substrates, commonly through interaction with

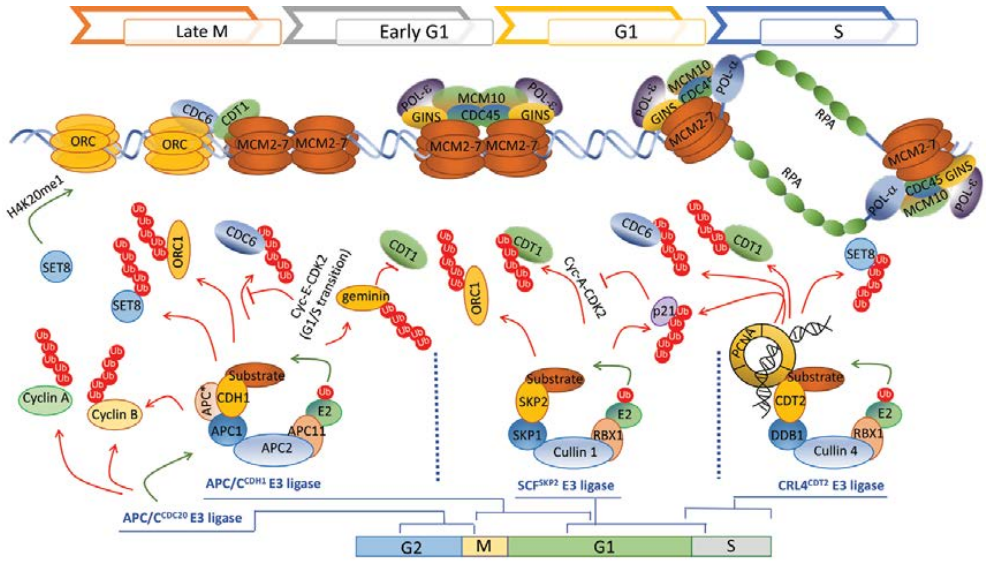


Figure 14.3 Regulation and restraint of origin licensing via the UPS. A schematic illustrating the various steps involved in origin licensing through the cell early part of the cell cycle and their regulation via the UPS. Three E3 ubiquitin ligases [APC/C^{CDH1} (left) SCF^{SKP2} (centre), and $CRL4^{CDT2}$ (right)] ensure the ordered but restricted assembly of the various pre-RC components in late M and early G1 phase of the cell cycle. APC/C^{CDC20} (not represented schematically) helps promote mitotic cyclin degradation and helps assembly of the AP ligase APC/C^{CDH1}. These E3 ligases are activated at distinct phases of the cell cycle (represented below). Distinct substrate receptors, CDC20 or CDH1, an F-box protein (SKP2), or a DCAF (CDT2) is critical for bridging the substrates for polyubiquitination by their cognate E3 ligases (APC/C, SCF, and CRL4 ligase, respectively). The $CRL4^{CDT2}$ ligase recognizes its substrates only when they interact with chromatin-bound PCNA, and thus, only targets chromatin-bound proteins for degradation. Other substrates are targeted for ubiquitination only in their soluble form (see text for details). M: mitosis. G1/S/G2: First gap, DNA synthesis and second phases of the cell cycle, respectively. APC* multiple subunits that together function as adaptor proteins

phosphorylated residues within small consensus 'degron' motifs in these substrates (Kipreos and Pagano, 2000; Cardozo and Pagano, 2004; Skaar *et al.*, 2013; Wang *et al.*, 2014; Heo *et al.*, 2016).

Ubiquitination is reversible and protein abundance is controlled by a set of deubiquitinases or DUBs (Fig. 14.2). DUBs play pivotal roles in the regulation of protein turnover, protein or enzymatic activation, protein–protein interaction, protein recycling, and cellular localization (Mukhopadhyay and Dasso, 2007; Komander *et al.*, 2009; Reyes-Turcu *et al.*, 2009; Hickey *et al.*, 2012), and are increasingly recognized as attractive therapeutic targets for cancer therapy (Hoeller and Dikic, 2009; Crosas, 2014; D'Arcy *et al.*, 2015; Pfoh *et al.*, 2015; Lei *et al.*, 2017; Harrigan *et al.*, 2018). Biochemically, DUBs hydrolyse the isopeptide bonds between the ε amino group of Lys side chains of the target substrate and the C-terminal group of ubiquitin, or the peptide bond between the α amino group of the target protein and the C-terminus of ubiquitin (Wilkinson, 1997).

Regulation of cell cycle control of replication via the UPS

The SCF^{SKP2} E3 ubiquitin ligase (Fig. 14.3), composed of the core SCF complex and the substrate receptor SKP2 (S-phase kinase-associated protein 2), is one of the best characterized SCF ligases and best known for its role in promoting cell cycle progression through the activation of CDKs (Nakayama and Nakayama, 2005; Skaar *et al.*, 2013). CDK activity controls replication initiation, and the SCF^{SKP2} ligase is critical for increasing CDK activity in G1 and in early S phase, by promoting the ubiquitination CDK inhibitors $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$ (Nakayama and Nakayama, 2005; Skaar *et al.*, 2013). SCF^{SKP2} also promotes progression through G2 phase, primarily through its ability to promote the ubiquitin-dependent proteolysis of cyclin A. The degradation of cyclin A in late S-phase ensures the availability of sufficient CDK1 molecules to assemble cyclin B–CDK1 complexes essential for progression through G2. Progression through S phase also requires the availability of sufficient CDK2 molecules for assembly with cyclin A, and this is mediated, at least in part, through the activity of the SCF^{FBXW7} ligase, which utilizes FBXW7 as a substrate receptor to degrade CDK2-phosphorylated cyclin E following entry into S phase (Clurman *et al.*, 1996; Koepp *et al.*, 2001).

CDK activity must be kept low during mitosis and in early G1, and this is facilitated by the multisubunit APC/C (anaphase promoting complex/cyclosome) ubiquitin ligase (Fig. 14.3), which promotes the ubiquitination and degradation of cyclin A and cyclin B (den Elzen and Pines, 2001). APC/C complex is the largest E3 ubiquitin ligase in mammals that is built around the APC2 cullin-like scaffold and utilizes the CDH1 (Hct1 in yeast) or CDC20 substrate receptors for recognizing and promoting the polyubiquitination (both Lys-48- and Lys-11-linked ubiquitin conjugation) of key drivers of the cell cycle (Visintin *et al.*, 1997; Zachariae and Nasmyth, 1999; Pines, 2006; van Leuken *et al.*, 2008). The specificity of the APC/C ligases is based on the substrate receptors CDH1/CDC20 ability to recognize degron motifs (destruction D-boxes and KEN-boxes) within the targeted substrates (Pfleger and Kirschner, 2000; Pfleger *et al.*, 2001). The APC/C^{CDC20} is activated in G2 and in early mitosis in a cyclin B–CDK1-dependent manner, and this is critical for the initial degradation of mitotic cyclins (cyclin A in prometaphase and cyclin B in metaphase) (Rahal and Amon, 2008). The $SCF^{\beta TRCP1}$ ligase utilizing the substrate receptor β-transducin-repeat-containing protein 1 (βTRCP1) aids in activating APC/C^{CDC20} both by stimulating CDK1 activity through enhancing the ubiquitination and degradation of the CDK1 tyrosine kinase inhibitor Wee1, and by relieving inhibition of the APC/C^{CDC20} via promoting the degradation of the F-box protein and early mitotic inhibitor 1 (EMI1), which is an endogenous inhibitor of APC/C (Guardavaccaro *et al.*, 2003; Watanabe *et al.*, 2004). Cyclin B–CDK1 subsequently phosphorylates the APC3 and APC1 subunits of the APC/C ligase, thereby facilitating the docking of CDC20 onto the APC/C ligase and the assembly of the active ligase complex (Fujimitsu *et al.*, 2016). Cyclin B–CDK1 additionally phosphorylates CDH1, resulting in conformational changes in CDH1 that preclude the assembly of an active APC/C^{CDH1} ligase.

