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Review

Ubiquitylation at Stressed Replication Forks: Mechanisms and Functions

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Accurate duplication of chromosomal DNA is vital for faithful transmission of the genome during cell division. However, DNA replication integrity is frequently challenged by genotoxic insults that compromise the progression and stability of replication forks, posing a threat to genome stability. It is becoming clear that the organization of the replisome displays remarkable flexibility in responding to and overcoming a wide spectrum of fork-stalling insults, and that these transactions are dynamically orchestrated and regulated by protein post-translational modifications (PTMs) including ubiquitylation. In this review, we highlight and discuss important recent advances on how ubiquitin-mediated signaling at the replication fork plays a crucial multifaceted role in regulating replisome composition and remodeling its configuration upon replication stress, thereby ensuring high-fidelity duplication of the genome.

Highlights

Ubiquitin modifications dynamically impact the composition and functionality of both unperturbed and stressed replication forks.

Ubiquitylation of major replisome platforms including proliferating cell nuclear antigen (PCNA), replication protein A (RPA), and FANCI–FANCD2 (Fanconi anemia group I protein–Fanconi anemia group D2 protein), enables recruitment of multiple effector proteins involved in overcoming diverse types of impediments to replication fork progression.

A range of E3 ubiquitin ligases and ubiquitin-binding proteins promote fork reversal and protection upon replication stress.

Both fork-stalling lesions that can be directly bypassed by the replicative CMG helicase and those requiring prior processing (e.g., interstrand and DNA-protein crosslinks) require ubiquitin signaling for preservation of replication integrity.

Ubiquitylation of CMG is essential for its unloading upon fork convergence during replication termination and DNA interstrand crosslink repair.

Replication Stress and Ubiquitin Signaling

Precise and complete replication of cellular DNA during the S phase of each cell cycle is essential for genome stability, cell proliferation, and organismal fitness. The DNA replication process commences prior to S phase, when replication origins are licensed by the loading of inactive double minichromosome maintenance complex (MCM)2–7 hexamers [1–3]. In S phase, origin firing converts these double hexamers into active, bidirectional replication forks, through the recruitment of CDC45 and the GINS complex, leading to formation of the replicative CDC45–MCM2–7–GINS (CMG) helicase that translocates on the leading strand to unwind the duplex DNA template [4,5]. Although eukaryotic DNA replication initiates from multiple replication origins, only a fraction of licensed origins fire during a normal S phase. However, when obstacles that hinder replication fork progression are encountered, activation of otherwise dormant nearby origins provides an important rescue mechanism for completing genome duplication [6]. In S phase, the active replisome, consisting of the CMG helicase, replicative DNA polymerases, the replication factor C (RFC)-loaded clamp proliferating cell nuclear antigen (PCNA), and auxiliary factors, regulates every aspect of bidirectional replication, which occurs continuously on the leading strand and discontinuously on the lagging strand [7].

Deregulation of DNA replication, giving rise to **replication stress** (see Glossary), is a hallmark of cancer cells and a recognized driver of genomic instability [8–10], representing an attractive target for clinical intervention. However, given the sheer size and complex organization of vertebrate genomes, low levels of replication stress occur naturally in most proliferating cells, arising due to obstacles including heterochromatin-imposed barriers (e.g., repetitive DNA sequences and G4 quadruplexes), replication-transcription collisions, ribonucleotide misincorporation, modified or mismatched nucleotides, and helix-distorting adducts that stall the advancing replication machinery [10]. Under normal conditions, such impediments are quickly resolved by replisome-associated factors, **mismatch and excision repair pathways**, or converging forks that complete replication downstream of blocked or inactive forks. Exacerbated replication stress, induced by oncogenes that deregulate the physiological DNA replication program, or exogenous agents,

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including mainstay chemotherapeutic drugs that drain essential DNA replication resources such as dNTP pools or generate replication fork barriers in the form of challenging DNA lesions [e.g., **DNA interstrand crosslinks (ICLs)** and **DNA-protein crosslinks (DPCs)**], may necessitate more extensive remodeling of replication fork structure and composition [10–12] (Figure 1A–C). Accordingly, cells employ sophisticated rescue mechanisms for dealing with the threats posed by a diverse range of genotoxic insults encountered by replication forks. These processes are dynamically orchestrated and regulated by protein PTMs, in particular phosphorylation and ubiquitylation. Protein modification by ubiquitin is a versatile regulatory mechanism that is mediated by an extensive network of enzymatic activities and impacts virtually all cellular processes (Box 1) [13]. While the indispensable roles of ubiquitin and the **small ubiquitin-like modifier (SUMO)** in the cellular response to DNA damage, particularly DNA double-strand breaks (DSBs), have been extensively characterized [14,15], their involvement in responses to replication stress has remained less well defined. However, a flurry of recent discoveries now paints a more detailed picture of the crucial functions of replisome-associated ubiquitylation processes, the factors involved, and their multifaceted underlying mechanisms in promoting protective responses to many types of fork-stalling insults (Figures 1 and 2 and Table 1).

In this review, we summarize and discuss important novel insights into how ubiquitin-dependent signaling regulates major features of the replication stress response in vertebrates to safeguard genome stability, and we highlight key outstanding questions in this area.

Replisome Composition and Replication Stress Signaling

The past decade has witnessed the development and application of innovative methods such as isolation of proteins on nascent DNA (iPOND), nascent chromatin capture (NCC), and CHROmatin MASS spectrometry (CHROMASS), which coupled with mass spectrometry have provided system-level insights into the composition of the replication machinery under both unperturbed and stressful conditions [16–18]. These analyses revealed that while ubiquitylation regulates multiple aspects of unperturbed replication [19], nascent chromatin associated with unchallenged replisomes harbors low levels of ubiquitylation but is enriched in SUMO modifications, relative to mature chromatin [20]. This ubiquitin/SUMO balance at the replisome may contribute to an environment permissive for efficient DNA replication and is in part maintained by the catalytic activity of the fork-associated deubiquitylating enzyme (DUB) USP7, which deubiquitylates SUMOylated proteins to prevent their displacement from forks and is critical for preserving proper replication fork and origin firing rates [21]. Extraction of ubiquitylated proteins from the replisome is fueled by the activity of the ubiquitin-selective ATPase **p97** [22]. Via its cofactors NPL4, UFD1, and FAF1/UBXN-3, p97 dynamically remodels replisome composition by promoting the turnover of several proteins, including the replication licensing factor CDT1, whose fork-associated ubiquitylation by the $CRL4^{CDT2}$ E3 ligase complex and subsequent proteasomal destruction are templated by their interaction with PCNA [23–25]. p97 is also instrumental for ubiquitin-dependent CMG unloading upon replication termination and DNA damage [26–30] as described later, and likely targets additional ubiquitylated client proteins at the replisome. Histone H2A monoubiquitylation constitutes one of the most abundant ubiquitin modifications in the cell, but may be present at low levels at nascent DNA given the delayed post-replicative chromatin reloading of its writers, the Polycomb complex E3 ligases RING1A/B and BMI1 [16], which could also contribute to a relatively ubiquitin-poor chromatin environment in the context of intact forks. In response to replication stress, however, replisomes are rapidly decorated with ubiquitin chains [17] via the concerted actions of E3 ligases that travel with the replication fork or are recruited upon fork-stalling insults [17,18,31]. Simultaneously, inactivation of replisome-associated DUBs such as USP1 [32] and enrichment of others, including the K63-selective DUB ZUFSP [33,34], may contribute to altering ubiquitin dynamics and conformations at replication stress