In late mitosis and through G1, CDC20 is exchanged for CDH1/Hct1 following the dephosphorylation and activation of CDH1 by the CDC14A phosphatase (Cdc14 in yeast), and the newly assembled $APC/C^{CDH1/Hct1}$ ligase complex

maintains low cyclin B levels (Jaspersen *et al.*, 1999; Donzelli *et al.*, 2002; Sullivan and Morgan, 2007; Robbins and Cross, 2010). APC/$C^{CDH1/Hct1}$ activation is facilitated by APC/C^{CDC20}, which mediates the release of the CDC14A from centrosomes (and yeast Cdc14 phosphatase from the nucleolus) through an unknown mechanism (Shirayama *et al.*, 1999; Bembenek and Yu, 2001; Kaiser *et al.*, 2002; Mocciaro *et al.*, 2010; Chen *et al.*, 2016). APC/C^{CDH1} ligase activity is critical for inactivating mitotic CDK and for exit from mitosis. This is accomplished via the APC/$C^{CDH1/Hct1}$-dependent polyubiquitination and degradation of not only mitotic cyclins, but also of CDC20, thereby stabilizing the APC/C^{CDC20} ligase ubiquitination substrate and the CDK inhibitor p21 (or its homologue in yeast, Sic1) (Shirayama *et al.*, 1999; Amador *et al.*, 2007). APC/C^{CDH1} maintains low CDK activity through early G1 by promoting the degradation of the SKP2 subunit of the SCF^{SKP2} ligase (Bashir *et al.*, 2004; Wei *et al.*, 2004). This prevents the premature degradation of the CDK inhibitors p21 and p27, which can bind to and inhibit CDK2 in G1 (Abbas and Dutta, 2009). At the G1/S transition, the APC/$C^{CDH1/Hct1}$ ligase is inactivated through the phosphorylation of the CDH1/Hct1 subunit by cyclin E-CDK2 (Cappell *et al.*, 2016). Further inhibition of CDH1 (and CDC20) is mediated by EMI1, and this has been proposed to mark a 'point of no return' for entry into S-phase (Reimann *et al.*, 2001; Cappell *et al.*, 2016). Stabilization of mitotic cyclins is essential for the completion of DNA synthsis and for progression throguh G2 (Di Fiore and Pines, 2007).

Ubiquitin-dependent restraint of origin licensing

One of the most important features of regulating DNA replication in eukaryotes is the uncoupling of origin licensing, which takes place in late M and early G1, from origin firing in S-phase (Fig. 14.3). This ensures that replication initiates from individual origins of replication during S phase and is prevented from firing again until nuclear division is completed. The fluctuating CDK activity during the cell cycle, which is largely dependent on the ubiquitin-dependent proteolysis described above, is essential for this uncoupling process. The rising CDK activity in S phase is incompatible for origin licensing as many of the origin licensing proteins are phosphorylation substrates for CDK. CDK-dependent phosphorylation of certain replication licensing proteins suppresses origin licensing, either because this triggers their ubiquitination and proteolytic degradation or results in their exclusion from the nucleoplasm (Blow and Dutta, 2005; Abbas and Dutta, 2017). For example, CDK-phosphorylated human ORC1 protein, the largest subunit of the ORC complex, undergoes ubiquitin-dependent proteolysis specifically in S phase cells, and this is mediated by the SCF^{SKP2} ubiquitin ligase (Méndez *et al.*, 2002; Tatsumi *et al.*, 2003). Unlike human ORC1, ORC1 from Drosophila undergoes ubiquitin-dependent proteolysis via the APC/$C^{FZr/CDH1}$ E3 ligase as soon as cells exit mitosis and requires a domain in the N-terminus of Drosophila ORC1 that is non-conserved in human ORC1 (Araki *et al.*, 2003, 2005; Narbonne-Reveau *et al.*, 2008).

The replication licensing protein CDC6 is also targeted for proteolysis through the UPS, and this ensures that replication occurs only once during each division cycle. Although yeast Cdc6, a factor essential for loading Mcm2–7 onto replication origins is ubiquitinated through the SCF^{Cdc4} E3 ubiquitin ligase, mammalian CDC6 was previously shown to be shuttled outside the nucleus through the rising CDK activity at the G1/S transition, and this was sufficient to prevent origin relicensing (Aparicio *et al.*, 1997; Tanaka *et al.*, 1997; Saha *et al.*, 1998; Fujita *et al.*, 1999; Jiang *et al.*, 1999; Petersen *et al.*, 1999; Alexandrow and Hamlin, 2004). However, recent evidence suggests that mammalian CDC6 also undergoes ubiquitin-dependent proteolysis. Three E3 ubiquitin ligases are implicated in restricting the expression of mammalian CDC6 to late mitosis and early G1. In G1 cells, and in cells exiting the cell cycle into quiescence, mammalian CDC6 is ubiquitinated and degraded via the APC/C^{CDH1} ligase, and this is dependent on an interaction between the substrate receptor CDH1 and CDC6 (Petersen *et al.*, 2000). This ubiquitin-dependent degradation of CDC6 ensures that origin licensing is completed before cells transverse through G1 phase and is dependent on the D-box and KEN-box motifs of CDC6, since a combination of point mutations of these motifs stabilizes CDC6 both in G1 and in quiescent cells. In cells entering the cell cycle from quiescence, and as cyclin E-CDK2 activity builds up, CDC6 is phosphorylated by cyclin

E-CDK2, and this prevents CDC6 recognition by CDH1, and promotes origin licensing before entry into S phase (Mailand and Diffley, 2005). In S and in G2 phases of the cell cycle, mammalian CDC6 is ubiquitinated and degraded via the activity of the $CRL4^{CDT2}$ and the $SCF^{CyclinF}$, respectively, and both of these activities are essential for preventing origin relicensing and rereplication (Clijsters and Wolthuis, 2014; Walter *et al.*, 2016).

The role of $CRL4^{CDT2}$ and the APC/C ubiquitin ligases in restraining origin licensing

In addition to CDC6, CDT1 is another major ubiquitination and degradation substrate for the $CRL4^{CDT2}$ ubiquitin ligase (Fig. 14.3). The multi-subunit CRL4 ligase complexes share common features with SCF ligases but utilize a different set of substrate adaptors collectively known as DCAFs (DDB1 and Cullin 4 associated factors) (Angers *et al.*, 2006; Higa and Zhang, 2007). DCAFs include at least 49 family members of WD motif-rich proteins that, similar to the F-box protein substrate receptors of the CRL1 ligases, recognize and recruit substrates for polyubiquitination by the CRL4 ligase (Angers *et al.*, 2006; He *et al.*, 2006; Higa *et al.*, 2006; Jin *et al.*, 2006). The core CRL4 complex is comprised of one of two paralogues, cullin 4A or cullin 4B, that binds DDB1 (DNA damage-specific protein-1) through its N-terminus (Fig. 14.3). DDB1 is an adaptor protein that is analogous to the SKP1 subunit in the SCF ligases, and functions to bridges one of the DCAFs to the cullin subunit. The C-terminus of the cullin 4 subunit binds to RBX1 or RBX2, which are required for the recruitment of E2 UBCs, necessary for polyubiquitination. The DCAF CDT2 assembles with CRL4 to form a rather unique E3 ubiquitin ligase that appears to recognize its substrates when they interact with the DNA polymerase δ processivity factor proliferating cell nuclear antigen (PCNA) through a specialized PCNA-interacting protein motif or PIP-box, and only when PCNA is loaded onto chromatin (Arias and Walter, 2006; Senga *et al.*, 2006). This likely restricts the $CRL4^{CDT2}$ activity to S and early G2 phases of the cell cycle as well as during the repair of certain DNA lesions that requires PCNA (e.g. nucleotide excision repair) (Higa *et al.*, 2003; Abbas and Dutta, 2011; Havens and Walter, 2011; Abbas *et al.*, 2013). The PIP-box contained within $CRL4^{CDT2}$ substrates, commonly referred to as the 'PIP degron', is a variant of the PIP-box motif that is commonly used by many proteins to interact with PCNA, and contains, in addition to the canonical sequence [Q-X-X-(I/L/M)-X-X-(F/Y)-(F/Y)], conserved Thr and Asp acid residues at positions 5 and 6 respectively, as well as a basic amino acid residue c-terminal of the PIP-box (at position +4), as well as a second basic amino acid at position +3 or +5 (or both) (Havens and Walter, 2009, 2011; Abbas *et al.*, 2010; Michishita *et al.*, 2011).

The ability of $CRL4^{CDT2}$ to prevent origin relicensing and rereplication was initially attributed to its ability to specifically target CDT1 for proteolysis during S phase (Arias and Walter, 2006; Jin *et al.*, 2006; Nishitani *et al.*, 2006; Senga *et al.*, 2006). In fact, in various eukaryotes, with the exception of budding yeast where Cdt1 is exported to the cytoplasm along with the Mcm2–7 complex (Devault *et al.*, 2002; Tanaka and Diffley, 2002), deficiency in cullin 4, DDB1 or in CDT2 induces rereplication and genomic instability reminiscent of that seen following CDT1 overexpression (Vaziri *et al.*, 2003; Zhong *et al.*, 2003; Jin *et al.*, 2006; Lovejoy *et al.*, 2006; Sansam *et al.*, 2006; Tatsumi *et al.*, 2006; Kim *et al.*, 2008). Rereplication induced by $CRL4^{CDT2}$ inactivation results in the accumulation of DSBs, presumably due to the accumulation of replication intermediates and replication fork stalling/collapse, and activates DNA damage checkpoints, both of which can be partially suppressed through the co-depletion of CDT1 (Zhu *et al.*, 2004; Lovejoy *et al.*, 2006; Zhu and Dutta, 2006). We now know that $CRL4^{CDT2}$ prevents rereplication through promoting the polyubiquitination and degradation of multiple proteins involved in origin licensing during S and G2 (Fig. 14.3). These include not only CDC6 and CDT1, but also SET8 and p21, both of which bind PCNA through PIP degrons (Abbas *et al.*, 2010; Abbas *et al.*, 2008; Centore *et al.*, 2010; Clijsters and Wolthuis, 2014; Jørgensen *et al.*, 2011; Kim *et al.*, 2008; Nishitani *et al.*, 2008; Oda *et al.*, 2010; Tardat *et al.*, 2010). The *Drosophila melanogaster* E2f1 transcription factor is another ubiquitination substrate for $CRL4C^{DT2}$ whose degradation in S-phase is critical for rereplication suppression and is dependent on the interaction between E2f1 and PCNA through a PIP degron that is absent in the human protein (Shibutani *et al.*, 2008).

The PCNA-dependent and $CRL4^{CDT2}$ catalysed polyubiquitination and degradation of chromatin-bound p21 in S phase is important for sustaining elevated CDK2 activity essential for S phase progression and for freeing PCNA from inhibitory p21 (Abbas and Dutta, 2009). Increased stability of p21 following $CRL4^{CDT2}$ inhibition contributes to the rereplication phenotype observed in these cells, presumably because of inhibition of CDK activity, a condition compatible for origin licensing, but not likely to be sufficient to do so in the absence of stabilized CDT1 and SET8. This is evident by the fact that the expression of PCNA binding-deficient mutant of p21 ($p21^{\Delta PIP}$), which is resistant to $CRL4^{CDT2}$-mediated polyubiquitination and degradation induces robust senescence but is only associated with minor rereplication (Kim *et al.*, 2008; Benamar *et al.*, 2016). This is contrary to the role of stabilized SET8 in rereplication induction in cells with inactivated $CRL4^{CDT2}$, which is both necessary and sufficient to induce rereplication (Abbas *et al.*, 2010; Tardat *et al.*, 2010; Benamar *et al.*, 2016). It is important to note that both p21 and CDT1 are also necessary for rereplication induction in cells expressing $CRL4^{CDT2}$-resistant mutant SET8 protein ($SET8^{\Delta PIP}$) (unpublished observations). Although the role of SET8 in promoting rereplication when stabilized in S phase is not entirely clear, it is likely to be dependent on its ability to monomethylate H4K20 and the subsequent conversion of this histone mark to H4K20me2/3 at replication origins (Abbas *et al.*, 2010; Tardat *et al.*, 2010 Beck *et al.*, 2012a;).

Unlike chromatin-bound CDT1, p21 and SET8, soluble forms of these proteins are targeted for ubiquitination and proteolysis both in late G1 and/or S phase by other ubiquitin ligases, most notably, the SCF^{SKP2} ligase (Fig. 14.3). This E3 ligase targets CDT1 for ubiquitination and degradation following its phosphorylation at Thr-29 by cyclin A-CDK2 in late G1 and in S phase (Li *et al.*, 2003; Liu *et al.*, 2004; Takeda *et al.*, 2005). Similarly, p21 is phosphorylated at Ser-130 by CDK2 and this promotes the degradation of soluble p21 at the G1/S transition and in S phase (Bornstein *et al.*, 2003). Soluble SET8 was also suggested to be targeted for ubiquitination via the SCF^{SKP2} ligase in S phase, although it is not clear whether this requires SET8 phosphorylation (Yin *et al.*, 2008; Oda *et al.*, 2010). However, depletion or deletion of SKP2, unlike $CRL4^{CDT2}$ inactivation, does not induce rereplication, suggesting that even in the presence of increased soluble fractions of these proteins, the $CRL4^{CDT2}$ is sufficient to prevent origin licensing by efficiently removing the chromatin-bound forms of these proteins.

Although CDT1, p21 and SET8 are largely undetectable in late G1 and throughout most of S-phase, they reappear in late S phase and in G2 (Abbas *et al.*, 2010). In the case of CDT1, this accumulation is critical for progression through G2, but this is not likely to be dependent on CDT1 ability to bind chromatin. This conclusion stems from the observation that CDT1 is phosphorylated by CDK1 in late S and early G2, and this prevents CDT1 from binding to chromatin, and that abolishing CDK1-dependent phosphorylation of CDT1 inhibits cell cycle progression (Rizzardi *et al.*, 2015). CDT1 is additionally phosphorylated by the stress-activated mitogen-activated protein kinases (MAPK) p38 and JNK and this too, precludes recognition by $CRL4^{CDT2}$ (Chandrasekaran *et al.*, 2011). A recent study suggested that CDT1 is also ubiquitinated and degraded in G2 cells via the SCF^{FBXO31} ubiquitin ligase, and that inactivation of this pathway results in minor rereplication (Johansson *et al.*, 2014). It is unclear from this study however, how the stabilized CDT1 in G2 cells with inactivated SCF^{FBXO31} gains access to chromatin in the presence of elevated CDK1 activity. The reaccumulation of SET8 in G2, similar to CDT1, is critical for cell cycle progression, and this is thought to be mediated through its ability to promote histone H4 methylation needed for chromatin condensation prior to entry into mitosis (Beck *et al.*, 2012b; Jørgensen *et al.*, 2013). Following the accumulation of methylated H4K20, and from prophase to early anaphase, cyclin B/CDK1 phosphorylates SET8 on Ser-29, and this removes SET8 from chromatin, without inhibiting its methyltransferase activity (Wu *et al.*, 2010). The dephosphorylation of SET8 in late M-phase by the CDC14 phosphatase primes the SET8 protein for proteolytic degradation via the APC/C^{CDH1} ligase (Wu *et al.*, 2010). The importance of p21 reaccumulation in G2 on the other hand, is not clear, but may be important to restrict cyclin A-CDK2 activity.