Glossary

DNA damage tolerance: DNA damage bypass pathways enabling replication past lesions that cannot be copied by replicative DNA polymerases and therefore stall replication. DNA damage tolerance pathways include translesion DNA synthesis (TLS) and template switching (TS), stimulated by PCNA monoubiquitylation and polyubiquitylation, respectively. These processes can be engaged at or behind the replication fork.

DNA interstrand crosslinks (ICLs): highly genotoxic lesions that covalently link the two DNA strands, preventing strand separation to impede DNA-associated transactions, including DNA replication and transcription.

DNA-protein crosslinks (DPCs): covalently trapped proteins on DNA that form roadblocks to essential DNA transactions, including DNA replication and transcription. DPCs can be generated endogenously by reactive metabolites (e.g., aldehydes), the abortive action of Topoisomerases, and by many mainstay chemotherapeutic agents.

Fanconi anemia (FA): rare recessive disorder characterized by bone-marrow failure, cancer predisposition, infertility, and congenital abnormalities. The 22 genes currently known to be mutated in FA patients encode proteins involved in DNA replication-dependent ICL repair.

Mismatch and excision repair pathways: cells employ multiple DNA repair pathways to remove DNA lesions before arrival of the replication fork and enable unhindered fork progression. Mismatch repair (MMR) corrects wrongly incorporated bases caused by infrequent replicative polymerase errors and frameshift mutations arising due to polymerase slippage. Base excision repair (BER) repairs damaged bases, ssDNA nicks, and abasic sites. Nucleotide excision repair (NER) resolves helix-distorting, bulky adducts, such as UV-induced damage.

Nucleolytic degradation: stalled replication forks contain exposed ssDNA stretches that are susceptible to nucleolytic degradation by several nucleases. Tightly controlled, limited nucleolytic processing of nascent DNA may facilitate fork restart. However, extensive nucleolytic degradation of ssDNA at replication forks results in aberrant structures that are prone to

sites. Collectively, these replication stress-induced events trigger robust ubiquitin-dependent modifications of key replisome components, including PCNA, replication protein A (RPA), and FANCI–FANCD2 (Fanconi anemia group I protein–Fanconi anemia group D2 protein), thereby generating recruitment platforms for the dynamic assembly of a range of ubiquitin-binding effector proteins, that mount tailored responses to different types of replication stress (Figure 1B,C), as described in subsequent sections.

Unlike the intolerance of replicative DNA polymerases for modified DNA templates, the CMG helicase can bypass most types of DNA lesions. Accordingly, fork encounters with such obstacles lead to functional uncoupling of replicative helicase and polymerase activities, generating extended stretches of single-stranded (ss)DNA that are rapidly bound and protected by the ssDNA-binding protein RPA (Figure 1A,B) [35]. This triggers replication stress signaling by providing binding sites for the ATR–ATRIP kinase complex, a master organizer of the replication stress response, as well as many other RPA-binding proteins [36,37]. Once activated at RPA-bound ssDNA, ATR phosphorylates a plethora of targets to halt replication and cell cycle progression, protect and reconfigure the replisome, and inhibit the firing of new origins globally while stimulating local origin firing to mitigate the adverse impact of replication stress [37]. Ubiquitylation of the RPA platform has an important role in facilitating fork stalling-associated transactions (Figure 1B). This is mediated by the RPA-binding E3 ligase RFWD3, which promotes non-proteolytic ubiquitylation targeting multiple sites across all three RPA subunits [38]. Another RPA-binding E3 ligase, PRP19, may also contribute to RPA ubiquitylation after DNA damage [39]. RFWD3 travels with the replisome under physiological conditions and its depletion leads to inefficient homologous recombination (HR)-dependent fork restart, prolonged S phase, sensitivity to replication stress, and persistent RPA foci [38,40,41]. These defects could be a consequence of impaired p97-dependent dissociation of polyubiquitylated RPA from damaged DNA [42] but require further mechanistic studies, as RFWD3-deficient cells display no overt defect in recruitment of the recombinase RAD51 [40], even though this ordinarily entails replacement of RPA at ssDNA. Recent studies point to a clinical significance of RFWD3 dysfunction. First, RFWD3 depletion in BRCA2-deficient cells stabilizes stalled forks and rescues their sensitivity to replication-stalling agents, suggesting that tumors with *BRCA2* mutations could acquire resistance to such compounds by inactivating RFWD3 [43]. Second, RFWD3 is critical for ICL repair, and a heterozygous patient-associated mutation disrupting its binding to RPA gives rise to **Fanconi anemia (FA)** [40,44], a rare recessive disorder resulting from defective replication-coupled ICL repair (see later) [11]. Further insights into the genome-protective mechanisms and functions of RFWD3-mediated ubiquitin signaling at replication obstacles are thus warranted. To this end, recent work showed that RFWD3 promotes ubiquitylation at ssDNA regions to trigger recruitment of multiple genome caretakers including **DNA damage tolerance** factors, facilitating replication across polymerase-stalling lesions including ICLs and DPCs [45]. Like RFWD3, the homologous ubiquitin-binding proteases DDI1 and DDI2 promote fork restart after replication stress. This is mediated by displacement of the functionally uncharacterized replisome component RTF2 and may involve the recently discovered ability of DDI2 to serve as a ubiquitin-directed protease, facilitating proteasomal degradation of ubiquitylated targets [46,47]. In general, proteasome activity regulates the abundance of many components of replicative stress responses. As an illustrative example, multiple E3 ligases target the key ATR effector kinase CHK1 for ubiquitin-mediated proteolysis, limiting the magnitude and duration of replication stress signaling [48–51].