In addition to the mechanisms by which CDT1 is targeted for proteolysis in late G1 and in S phase, metazoans evolved another mechanism

to suppress CDT1 activity through the expression of a small protein inhibitor of CDT1 called geminin (Wohlschlegel *et al.*, 2000; Tada *et al.*, 2001). Geminin is under the transcriptional control of E2F1, which transactivates dozen other genes essential for S-phase progression, including cyclin E (Wong *et al.*, 2011). Geminin, however, undergoes ubiquitin-dependent degradation in late mitosis and early G1 via the APC/C^{CDH1} E3 ligase activity (McGarry and Kirschner, 1998). In late G1 and early S-phase, geminin is phosphorylated by cyclin E-CDK2, and this prevents its recognition by CDH1, stabilizing the protein, which directly binds CDT1 and sterically hinders its ability to recruit MCM2–7 complexes to replication origins (Tada, 2007; Caillat and Perrakis, 2012). At the same time, cyclin E-CDK2 phosphorylates CDH1, thereby inactivating APC/C^{CDH1} (Cappell *et al.*, 2016). In addition, residual CDH1 is inhibited by EMI1, marking a 'no return' decision to enter S-phase (Cappell *et al.*, 2016). EMI1 also binds CDC20 and inhibits the APC/C^{CDC20} ligase in S-phase, thereby stabilizing mitotic cyclins A and B, which are essential for the completion of DNA synthesis and G2 progression (Reimann *et al.*, 2001; Di Fiore and Pines, 2007; Cappell *et al.*, 2016). As mentioned above, in S phase, the SCF^{SKP2} ligase cooperates with $CRL4^{CDT2}$ to promote the degradation of soluble and chromatin-bound CDT1, respectively. The former pathway is aided by cyclin A-CDK2, which phosphorylates CDT1 at Thr-29, and requires EMI1 for suppressing APC/C^{CDC20}, which would otherwise ubiquitylate and degrade not only cyclin A, but also geminin. Whereas suppressing geminin initiates rereplication in certain cell types, it is insufficient to do so in some other cancer cell types or in non-malignant cells (Machida and Dutta, 2007; Zhu and Depamphilis, 2009; Benamar *et al.*, 2016). Activation of S phase APC/C ligase on the other hand (e.g. by depleting EMI1), is sufficient to initiate rereplication in the majority of mammalian cells examined (Machida and Dutta, 2007; Benamar *et al.*, 2016). Together, these findings highlight the importance of $CRL4^{CDT2}$ and EMI1 for restraining origin licensing in S phase by preventing the accumulation of chromatin-bound and active CDT1, as well as other replication licensing proteins.

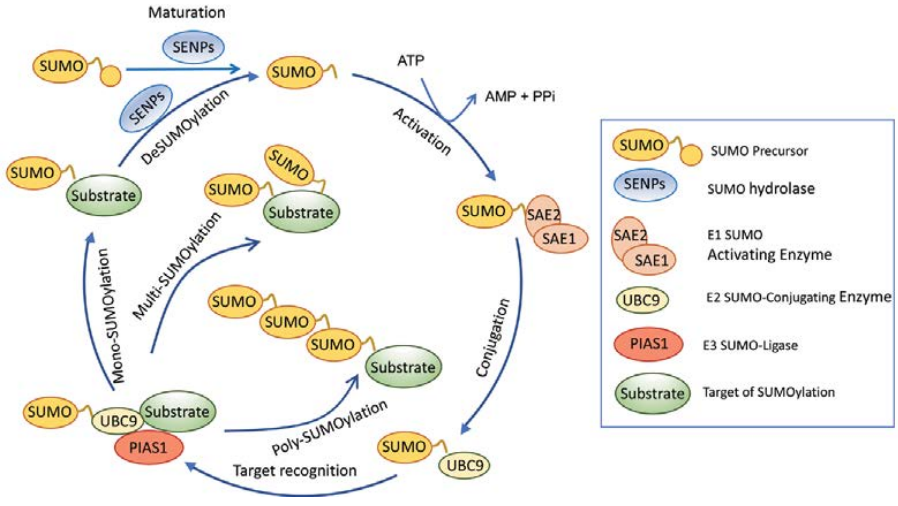


Figure 14.4 Posttranslational modification by SUMOylation. A schematic representing the various steps involved in the SUMOylation and deSUMOylation cycle. SUMO E1, E2 and E3 enzymes (mammalian representative of these enzymes is shown) promote the conjugation of SUMO to substrate proteins. DeSUMOylation is catalysed by SUMO-specific proteases [mammalian SENPs (Sentrin/SUMO-specific proteases) is shown as a representative example] and is involved both in SUMO maturation and in the removal of SUMO moieties from protein substrates.

SUMO-dependent regulation of replication initiation

Overview of the SUMOylation process

Modification via the small ubiquitin-like molecule SUMO (Fig. 14.4) also plays important roles in the regulation of eukaryotic DNA replication as well as the regulation of multiple other cellular activities including DNA repair, transcription, nuclear transport, and protein quality control (Sarangi and Zhao, 2015; Jalal *et al.*, 2017; Zilio *et al.*, 2017). Similar to ubiquitination, sumoylation involves the covalent conjugation of SUMO or SUMO chains to the ε amino-group Lys residue of substrates, and requires the sequential action of E1 activating, E2 conjugating, and E3 ligase enzymes (Johnson, 2004; Gareau and Lima, 2010; Lamoliatte *et al.*, 2014), reminiscent of that involved in protein ubiquitination. SUMO, like ubiquitin, is usually conjugated to Lys side chains of substrate protein and can be conjugated at single Lys in the substrate proteins (mono-sumoylation), at multiple Lys residues of the substrate proteins (multi-sumoylation), or form various length chains at single Lys in the protein substrates (poly-sumoylation) (Fig. 14.4).

Like ubiquitination, protein modification by sumoylation is reversible and is regulated by a set of SUMO-specific cysteine proteases (Mukhopadhyay and Dasso, 2007; Hickey *et al.*, 2012). SUMO proteases deconjugate SUMO proteins using their isopeptidase activity, cleaving between the terminal Gly of SUMO and the substrate Lys (Hickey *et al.*, 2012). The first described SUMO protease, the *S. cerevisiae* protein U1p1 (UBL-specific protease 1), exhibits distant similarity to certain viral proteases but is unrelated to any known deubiquitinating enzyme (Li and Hochstrasser, 1999). Mammalian cells express at least six SUMO-specific proteases, known as SENPs or Sentrin/SUMO-specific proteases (SENP1-SENP3 and SENP5-SENP7), that share significant sequence homology with U1p1, and can be broadly classified into three subfamilies based on their sequence homology, subcellular localization and substrate specificity (Mukhopadhyay and Dasso, 2007; Hickey *et al.*, 2012). Three additional SUMO-specific proteases, DESI1 (deSUMOylating isopeptidase 1), DES12 and USPL1 (ubiquitin-specific protease-like) exist in mammalian cells and share only little sequence similarity to U1P or SENPs (Schulz *et al.*, 2012; Shin *et al.*, 2012). Some SUMO-specific proteases are also important for SUMO maturation, as they cleave the precursor or inactive form of SUMO at the c-terminus to expose two glycine residues. SUMO proteases play important roles in protein–protein interaction and in regulating cellular localization, and significant effort is dedicated for the development of pharmacological inhibitors of this class of proteases for therapeutic purposes (Mukhopadhyay and Dasso, 2007; Hickey *et al.*, 2012; Kumar and Zhang, 2015; Bialik and Woźniak, 2017).

Regulation of replication initiation proteins via SUMOylation

As is the case for ubiquitination, modification of replication initiation proteins by sumoylation helps restrict origin licensing to late mitosis and early G1. Initial studies in budding yeast demonstrated that multiple subunits of the ORC complex undergo sumoylation, although the functional significance of these modifications is not entirely clear (Cremona *et al.*, 2012). Studies of human ORC2 demonstrated that this subunit is sumoylated in G2/M. ORC2 sumoylation restricts the ORC complex to centromeric regions within the genome and enhances the demethylation of histone H3 lysine 4 (H3K4) in centromeric chromatin via the recruitment of the H3K4 demethylase KDM5A (Craig *et al.*, 2003; Prasanth *et al.*, 2004; Lee *et al.*, 2012; Huang *et al.*, 2016). Inhibition of ORC2 sumoylation results in rereplication, polyploidy and DNA damage at centromeric chromatin that correlate with the accumulation of H3K4 trimethylation (H3K4me3) in centromeric chromatin, reduced transcription from centromeric α-Satellites, and replication from decondensed pericentric heterochromatin (Huang *et al.*, 2016). It remains to be seen whether the sumoylation of other ORC subunits or ORC2 from the other eukaryotes play a specialized role in the regulation of origin licensing as that seen for human ORC2 or exhibit similar, generalized, and, possibly, redundant function in preventing relicensing of replication origins from centromeric heterochromatin through promoting epigenetic changes.

Similar to ORCs, multiple subunits of the MCM2–7 complex from various eukaryotes were found to be sumoylated (Golebiowski *et al.*, 2009; Elrouby and Coupland, 2010; Cremona *et al.*, 2012; Hendriks *et al.*, 2014; Ma *et al.*, 2014; Schimmel *et al.*, 2014; Tammsalu *et al.*, 2014; de Albuquerque *et al.*, 2016; Wei and Zhao, 2016). MCM2–7 sumoylation appears to also negatively regulate replication initiation. This is supported by the finding that, in both man and yeast, the sumoylation of the six-subunit complex is detectable in G1, preceding DDK1-mediated phosphorylation of the MCM4 subunit, but is rapidly declined as cells enter S phase and remains undetectable until the G1 of the next cycle; the exception is with the yeast Mcm7 subunit, which persists throughout S phase and peaks with the completion of replication (Cremona *et al.*, 2012; Schimmel *et al.*, 2014; de Albuquerque *et al.*, 2016; Wei and Zhao, 2016). Studies in yeast demonstrate that Mcm2–6 sumoylation increases its association with the PP1 phosphatase, thereby preventing premature phosphorylation of Mcm4, an essential step for CMG formation and origin firing (Davé *et al.*, 2014; Hiraga *et al.*, 2014; Mattarocci *et al.*, 2014; Wei and Zhao, 2016). At the G1/S transition, and as cells enter S phase, the DDK kinase activity rises, and this, combined with Mcm2–6 desumoylation, potentially via the Ulp2 protease (de Albuquerque *et al.*, 2016; Wei and Zhao, 2016), aid in Mcm4 phosphorylation, CMG activation, and origin firing (Wei and Zhao, 2016). A further evidence in support of a negative role for sumoylation in the regulation of replication initiation in eukaryotes is obtained from a study in *Xenopus*, where the expression of SUMO-specific proteases or a dominant-negative SUMO E2 was found to increase origin firing (Bonne-Andrea *et al.*, 2013). Because the PPI-DDK-mediated regulation of MCM2–7 activation is conserved across eukaryotic species (Wotton and Shore, 1997; Lee *et al.*, 2003; Cho *et al.*, 2006; Masai *et al.*, 2006; Montagnoli *et al.*, 2006; Tsuji *et al.*, 2006; Cornacchia *et al.*, 2012; Hayano *et al.*, 2012; Yamazaki *et al.*, 2012), these studies support the conclusion that negative regulation of MCM2–7 phosphorylation through sumoylation is an evolutionary conserved mechanism that regulates replication initiation in eukaryotes.

Ubiquitin and SUMO regulation of DNA synthesis

Proteolytic and non-proteolytic roles for ubiquitin and SUMO at the replisome

Emerging evidence support important roles for protein ubiquitination and sumoylation in the regulation of unperturbed DNA synthesis (Fig. 14.5), as well as in coordinating DNA synthesis with chromatin dynamics (Almouzni and Cedar, 2016; García-Rodríguez *et al.*, 2016; Henikoff, 2016; Talbert and Henikoff, 2017). Proteomic analysis demonstrated that many of the components of the replisome were found to be ubiquitinated (Wagner *et al.*, 2011). Although ubiquitination plays both proteolytic and non-proteolytic functions during DNA synthesis, sumoylation of replisome components almost invariably plays only non-proteolytic regulatory roles. The non-proteolytic regulatory functions of ubiquitin and SUMO are not always apparent, although in some cases their role is beginning to be appreciated. For example, the catalytic subunit of polymerase δ in the fission yeast *Saccharomyces Pombe* is stable despite undergoing ubiquitination in unperturbed S phase (Roseaulin *et al.*, 2013). Pol2, the catalytic subunit of DNA polymerase ε, however, is ubiquitinated and degraded via the SCF^{Pof3} ligase (Roseaulin *et al.*, 2013). This implies that the synthesis of the leading strand requires a 'fresh' supply of DNA polymerase, whereas the synthesis of the discontinuous lagging strand does not (Roseaulin *et al.*, 2013). In mammalian cells, both regulatory subunits of DNA polymerase δ (POL δ), p66 and p12, are ubiquitinated during S phase, and this modification appears to regulate protein–protein interactions either within the polymerase holoenzyme or with other replication factors (Liu and Warbrick, 2006). Interestingly, the suppression of fork progression in response to DNA damage is mediated, at least in part, through ubiquitin-dependent proteolysis of the p12 subunit via the $CRL4^{CDT2}$ ligase, which requires the interaction of p12 with PCNA (Terai *et al.*, 2013). This is only one of the several examples of the role of UPS in regulating DNA replication under replication stress or in response to DNA damage, which are described in greater details in several recent outstanding reviews (Sommers *et al.*,

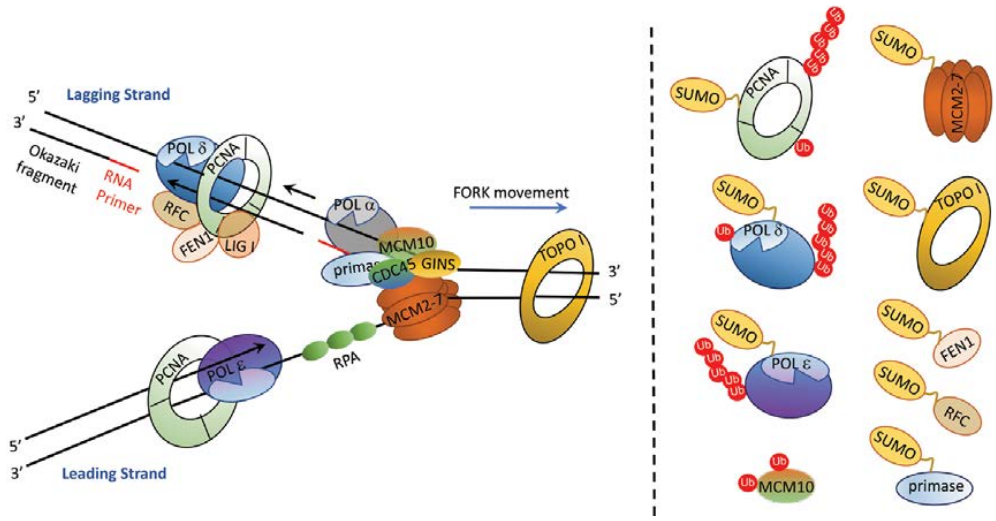


Figure 14.5 Ubiquitin and SUMO regulation of DNA synthesis. A schematic model of the replication fork during DNA synthesis in eukaryotic cells. The CMG replicative helicase (MCM2–7/GINS/CDC45) unwinds the duplex DNA ahead of the replication fork. Topoisomerase TOPO I is important for the relaxation of the positive supercoiling building ahead of the replication fork. TOPO II (not shown) can resolve the intertwining of the daughter DNA strands resulting from the fork rotation behind the replication fork. Single-stranded DNA (ssDNA) is coated by the ssDNA binding protein RPA (replication protein A). The replication factor C (RFC) loads PCNA and DNA polymerase ε (POL ε) to synthesize the leading strand (continuous replication). On the lagging strand, DNA polymerase α (POL α), which is stabilized by the Minichromosome maintenance protein 10 (MCM10), synthesizes short RNA/DNA primer. RFC subsequently displaces POL α, and polymerase δ (POL δ) synthesizes short DNA segments (Okazaki fragments). The flap structure-specific endonuclease FEN1 processes the 5′ ends of Okazaki fragments, and the DNA ligase I (LIG I) joins the DNA fragments (discontinuous replication). Many of these proteins (shown on right) are modified by ubiquitination and SUMOylation and this is important for the regulation of DNA synthesis (see text for details).

2015; García-Rodríguez *et al.*, 2016; Renaudin *et al.*, 2016).