DNA Damage Tolerance and Replication-Coupled Repair

The nature and position of obstacles interfering with DNA synthesis during replication fundamentally influence the ensuing replication stress response. Mismatch and excision repair pathways ensure that many common DNA lesions, including ribonucleotides, base lesions, abasic sites,

illegitimate recombination and often result in catastrophic genomic instability.
p97: hexameric ATPase also known as Valosin-containing protein (VCP) that extracts proteins from macromolecular complexes and cellular membranes. Via its numerous cofactors and adaptors, p97 binds to and unfolds polyubiquitylated proteins, promoting their proteasomal degradation or recycling.
Replication fork reversal: the conversion of the replication fork from a three-way junction into a four-way junction 'chicken foot' structure involving annealing of the nascent DNA strands. Reversed forks retain replisomes that are poised to restart upon removal or bypass of fork-stalling insults and protect against unscheduled nucleolytic degradation.
Replication stress: any condition that causes a deviation from physiological replication fork progression, including fork stalling, slowing, and acceleration. Sources of replication stress include difficult-to-replicate genomic regions, DNA damage, replication-transcription conflicts, and oncogene activation.
Small ubiquitin-like modifier (SUMO): ubiquitin-like polypeptide that is covalently conjugated to numerous cellular proteins via a three-step enzymatic cascade, similar to that operating for ubiquitin (Box 1), and plays an important role in cellular stress responses. Three SUMO isoforms (SUMO1–3) are expressed in vertebrates.

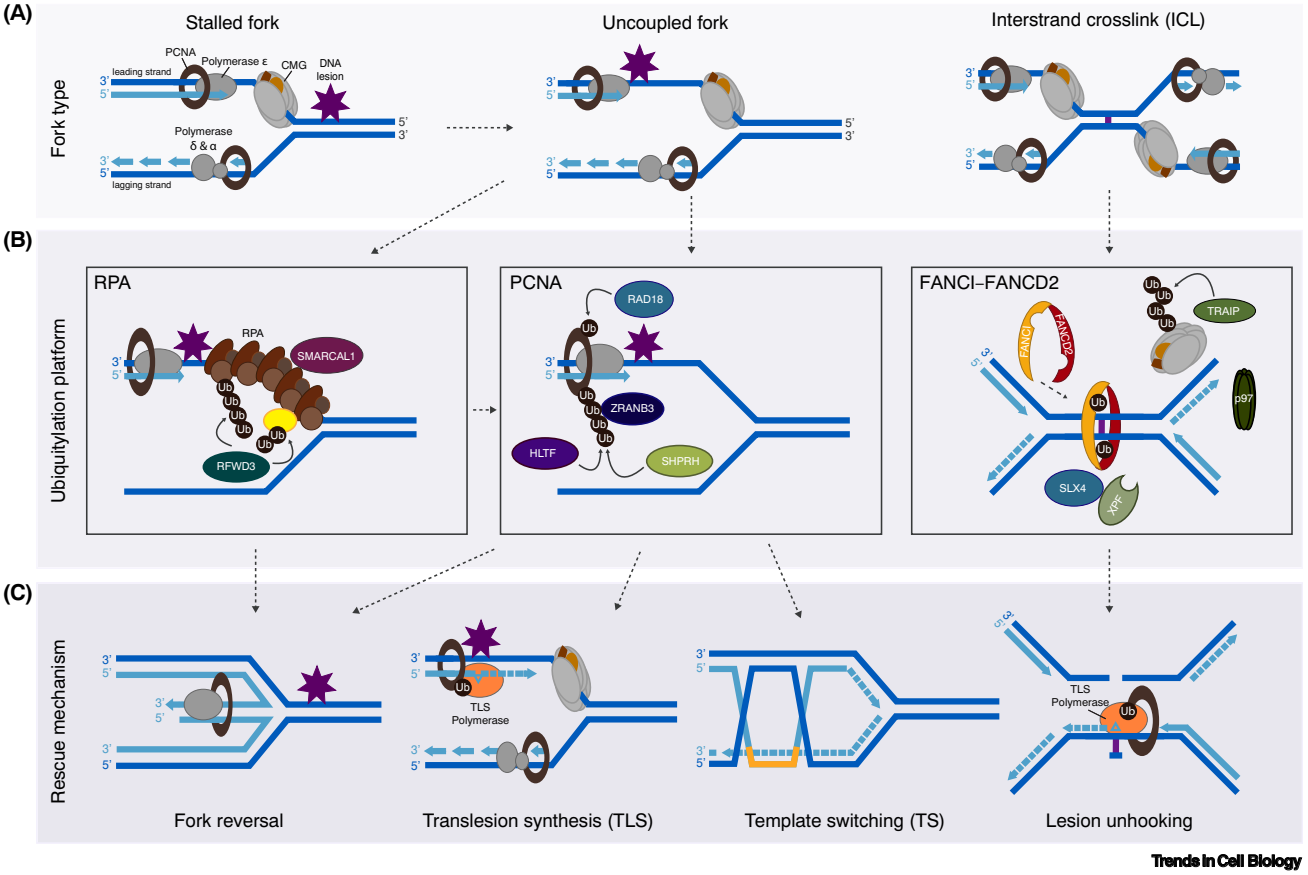

Figure 1. Ubiquitylation Platforms at the Replisome Govern the Replication Stress Response. Replication impediments alter fork conformation (A) and trigger ubiquitylation of major replisome platforms (B) to mount effector responses (C) promoting lesion removal or bypass and thus continuation of ongoing DNA synthesis. Unlike high-fidelity replicative DNA polymerases, the CMG helicase can bypass most types of DNA lesions. This leads to functional uncoupling of replicative helicase and polymerase activities, generating extended stretches of RPA-coated ssDNA, that activate ATR signaling and provide recruitment platforms for multiple effector proteins, including the annealing helicase SMARCAL1, which promotes fork reversal. RPA is polyubiquitylated by RFWD3 in a non-proteolytic manner, which may impact RPA-protein interactions and facilitate its p97-dependent extraction from chromatin. Replication obstacles also trigger PCNA ubiquitylation to promote DNA damage tolerance pathways. RAD18-mediated PCNA monoubiquitylation stimulates TLS-mediated replication past lesions by facilitating transient polymerase switching to a damage-tolerant but error-prone TLS polymerase. For DPCs, the protein adduct must be proteolytically trimmed in a ubiquitin-driven manner in order for TLS-mediated bypass to occur (not shown). PCNA monoubiquitylation can be extended into a K63-linked polyubiquitin chain by the RAD5-like E3 ligases HLTF and SHPRH, promoting error-free damage bypass via TS or fork reversal by the helicase ZRANB3. Reversed forks retain active replisomes, which are protected from nucleolytic degradation and poised to restart once the lesion has been removed or bypassed. Fork convergence on helicase-blocking ICLs activate the intricate, multistep FA pathway via monoubiquitylation of the FANCI–FANCD2 platform by the FA core complex, which in conjunction with TRAIP-dependent ubiquitylation and unloading of one of the converging CMGs stimulates nucleolytic incisions by XPF–SLX4 to unhook the ICL and promote downstream TLS and HR repair steps. Abbreviations: CMG, CDC45–MCM2–7–GINS; DPCs, DNA-protein crosslinks; FA, Fanconi Anemia; FANCI–FANCD2, Fanconi anemia group I protein–Fanconi anemia group D2 protein); HLTF, helicase-like transcription factor; HR, homologous recombination; ICLs, interstrand crosslinks; PCNA, proliferating cell nuclear antigen; RPA, replication protein A; SHPRH, SNF2 histone-linker PHD-finger RING-finger helicase; TLS, translesion DNA synthesis; TS, template switching.

and bulky adducts, are efficiently removed prior to the arrival of a replication fork. When lesions that block polymerase α or δ on the lagging strand are occasionally encountered by the replisome, its progression seldomly stalls, as such insults can be simply bypassed by the creation of new Okazaki fragments and dealt with post-replicatively. A similar DNA damage tolerance strategy for lesions obstructing polymerase ε on the leading strand feeds on fork restart downstream of the lesion by the specialized DNA primase/polymerase enzyme PrimPol [52]. These DNA damage bypass processes generate ssDNA gaps behind the replication fork that can be filled via DNA damage tolerance mechanisms. This critically relies on PCNA ubiquitylation, with RAD18-dependent PCNA monoubiquitylation promoting translesion DNA synthesis (TLS) and

Box 1. The Ubiquitin System

Covalent modification of cellular proteins with the small and highly conserved polypeptide ubiquitin, termed ubiquitylation, is a universal signaling mechanism affecting virtually all aspects of cell biology. Conjugation of ubiquitin to lysine residues in target proteins proceeds via a three-step relay involving numerous combinations of E1 ubiquitin-activating (Figure IA), E2 ubiquitin-conjugating (Figure IB), and RING- or HECT-type E3 ligase (Figure IC) enzymes, which act sequentially to catalyze the modification of tens of thousands of lysine residues distributed among a sheer number of substrates in human cells [13,114]. The resulting ubiquitin marks can be removed by deubiquitylating enzymes (DUBs) (Figure ID), rendering ubiquitylation a highly dynamic and reversible protein modification [115]. Adding further complexity to the versatility of ubiquitin-mediated signaling processes, ubiquitin is not only attached as single moieties (monoubiquitylation) but can also be conjugated to any of the seven lysine residues or the N-terminal methionine within ubiquitin itself, giving rise to eight possible distinct polyubiquitin chain conformations, all of which are formed in cells and serve defined, but not in all cases well understood, cellular functions. For instance, K48- and K11-linked ubiquitin chains are major signals for degradation via the 26S proteasome, whereas K63-linked ubiquitylation is a non-proteolytic modification with critical regulatory roles in many cellular processes [13,114]. These complex modifications underlie a cellular 'ubiquitin code' that is read and decoded by hundreds of proteins containing ubiquitin-binding domains (UBDs) (Figure IE), coupling specific ubiquitin modifications to downstream effector pathways [116]. Approximate numbers of different classes of ubiquitin signaling enzymes encoded by human cells are indicated in red.

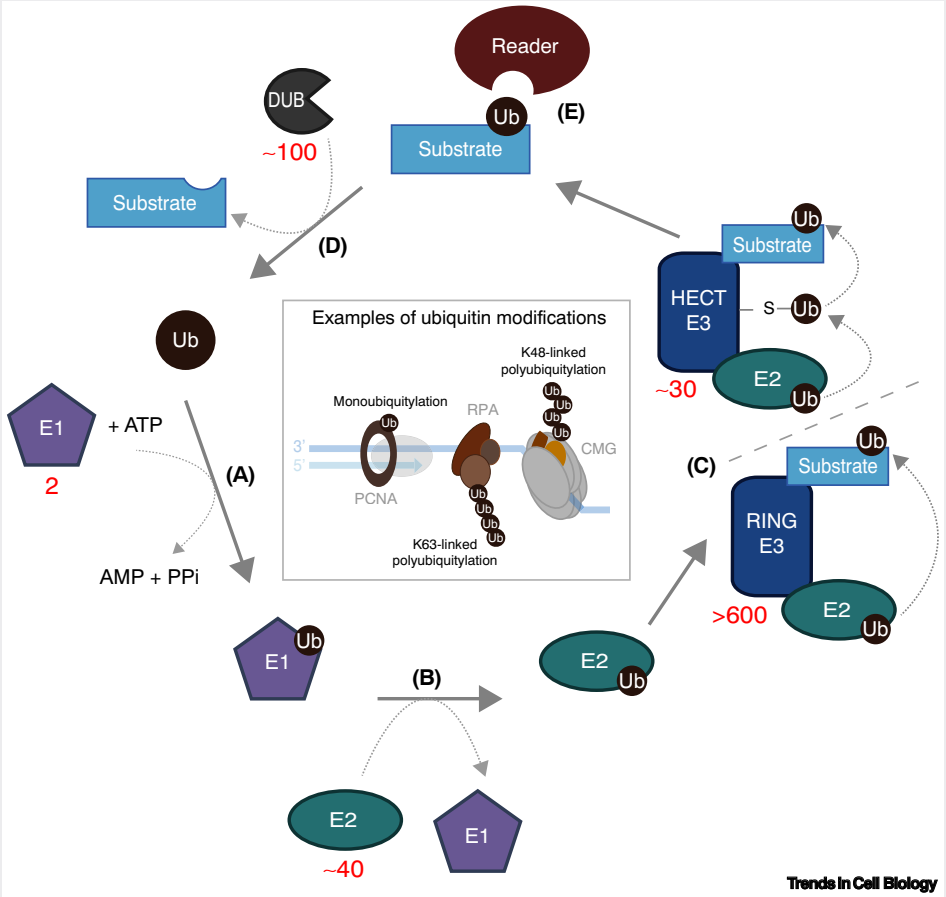

Figure I. The Ubiquitin Modification Cycle.