Another replisome protein that undergoes both proteolytic and non-proteolytic ubiquitination is the minichromosome maintenance protein 10 (MCM10). Mcm10 was first identified by Lawrence Dumas and colleagues in a screen for temperature-sensitive mutants for S phase progression defects in *S. cerevisiae* and denoted as *dna43* (Dumas *et al.*, 1982). *MCM10* was subsequently identified (and the gene sequenced) in an independent study aimed at identifying replication initiation mutants that are defective in the maintenance of minichromosomes (Merchant *et al.*, 1997). MCM10 is an essential DNA replication factor and is conserved in all eukaryotes but is absent in bacteria and archaea. The protein functions primarily as a scaffold protein with DNA binding properties but lacks enzymatic functions. Initial studies in fission yeast demonstrated that Mcm10/Cdc23 plays a role in replication initiation through facilitating Cdc45 chromatin binding, an essential step in CMG activation (Gregan *et al.*, 2003). Subsequent studies showed that Mcm10 facilitates the initial strand separation through its binding to origins through its Zink finger-dependent DNA binding activity (Kanke *et al.*, 2012; van Deursen *et al.*, 2012; Thu and Bielinsky, 2013). In budding yeast, Mcm10 appears to play an additional role in replication elongation through interacting with and stabilizing the catalytic subunit of DNA polymerase α (Pol1) (Ricke and Bielinsky, 2004). In G1 and in S phase, the budding yeast Mcm10 undergoes mono-ubiquitination at two Lys residues (diubiquitination) and this was shown to be essential for its interaction with PCNA and for cell growth (Das-Bradoo *et al.*, 2006; Thu and Bielinsky, 2013). Similar to budding yeast, mammalian MCM10 interacts with and stabilizes the catalytic subunit of DNA POL α (p180) (Fig. 14.5), and this is important for efficient DNA

synthesis (Chattopadhyay and Bielinsky, 2007; Zhu *et al.*, 2007). Whether mammalian MCM10 undergoes di-ubiquitination, and if this regulates its ability to interact with PCNA or with other components of the replisome is not known. Mammalian MCM10 however, was shown undergo ubiquitin-dependent degradation both in unperturbed cells and following exposure to of cells to ultraviolet radiation (UV) (Kaur *et al.*, 2012; Romani *et al.*, 2015). Although MCM10 degradation following DNA damage may be important to halt DNA synthesis in the face the bulky DNA lesions induced by UV, the significance of its proteolytic degradation during unperturbed S phase remains to be determined.

Systematic and proteome-wide proteomic studies demonstrate that many of the replisome proteins that are regulated via the UPS are also sumoylated (Cremona *et al.*, 2012; Tammsalu *et al.*, 2014; Bursomanno *et al.*, 2015). Similar enrichment for poly-sumoylated proteins during DNA synthesis is also observed using an *in vitro* replication assay in Xenopus egg extract (Bonne-Andrea *et al.*, 2013). Additional studies utilizing a method of isolating proteins on nascent DNA coupled with mass spectrometry (iPOND-MS) also demonstrate that chromatin isolated within the vicinity of the replisome is significantly enriched for sumoylated proteins (Lopez-Contreras *et al.*, 2013; Dungrawala *et al.*, 2015). These studies also demonstrated a relative depletion of ubiquitination events, suggesting an interaction between ubiquitination and sumoylation at the replisome. The identity of the E3 SUMO ligase responsible for protein sumoylation at the replisome is not known, but PIAS1 is a good candidate given its enrichment at these active replicating sites (Lecona *et al.*, 2016). The USP7/HAUSP (Herpesvirus-associated ubiquitin-specific protease) DUB is another protein that is enriched at active DNA synthesis sites and may be responsible for the observed depletion of ubiquitinated proteins (Lecona *et al.*, 2016). USP7 is a SUMO-DUB (SDUB), and is one of only two DUBs (the other is USP11) that have been shown to deubiquitylate sumoylated proteins (Hendriks *et al.*, 2015; Lecona *et al.*, 2016). Pharmacological inhibition of USP7 slows replication fork progression, inhibits new origin firing, and reverses the high-SUMO and low-ubiquitin chromatin environment observed at or near the replisome (Bonne-Andrea *et al.*, 2013; Lopez-Contreras *et al.*, 2013; Lecona *et al.*, 2016). How USP7 regulates new origin firing and replication progression is not entirely clear, but likely dependent on the stabilization of sumoylated replisome components that are essential for the replisome activity (Lecona *et al.*, 2016; Wei and Zhao, 2016). This conclusion is substantiated by the reduced replication progression in SUMO E2 and E3 mutants as well as by the prolonged S phase progression seen in human cells with inactivated UBC9 SUMO-conjugating enzyme (Cremona *et al.*, 2012; Schimmel *et al.*, 2014; Hang *et al.*, 2015). These studies, however, do not exclude the possibility that the accumulation of ubiquitinated proteins (or their ubiquitin-dependent degradation) upon USP7 inhibition may contribute to the inhibition of replication progression or new origin firing.

One of the most notable examples of replisome proteins that is regulated by sumoylation is the budding yeast Pol2. Pol2 sumoylation is mediated by the Nse2/Mms21 SUMO ligase, and this sumoylation, as well as the sumoylation of Mcm6, is reduced not only in cells with mutations in Nse2, but also in cells deficient in Rtt107, a multi-functional scaffolding protein that plays multiple roles in replication progression (Hang *et al.*, 2015). Although the main function of Pol2 sumoylation is not entirely clear, it is tempting to speculate that it may have important regulatory role for controlling DNA polymerase activity during replication fork progression. Significantly, the Nse2/Mms21 SUMO ligase, along with the Ubc9 SUMO-conjugating enzyme, also plays a role for the sumoylation of Smc5 and Smc6 subunits of the SMC (structural maintenance of chromosomes) SMC5/6 complex, and this is important for the repair of collapsed replication forks and for counteracting recombinogenic events at damaged replication forks (Ampatzidou *et al.*, 2006; Branzei *et al.*, 2006; Chen *et al.*, 2009; Xue *et al.*, 2014). Interestingly, Rtt107, which plays an important role in cellular response to replication stress to reduce replication-associated recombination, forms two additional and distinct complexes with the cullin 4 E3 ubiquitin ligase $Rtt101^{Mms22}$ (Collins *et al.*, 2007; Hang and Zhao, 2016; Xue *et al.*, 2014), and with the Slx4 scaffolding protein (Hang and Zhao, 2016). The $Rtt101^{Mms22}$ ubiquitin ligase ubiquitylates acetylated histone H3, and this facilitates nucleosome assembly during replication

(Han *et al.*, 2013). The Rtt107–Slx4 complex on the other hand, is critical for controlling recombination during DNA replication, particularly under conditions of replicative stress (Chin *et al.*, 2006; Roberts *et al.*, 2006; Ohouo *et al.*, 2010). The Ataxia telangiectasia related protein kinase ATR and the checkpoint protein CHK1 play important roles in stabilizing stalled replication forks and for preventing their collapse into DSBs. In mammalian cells, the generation of DSBs following ATR inhibition is dependent on the SLX4 scaffold endonuclease, and requires the activity of the RNF4 E3 ubiquitin ligase that promotes the ubiquitin-dependent degradation of sumoylated proteins at stalled replication forks (Ragland *et al.*, 2013). Interestingly, RNF4 also promotes the polyubiquitination of activated Fanconi anaemia proteins FANCD2 and FANCI following their ATR-dependent sumoylation by the SUMO E3 ligases PIAS1/PIAS4 at stalled replication forks (Gibbs-Seymour *et al.*, 2015). Ubiquitinated FANCD2 and FANC1 are subsequently removed from the stalled replication sites through the activity of the DVC1-p97 segregase complex, and inactivation of FANCD2/FNACI sumoylation compromises cell survival in response to replication stress (Gibbs-Seymour *et al.*, 2015). This example highlights the interplay between sumoylation and ubiquitination in the regulation of DNA replication at active replication sites both during normal replication and in response to replication stress.

In addition to undergoing mono-ubiquitinated, the p66 subunit of the mammalian DNA POL δ is also mono-sumoylated by SUMO3, and this modification likely regulates protein–protein interaction or impacts the polymerase function (Liu and Warbrick, 2006). Other proteins involved in the synthesis of DNA lagging strand, such as the flap endonuclease 1 protein (FEN1), also undergoes sumoylation. FEN1 sumoylation in human cells is mediated by SUMO3 and begins in S phase and peaks in G2/M (Guo *et al.*, 2012). FEN1 sumoylation promotes its ubiquitination and degradation via the PRP19 E3 ligase, which interacts with sumoylated FEN1 at least in part through its SIM (sumo-interacting motif) motif (Guo *et al.*, 2012). Interestingly mutation of Ser-187 in FEN1 to Ala abrogates the phosphorylation at this site and precludes FEN1 sumoylation resulting in cell cycle delay and polyploidy (Guo *et al.*, 2012).