K63-linked PCNA polyubiquitylation facilitating template switching (TS) via HR (Figure 1B,C). The central role of PCNA ubiquitylation in DNA damage tolerance has been comprehensively covered in previous reviews [53,54] and will not be extensively discussed here. Briefly, specialized TLS polymerases are recruited to monoubiquitylated PCNA via dual PCNA-interacting protein (PIP)

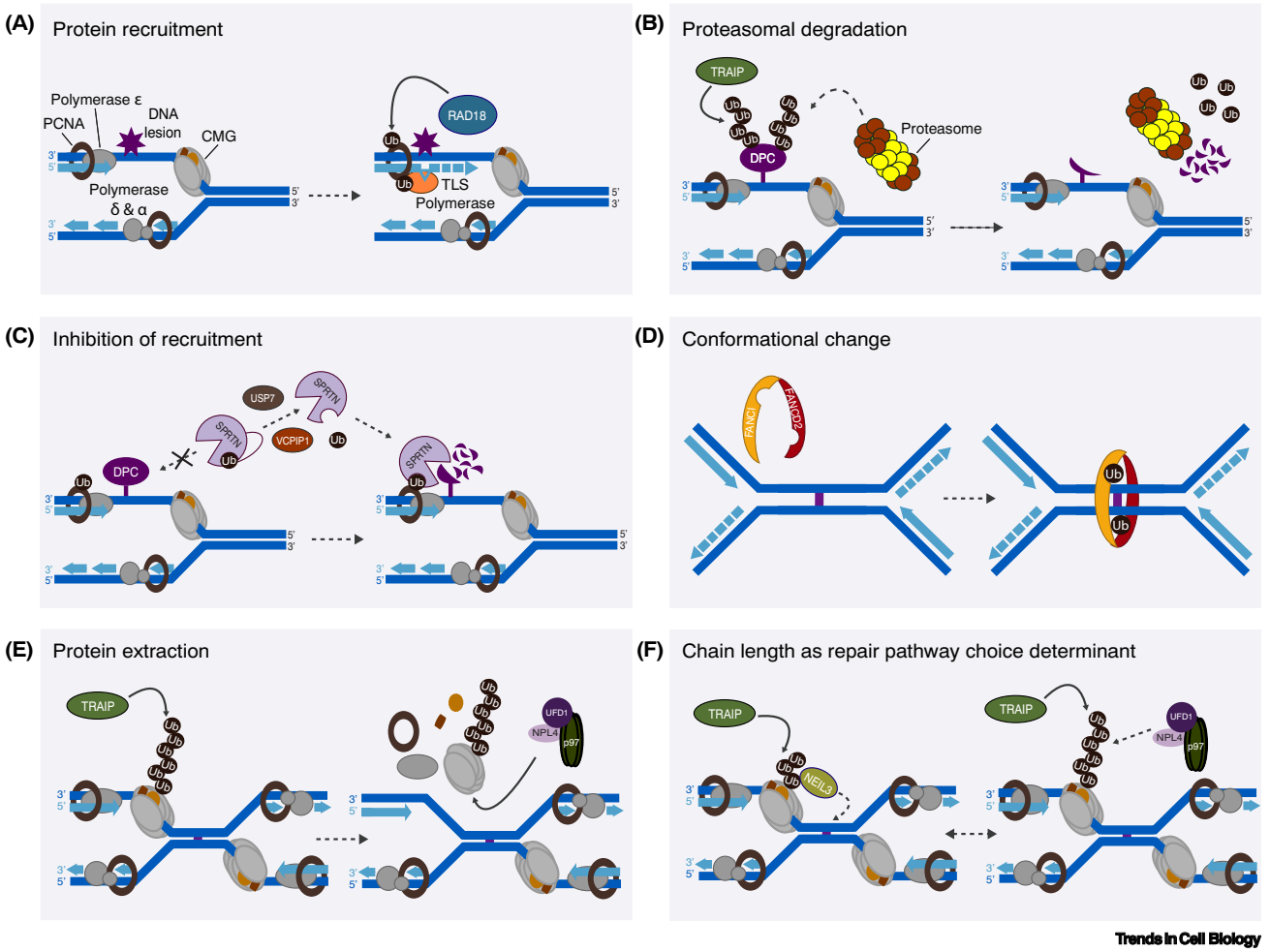

Figure 2. Regulatory Principles of Ubiquitylation at Stressed Replication Forks. Ubiquitylation orchestrates and regulates protein interactions with stressed replication forks via several regulatory principles to promote responses to fork-stalling insults. Fork-associated ubiquitylation processes generate recruitment platforms for a range of effector proteins, exemplified by PCNA monoubiquitylation-dependent recruitment of Y-family TLS polymerases (A). Ubiquitylation also plays active roles in removing proteins residing in the context of the replisome and antagonizing protein interactions with the fork (e.g., via proteolytic cleavage of polymerase-blocking DPCs) (B) or ubiquitin-dependent shielding of protein interaction modules such as a UBD in SPRTN, thereby blocking its access to chromatin (C). Ubiquitylation can also serve a structural role in overcoming fork-stalling lesions (e.g., by locking the FANCI–FANCD2 complex on DNA in a pin-like fashion) (D). p97 extracts many ubiquitylated replisome components including CMG from the fork (E), and the length and conformation of ubiquitin conjugates can centrally influence repair pathway choice at stalled forks, such as during ICL repair where short TRAIP-generated ubiquitin chains on CMG are recognized by NEIL3 for direct ICL reversal, whereas longer chains enable p97 recruitment to promote CMG unloading and ICL repair via the FA pathway (F). Abbreviations: CMG, CDC45–MCM2–7–GINS; DPCs, DNA-protein crosslinks; FA, Fanconi Anemia; FANCI–FANCD2, Fanconi anemia group I protein–Fanconi anemia group D2 protein); ICLs, interstrand crosslinks; PCNA, proliferating cell nuclear antigen; SPRTN, SprT-like N-terminal domain; TLS, translesion DNA synthesis; UBD, ubiquitin-binding domain.

box and ubiquitin-binding domain (UBD) motifs (Figures 1C and 2A), enabling replication past DNA lesions by virtue of their permissive catalytic sites, albeit in an error-prone manner. Extension of the PCNA monoubiquitylation mark into a K63-linked polyubiquitin chain by the E3 ligase RAD5 promotes error-free damage bypass via TS, which utilizes the undamaged sister chromatid and is an important genome protection mechanism in yeast (Figure 1B,C) [53,54]. To which extent TS is operational in vertebrate cells remains unclear. While the mammalian RAD5 orthologs helicase-like transcription factor (HLTF) and SNF2 histone-linker PHD-finger RING-finger helicase (SHPRH) can both catalyze PCNA polyubiquitylation *in vitro*, cells lacking both of these ubiquitin ligases show residual PCNA polyubiquitylation and are not sensitive to DNA damage [55–59].

Table 1. Writers, Readers and Erasers of Replication Fork-Associated Ubiquitylation

Factor	Function	Refs
Writers		
TRAIP	Ubiquitylates CMG on the leading strand in the presence of helicase-blocking obstacles including ICLs and DPCs. Defines a mitotic backup pathway for CMG unloading	[30,61,103–106]
RFWD3	Ubiquitylates ssDNA-bound RPA complexes in a non-proteolytic manner to promote DNA damage bypass and fork restart	[38,40–45]
RAD18	Monoubiquitylates PCNA to stimulate TLS	[53,54]
HLTF	Promotes PCNA polyubiquitylation to promote error-free fork restart. Recognizes free 3′-OH ends in DNA and catalyzes fork reversal	[56,58,85–88]
SHPRH	Promotes PCNA polyubiquitylation	[56,57,59]
$CRL2^{LRR1}$	Ubiquitylates MCM7 to promote p97-dependent CMG unloading upon fork convergence during replication termination	[27,29,101]
FANCL	Component of the FA core complex that monoubiquitylates FANCD2 and FANCI in conjunction with the E2 enzyme UBE2T	[11,71]
RNF168	Ubiquitylates H2A-type histones on K13 and K15 to promote recruitment of 53BP1, RNF169, and the BRCA1–A complex	[15,98]
FBH1	Accumulates at stalled forks and functions as a negative regulator of HR by disrupting RAD51 nucleofilaments	[90–93]
PRP19	Contributes to RPA ubiquitylation upon DNA damage	[39]
Readers		
Y-family TLS polymerases	Interact with monoubiquitylated PCNA and enable error-prone replication past DNA lesions due to their flexible active sites	[53,54]
ZRANB3	Translocase that recognizes K63-polyubiquitylated PCNA and promotes fork reversal	[82–84]
p97 and cofactors (UFD1-NPL4-FAF1)	Recognize ubiquitylated client proteins to promote their displacement from replication forks	[22–30,42]
WRNIP	Accumulates at stalled forks via binding to ubiquitylated PCNA and stabilizes RAD51 nucleofilaments	[94–96]
SPRTN	Promotes replication-coupled DPC proteolysis by recognizing ubiquitylated factors at DPC sites	[61–63]
26S proteasome	Recognizes and proteolytically degrades ubiquitylated DPCs	[61]
53BP1	Reader of RNF168-dependent H2A(X) ubiquitylation that protects stalled or reversed forks from nucleolytic degradation by MRE11	[97–99]
NEIL3	Glycosylase that recognizes short ubiquitin chains on CMG and directly cleaves ICLs	[30,108]
Erasers		
USP7	Deubiquitylates SUMOylated proteins at unstressed forks to prevent their displacement from the replisome. Deubiquitylates SPRTN	[21,66]
USP1	Deubiquitylates monoubiquitylated FANCD2 and PCNA	[32,79]
ZUFSP	Recognizes and deubiquitylates long K63-linked ubiquitin chains at replication stress sites	[33,34]
VCPIP1	Reverses SPRTN monoubiquitylation to promote its chromatin access	[65]

Conversely, K63-linked PCNA polyubiquitylation is important for promoting **replication fork reversal** and subsequent RAD51-dependent fork restart, as described later, and additional E3 ligases targeting PCNA may await discovery.

Ubiquitylation is also instrumental for repair of CMG helicase-blocking lesions such as DPCs and ICLs. DNA replication-coupled repair of DPCs via proteases, including SprT-like N-terminal

domain (SPRTN) and the proteasome, offers an important means of recognizing and resolving this bulky and highly heterogenous class of DNA lesion [12]. While the CMG is ultimately able to bypass DPCs on the leading strand in a manner that requires the helicase regulator of telomere length 1 (RTEL1) [60], it temporally stalls at these lesions, triggering DPC ubiquitylation by the replisome-associated ubiquitin ligase TRAF-interacting protein (TRAIP), which appears to promiscuously ubiquitylate proteins ahead of the CMG [61]. This enables DPC proteolysis via the proteasome (Figure 2B). Despite harboring UBDs, SPRTN does not require DPC ubiquitylation to proteolytically cleave the protein adduct. By contrast, SPRTN recruitment to DPCs is strictly dependent on ubiquitylation of proteins that reside at the damage sites, but whose identity remains unclear [61–63]. Moreover, SPRTN is subject to tight regulatory control of its protease activity that is at least partially mediated by its UBD-coupled monoubiquitylation, which inhibits SPRTN chromatin association (Figure 2C) [62,64]. Recent studies identified VCPIP1 and USP7 as DUBs regulating SPRTN monoubiquitylation status and chromatin access [65,66]. Despite the threat they pose to replication integrity and genome stability, DPCs can also serve a genome-protective function. Specifically, it was recently shown that the fork-associated protein HMCES forms DPCs at abasic sites to prevent their error-prone processing, with HMCES protein adducts being subsequently resolved via ubiquitin-dependent proteolysis [67]. Outside the context of DNA replication, when DPCs cannot be resolved via the replisome, SUMOylation is indispensable for DPC removal in a manner that may involve downstream processing of the adducted protein via ubiquitylation and the proteasome [12,68,69].