In addition to the various components of the replicative helicases and polymerases, other components of the replisomes, including topoisomerases, DNA primase, the clamp loader RFC complex, as well as the nucleosome remodelling factor FACT were also found to be sumoylated (Golebiowski *et al.*, 2009; Elrouby and Coupland, 2010; Cremona *et al.*, 2012; Ma *et al.*, 2014; Tammsalu *et al.*, 2014). Among these, the sumoylation of DNA topoisomerase (TOP1) is best understood. The PIAS1 SUMO ligase was recently shown to sumoylate TOP1, and this is essential for reducing R-loop-mediated stalling of replication forks (Li *et al.*, 2015). Biochemically, TOP1 sumoylation inhibits its catalytic activity, thereby reducing the nicking of DNA at transcriptionally active sites (Li *et al.*, 2015). TOP1 sumoylation also enhances its binding to active RNA polymerase II, resulting the recruitment of splicing factors to suppress R-loop formation (Li *et al.*, 2015). The role of sumoylation and/or ubiquitination in the regulation of other replisome components as well as other complexes involved in replication progression, such as components of the SMC complex (e.g. cohesin, condensin), is less understood, although emerging evidence support an important role for sumoylation in cohesion establishment (Rudra and Skibbens, 2013).

PCNA: A central hub for ubiquitination and SUMOylation signalling

One of the best examples for the role of ubiquitination and sumoylation in the regulation of DNA replication progression involves PCNA (Fig. 14.5). The homotrimeric DNA polymerase sliding-clamp coordinates the activity of many proteins involved in DNA replication, DNA repair and other chromatin-related transactions (Choe and Moldovan, 2017; Ulrich and Takahashi, 2013). Although PCNA can be ubiquitinated at multiple Lys residues (McIntyre and Woodgate, 2015), only the mono-ubiquitination of PCNA at a conserved Lys residue (Lys-164 in human PCNA) is well understood. This particular modification is carried out by the Rad6-Rad18 E2-E3 ubiquitin conjugating enzyme/ligase and is one of the best understood posttranslational modifications of this protein. Such modification impacts the affinity of PCNA for different DNA polymerases, and is essential for error-prone translesion DNA synthesis (TLS) through the recruitment

of translesion Y-family DNA polymerases [e.g. polymerase eta (POL-η)] to replication factories to bypass replication-stalling DNA lesions (Yang *et al.*, 2013). This recruitment is dependent on the ubiquitin-binding domain of TLS polymerases, which has a high-affinity to mono-ubiquitinated PCNA (Bienko *et al.*, 2005; Plosky *et al.*, 2006). The $CRL4^{CDT2}$ and RNF8 ubiquitin ligases are two other E3 ligases that can substitute for Rad18 in promoting PCNA mono-ubiquitination (Zhang *et al.*, 2008; Terai *et al.*, 2010). Although this post-translational modification is significantly stimulated in cells exposed to bulky DNA lesions, such as those induced by UV (e.g. cyclobutane pyrimidine dimers), mono-ubiquitinated PCNA is detectable in normal proliferating cells in the absence of DNA damage, perhaps to aid in the replication of difficult to replicate DNA sequences or to cope with replication stress (Leach and Michael, 2005; Frampton *et al.*, 2006; Terai *et al.*, 2010).

In *S. cerevisiae*, the heterodimeric E2 ubiquitin conjugating enzyme, Ubc13-Mms2, which is recruited to chromatin by the RING-finger protein Rad5, can convert the mono-ubiquitinated Lys on PCNA to Lys-63-linked polyubiquitin chain to participate in gap-filling damage tolerance (Prakash, 1981; Hoege *et al.*, 2002; Torres-Ramos *et al.*, 2002; Branzei *et al.*, 2004; Haracska *et al.*, 2004) and in template switching, an error-free pathway of DNA that utilizes the newly replicated sister chromatid as a template for replication (Hoege *et al.*, 2002; Branzei *et al.*, 2008, 2011; Choi *et al.*, 2010; Hedglin and Benkovic, 2015).

In mammals, this biochemical activity is carried out by the SNF2 histone linker plant homeodomain RING helicase (SHPRH) or by the helicase-like transcription factor (HLTF), and this was shown to suppress PCNA-dependent TLS and mutagenesis (Motegi *et al.*, 2008; Unk *et al.*, 2008). Interestingly, RAD18 itself can be mono-ubiquitinated, and this PTM inhibits its ability to mono-ubiquitylate PCNA and, the same time, suppresses its interaction with SHPRH or HLTF (Lin *et al.*, 2011; Moldovan and D'Andrea, 2011; Zeman *et al.*, 2014). Template switching is further facilitated by USP7, which deubiquitinates and stabilizes both HLTF and RAD18 through enhancing the interaction between the non-ubiquitinated RAD18 and HLTF (Qing *et al.*, 2011; Zeman *et al.*, 2014). Under replicative stress (e.g. following treatment with the alkylating agent methyl methanosulfonate (MMS)), USP7 also deubiquitinates and stabilizes both RAD18 and POL-η, and this promotes TLS (Qian *et al.*, 2015; Zlatanou *et al.*, 2016). Under these conditions, the E3 ubiquitin ligase TNF receptor associated factor (TRAF)-interacting protein (TRIP) also facilitates TLS by promoting the Lys-63-polyubiquitination of POL-η, which is required for its focus formation at damage sites (Wallace *et al.*, 2014).

Several activities restrain TLS activity to reduce or prevent the mutagenic load caused by the low-fidelity polymerases. USP7 likely plays a role in this regulatory step by removing the mono-ubiquitin moiety on PCNA (Kashiwaba *et al.*, 2015). The isopeptidase USP1, however, plays a more prominent role in deubiquitinating PCNA and in turning off TLS (Huang *et al.*, 2006; Andersen *et al.*, 2008). TLS is also restrained under conditions of increased DNA damage by UV irradiation, and this is mediated by USP10, which also deubiquitinates PCNA (Park *et al.*, 2014). USP10-dependent PCNA deubiquitination requires the activity of EFP, an ISG15 E3 ligase, which ISGylate mono-ubiquitinated PCNA, thereby recruiting USP10 to deubiquitylate PCNA (Park *et al.*, 2014). Following the release of POL-η, PCNA is de-ISGylated by UBP43, and engages the replicative DNA polymerases to resume normal replication, and inactivation of this pathway increases mutagenesis. In yeast, however, increased PCNA mono-ubiquitination, for example through inactivating the PCNA deubiquitinase Ubp10, does not increase mutagenesis, suggesting the existence of other mechanisms to suppress TLS (Gallego-Sanchez *et al.*, 2012). The TLS polymerases themselves are subject to proteolytic degradation, and in the case of POL-η, this is mediated by MDM2 (Jung *et al.*, 2012). POL-η can also be mono-ubiquitinated by the PIRH2 E3 ligase, and this suppresses its interaction with mono-ubiquitinated PCNA (Jung *et al.*, 2010, 2011). Inactivation of TLS polymerases through ubiquitination is also conserved in yeast. For example, the *S. cerevisiae* homologue of POL-η, Rad30, as well as the Rev1 polymerase undergo proteolytic degradation, and for Rad30, this is mediated via the SCF^{Ufo1} ubiquitin ligase (Waters and Walker, 2006; Skoneczna *et al.*, 2007; Plachta *et al.*, 2015).

Sumoylation also plays important roles for regulating PCNA function. In fact, PCNA is sumoylated at the same Lys residue that is

subject to mono-ubiquitination, suggesting that both sumoylation and ubiquitination of the same residue on PCNA is tightly regulated for optimal activity of this important protein. In yeast, PCNA sumoylation on Lys-164 (and to a lesser extent on Lys-127) is cell cycle regulated, preceding the entry of cells into S-phase, and is robustly induced by severe or lethal DNA damage (Hoege *et al.*, 2002; Branzei, 2011; Hedglin and Benkovic, 2015). PCNA sumoylation, which is catalysed by the Ubc9 SUMO-conjugating enzyme, appears to interfere with PCNA-polymerase binding and with DNA repair, and is likely to be important for unloading PCNA during normal replication (Hoege *et al.*, 2002; Branzei, 2011; Hedglin and Benkovic, 2015). Inactivation of UBC9 function in human cell lines prolongs S-phase, but it is unclear whether this is due to suppression of PCNA sumoylation (Schimmel *et al.*, 2014). PCNA sumoylation is also important for the recruitment of the Srs2 helicase and anti-recombinase to suppress spontaneous and DNA damage-induced homologous recombination during S phase (Papouli *et al.*, 2005; Pfander *et al.*, 2005; Armstrong *et al.*, 2012; García-Rodríguez *et al.*, 2016; Zilio *et al.*, 2017). Inactivation of PCNA sumoylation was also shown to suppress post-replication repair associated with template switching (Branzei *et al.*, 2008), and this likely due to interference between SUMO–PCNA interaction with Srs2 and/or with Rad18, Rad5 and ELg1 (an alternative subunit of the RFC clamp loader) (Pfander *et al.*, 2005; Parnas *et al.*, 2010). Sumoylation of mammalian PCNA is less abundant (Gali *et al.*, 2012), reflecting the lower recombination activity in mammals.

Regulation of replication termination by ubiquitin and SUMO

How eukaryotic DNA replication is terminated is not entirely clear, but emerging evidence support important roles for ubiquitination in this process (Fig. 14.6). Studies in budding yeast and in Xenopus egg extracts show that the disassembly of the CMG complex is dependent on Lys-48-linked polyubiquitination of the MCM7 subunit of the MCM2–7 helicase (Maric *et al.*, 2014; Moreno *et al.*, 2014). Ubiquitinated MCM7 is recognized by the hexameric AAA+ adenosine triphosphatase (ATPase) and the segregase Cdc48/p97, which

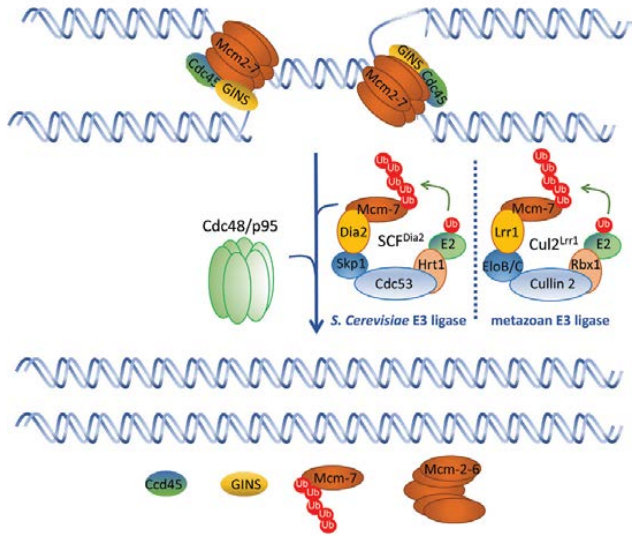


Figure 14.6 Ubiquitin-dependent regulation of replication termination in eukaryotes. A model depicting the termination of eukaryotic DNA replication at converging replication forks, and its regulation by the ubiquitination of the Mcm7 Subunit of the Mcm2–7 helicase complex leading to the disassembly of the CMG complex (Mcm2–7-GING-Cdc45). Mcm7 ubiquitination is promoted by the SCF^{Dia2} E3 ubiquitin ligase in *S. cerevisiae* and by the $CRL2^{Lrr1}$ E3 ubiquitin ligase in metazoans and is extracted through the activity of the p97 chaperon.

on ATP hydrolysis promotes protein unfolding (Barthelme *et al*., 2014; Maric *et al*., 2014; Moreno *et al*., 2014). This triggers MCM7 translocation through the Cdc48/p97 ring, with the consequent disassembly of the hexameric MCM2–7 complex and replication termination (Bell, 2014; Lengronne and Pasero, 2014). Mcm7 polyubiquitination in *S. cerevisiae* is mediated by the SCF E3 ligase and the F-box protein Dia2, and inactivation of this pathway prevents CMG disassembly resulting in replication defects, although Mcm2 proteolysis is not required for Mcm2–7 disassembly and replication termination (Maric *et al*., 2014; Moreno *et al*., 2014; Morohashi *et al*., 2009). Polyubiquitination of the MCM7 in *C. elegans* and in *Xenopus*, is carried out by the replisome associated ubiquitin ligase $CRL2^{Lrr1}$, which is similarly required for replication termination (Dewar *et al*., 2017; Sonneville *et al*., 2017). A role for protein sumoylation is replication termination is also beginning to emerge. In *S. cerevisiae* for example, the termination of DNA replication is associated with a specific reduction in Mcm7 sumoylation, which unlike the sumoylation of the other Mcm2–6 subunits, is concordant with the completion of DNA replication concurrent with increases in polyubiquitination of this subunit. (Wei and Zhao, 2016). It remains to be determined if the sumoylation of Mcm7 interferes with or is coordinated with the polyubiquitination of this subunit and with replication termination. As mentioned above, the Top2 DNA topoisomerase in budding yeast has been implicated in promoting replication across TERs, and this is important for the merging of the converging replication forks at these replication termination sites (Fachinetti *et al*., 2010). Top2 is also important for the decatenation of sister chromatids (Lee and Bachant, 2009). Interestingly, a subset of Topo II in various eukaryotes, including human TOPO II, is found to be sumoylated. In mitosis, the sumoylation of metazoan Topo II is essential for its recruitment to kinetochores, and interference with this sumoylation results in elevated frequency of segregation errors and aneuploidy (Lee and Bachant, 2009). A similar function for Topo II sumoylation in promoting replication termination is expected, but a concrete evidence for this prediction is yet to emerge.

Concluding remarks

Significant advances in our understanding of the molecular and biochemical activities that function to control DNA replication have been made in the last few decades. The identification and characterization of the various PTMs of the many proteins that are associated with almost every step of DNA replication enriched our appreciation of the complexity underlying this highly conserved and important biological activity. In particular, the covalent attachment of ubiquitin and/or the ubiquitin-related protein SUMO on specific Lys residues on replication and replication-related proteins to form monomers and polymers of ubiquitin or SUMO chains ensures the timely and efficient temporal and spatial control of replication both during normal proliferation and in response to various perturbations. Modification of replication proteins by ubiquitin and SUMO involves both proteolytic and non-proteolytic functions that operate cooperatively through convoluted feedback mechanisms that, together with other PTMs, provide rich and complex networks of protein-protein communications to control both the fidelity and robustness of DNA replication. The execution of these modifications by a diverse and highly specific set of E2–E3 pairs of ubiquitin and SUMO conjugating enzymes and ligases, as well as their reversal by an equally diverse and specific set of ubiquitin- and SUMO-proteases, adds a readily apparent new layer of complexity that will require significant more research to fully understand and appreciate. While we know a great deal about the mechanisms involved in the ubiquitination and sumoylation of replication proteins and their impact on replication, proteome-wide studies indicate that many more replication and replication-related proteins are modified by these versatile moieties, both during normal replication and in response to cellular stresses, particularly those that cause replication stress. For these, the challenge is to understand the functional significance of these additional modifications and to identify the biochemical activities underlying their regulation. It is expected that new breakthroughs will come to be soon realized given the recent development of novel state-of-art biochemical protocols and assays (e.g. iPOND-MS and proximity labelling

assays), gene-specific editing tools (e.g. CRISPR/Cas and TALENs) as well as new genetic screening and functional assays. Lessons from past research, as outlined in this review, indicate that few family members of the ubiquitin and SUMO conjugating and deconjugating enzymes, such as the SCF, APC/C and CRL4 ubiquitin ligases, the USP7 deubiquitinase, the UBC9 SUMO conjugating enzyme as well as the SUMO ligase PIAS1, play key role in the regulation of the various aspects of eukaryotic DNA replication. These will likely to dominate the scene in future research in this area.

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