As for DPCs, replication-dependent repair of ICLs crucially depends on ubiquitylation. The FA pathway, comprising at least 22 gene products whose mutation gives rise to FA, provides a major cellular pathway for replication-coupled ICL repair that proceeds via a complex series of highly interdependent and coordinated steps [11]. Central to this intricate process is the multisubunit FA core complex, which together with the E2 enzyme UBE2T forms a ubiquitin ligase that monoubiquitylates the FANCI–FANCD2 (ID2) complex on chromatin when replication forks stall at ICLs (Figure 1A,B) [11,70,71]. This promotes incisions around the ICL by the XPF–ERCC1 nuclease complex coordinated by the multiprotein scaffold SLX4 [72,73], unhooking the lesion to facilitate subsequent resolution of repair intermediates via TLS-mediated bypass of the adducted strand and HR-dependent repair of the DSB generated on the opposite strand (Figure 1C) [11,74,75]. Notwithstanding the undisputed key role of ID2 monoubiquitylation in ICL repair, readers of this modification that promote downstream repair events have remained elusive despite vigorous research efforts. While SLX4 contains UBDs whose mutation causes FA and thus seems an attractive candidate, available evidence collectively argues against this scenario [76]. Recent structural studies revealed that monoubiquitylation of DNA-bound ID2 acts in a pin-like fashion to induce a marked conformational change of the complex that allows it to tightly encircle DNA like a sliding clamp (Figure 2D) [77,78]. Importantly, the FANCD2-conjugated ubiquitin moiety is buried within the modified ID2 conformation and so may be effectively inaccessible to direct recognition by UBD-bearing factors. This raises the interesting possibility that readers of monoubiquitylated ID2 might recognize other interfaces in the complex that become exposed upon FANCD2 monoubiquitylation, as opposed to direct UBD-mediated binding to this mark *per se*, which could explain why such factors have so far evaded identification. However, whether endogenous chromatin-bound monoubiquitylated ID2 complexes in cells fully recapitulate the *in vitro* structures remains to be established. The notion that FANCD2 monoubiquitylation locks the ID2 complex on DNA also provides a prospective mechanistic rationale for why preventing FANCD2 deubiquitylation by knockout of the responsible DUB USP1 leads to FA [79], as impaired removal of monoubiquitylated ID2 from damaged DNA obstruct with subsequent repair steps. Future biochemical and cellular studies are needed to address the mechanistic significance of the ubiquitin-ID2 clamp in ICL repair and possibly at other challenged fork structures.

Replication Fork Stability and Remodeling

Stalled replication forks are dynamic structures that must be stabilized to allow for their timely rescue by a converging fork or recruitment of fork remodeling enzymes that promote fork restart. Whether stalled forks recover or eventually break and/or collapse depends on a meticulously choreographed process that entails their initial protection from **nucleolytic degradation**, removal of the blocking lesion or bypassing it via TS or repriming, fork reconfiguration by translocases, and ultimately RAD51-mediated replication restart [80,81]. Ubiquitylation plays a prominent role in regulating these intricate fork-associated events. More specifically, K63-linked polyubiquitin chains and PCNA polyubiquitylation are essential for replication fork reversal, a process that facilitates fork restart and preserves genome integrity when properly controlled [80,82]. Through a bivalent interaction mediated by its UBD and PIP box motifs, the annealing helicase zinc finger Ran-binding domain-containing protein 3 (ZRANB3) specifically recognizes K63-polyubiquitylated PCNA and utilizes its translocase activity to stabilize stalled forks, promote fork reversal, and limit excessive strand invasion (Figure 1B,C) [82,83]. ZRANB3 deficiency leads to hyper-recombination, increased genome instability, and lethality upon replication stress [83,84]. The E3 ubiquitin ligase HLTF, which is also involved in PCNA polyubiquitylation and TS in the context of DNA damage tolerance, acts in a complementary fashion to ZRANB3. HLTF rapidly accumulates at stalled replication forks through interaction with free 3′-OH ends in DNA by means of its HIP116 Rad5p N-terminal (HIRAN) domain [85]. This enables HLTF to restrain premature fork restart and facilitate fork reversal in a manner dependent on both its E3 ligase and translocase activities [85–87]. HLTF delays S phase progression and globally slows down replication, which may provide time and opportunities for fork reversal while holding off error-prone DNA damage bypass processes [88]. In contrast to ZRANB3 and SMARCAL1, another principal fork reversal enzyme recruited via, and regulated by, interaction with RPA [89], depletion of HLTF leads to increased resistance to replication stress [88], suggesting that these remodelers are not functionally redundant and may recognize distinct stalled fork intermediates in a manner that could be partially dictated by their interactions with different structures and recruitment platforms at the replisome.

A number of proteins in the ubiquitin signaling network modulate replication fork stability by regulating RAD51 nucleofilaments, which protect against unscheduled nucleolytic attacks by MRE11, DNA2, and other nucleases [80]. The FBH1 helicase, an F-box-containing SCF E3 ubiquitin ligase complex substrate recognition factor, is recruited to challenged replication forks via interaction with PCNA and its intrinsic helicase activity [90,91]. FBH1 catalyzes fork regression and acts as a negative regulator of HR by disrupting RAD51 filaments, possibly involving direct ubiquitylation of RAD51 [91–93]. Depletion of FBH1 rescues MRE11-mediated fork degradation resulting from loss of the ATPase WRNIP1, which interacts with ubiquitylated PCNA and accumulates at stalled forks via its UBD to stabilize RAD51 filaments [94–96]. Similar to the central role of canonical DSB repair factors including MRE11, BRCA2, and RAD51 in modulating fork stability [80], the DSB-responsive RNF8/RNF168-mediated ubiquitin signaling pathway [15] has also been implicated in replication fork protection, with a range of key components of this pathway including RNF168, RNF169, 53BP1, and BRCA1–BARD1 accumulating at stalled forks [17,97,98]. RNF168 and 53BP1 appear to protect reversed forks during the physiological duplication of hard-to-replicate regions of the genome in a manner dependent on H2A/H2AX [H2A(X)] ubiquitylation [98]. Whether this protective role is mediated by direct interactions with replisome components such as PCNA or via recognition of double-stranded DNA ends at arms of regressed forks remains to be seen. NCC experiments have shown that H2A(X) rapidly repopulates nascent DNA [16], raising the possibility that the arms of regressed forks undergo chromatinization, thus bearing structural resemblance to DSBs. This could allow RNF168 to ubiquitylate H2A(X) in its native nucleosomal environment and thereby promote recruitment of 53BP1, which may shield fork intermediates from unfettered nucleolytic

degradation, apparent upon depletion of RNF168 or 53BP1 [90]. Further supporting this scenario, 53BP1 has been shown to physically accumulate at and protect stalled replication forks against nascent strand degradation [97,99]. Interestingly, the fork protection defect arising from 53BP1 loss is dependent on fork reversal by FBH1 but not SMARCAL1, ZRANB3, or HLTF [99]. Further clarification of which fork structures canonical DSB signaling proteins are recruited to and how they regulate the replication stress response upstream of fork breakage will be important, as it is currently unclear which of these factors are critical for mounting a successful response.

Replication Termination and CMG Helicase Unloading

When converging replication forks meet and pass each other, the CMG helicase is unloaded from DNA and replication is locally terminated [100]. The mechanistic basis of this process has recently become clear and critically relies on ubiquitylation. In higher eukaryotes, CMG unloading is driven by K48-linked polyubiquitylation of the MCM7 subunit by the $CRL2^{LRR1}$ E3 ligase (SCF^{Dia2} in budding yeast) and subsequent extraction by the p97–UFD1–NPL4 complex [26–29]. Recent studies in yeast and vertebrates indicate that the DNA structure of elongating replication forks sterically blocks the interaction between CMG and $CRL2^{LRR1}$, thereby suppressing CMG ubiquitylation and unloading until fork convergence has occurred [101–103]. In the absence of $CRL2^{LRR1}$, TRAIP provides an alternative pathway for ubiquitin-driven CMG unloading during mitosis, which is critical for preserving viability when cells enter mitosis with incompletely replicated DNA [30,103,104]. Despite producing the same outcome, the $CRL2^{LRR1}$- and TRAIP-mediated CMG unloading pathways differ mechanistically in that TRAIP-dependent CMG ubiquitylation is not exclusive to MCM7, but targets multiple CMG components with heterotypic and/or branched polyubiquitin chains when forks converge at an ICL (Figure 2E) [30]. While TRAIP interacts directly with PCNA and travels with replication forks under both normal and perturbed conditions [105], its E3 ligase activity appears to be selectively directed towards replication obstacles, particularly on the leading strand where CMG translocates [30]. However, how TRAIP ligase activity is controlled remains poorly understood. In contrast to another replisome-associated ubiquitin ligase, RFWD3, TRAIP exhibits a mutually exclusive localization pattern with RPA [105] and might potentially be less active in the presence of RPA-ssDNA regions. This is apparent from both the absence of such structures in contexts that require TRAIP function and its limited ability to promote DPC ubiquitylation for efficient SPRTN- or proteasome-dependent removal following CMG bypass of the DPC, whereupon ssDNA generation necessitates a second E3 ligase for amplifying DPC ubiquitylation [61]. In cells, TRAIP loss or inactivation leads to aberrant accumulation of cells in S/G2 phase, impaired replication fork progression, hypersensitivity to replication stress, and chromosomal instability [103,105,106]. Some of these phenotypes clinically manifest in patients with primordial dwarfism and microcephaly, resulting from mutations in TRAIP that reduce its expression level and/or E3 ligase activity [106], while full knockout of *TRAIP* causes early embryonic lethality in mice [107].

It is becoming apparent that FA pathway-independent mechanisms for overcoming ICLs during replication exist, and TRAIP-dependent CMG ubiquitylation is emerging as a central decision point for choosing between these repair routes (Figure 2F). Following collision of the CMG with an ICL, the crosslink can be directly unhooked via a process that is mechanistically simpler than the FA pathway and involves direct cleavage of the ICL by the NEIL3 glycosylase [108], which via its UBD recognizes short ubiquitin chains on CMG generated by TRAIP [30]. If the ICL cannot be resolved by NEIL3, TRAIP further extends CMG polyubiquitylation to enforce its p97-dependent unloading and stimulate ICL repair via the FA pathway [30]. TRAIP thus emerges as a central regulator of critical fate decisions at the replisome via ubiquitin, and further in-depth studies of its multifaceted functions are therefore pertinent.

Concluding Remarks

It is now clear that replication fork-associated ubiquitylation processes are of paramount importance for achieving faithful genome duplication under stressful conditions, in line with the pervasive regulatory involvement of ubiquitin signaling throughout cell biology. This hinges on the central role of ubiquitin in governing replisome composition during both normal and perturbed DNA replication, entailing recruitment of key effectors via ubiquitylation of central replisome platforms, such as PCNA, RPA, and FANCI–FANCD2, as well as removal of fork components and impediments (e.g., CMG unloading and DPC proteolysis) (Figures 1 and 2). The growing insights into the crucial roles of ubiquitin in replication stress signaling go hand in hand with many recent breakthroughs in the mechanistic understanding of how cells respond to and overcome multiple types of fork-stalling insults. Despite these advances, much remains to be gleaned about how ubiquitylation dynamics, specificity, and architecture exert regulatory control of stress-induced replisome transactions in vertebrates (see Outstanding Questions). Whereas some ubiquitin ligases, such as RAD18 and the FA core complex, display exquisite substrate specificity, other key replication stress-responsive E3s, including TRAIP and RFWD3, appear to function more promiscuously by non-specifically targeting proteins residing at replication stress sites, in a manner that clearly necessitates tight control of their ligase activity but is not yet fully understood. In general, only a few substrates of ubiquitin ligases and DUBs with well-established roles at the replication fork have been identified thus far, while for others (such as BRCA1–BARD1) it is unclear whether or not their enzymatic activity impacts replisome-associated processes. Future efforts are likely to reveal additional important targets of ubiquitin signaling enzymes at stressed forks. For instance, ubiquitylation of cohesin has been linked to protection of stalled forks in yeast [109–111], and proteomic surveys of fork-associated protein landscapes in human cells suggest the presence of additional E3 ligases and DUBs [31], whose potential functions and substrates in this context have yet to be established. The relative importance of different ubiquitin chain linkages and topologies in promoting fork-associated responses also awaits further clarification. For some processes, such as fork reversal mediated by K63-polyubiquitylated PCNA, ubiquitin chain specificity is clearly critical, whereas in other cases, such as RFWD3-mediated RPA polyubiquitylation involving multiple linkage types, more flexibility appears to be tolerated [38,83]. Whether ubiquitin linkages other than K48- and K63-linked chains have direct roles in the replication stress response is another lingering question, subject to the general challenges associated with probing low-abundant, atypical ubiquitin chain types and their functions. In addition, the identities of key readers of major ubiquitylation targets at the replisome, including FANCI–FANCD2 and RPA, are still uncertain, representing notable gaps in our understanding of critical responses to fork-stalling insults. Genome stability maintenance processes are often coregulated by ubiquitin and SUMO, and the replication stress response in all likelihood is no exception. Indeed, SUMOylation is an abundant replisome-associated PTM, but as of yet its precise roles in this context are not well understood, although there is at least some evidence to suggest that SUMO modifications can promote protein displacement from stressed forks, possibly via direct crosstalk with ubiquitylation. The recent development of a potent small molecule inhibitor of SUMOylation [112], should greatly facilitate efforts at deciphering the workings of SUMO in the replication stress response.

The heavy burden of replication stress typically facing cancer cells provides an attractive target for therapeutic exploitation, and inhibitors of ATR and CHK1 have shown promising potential in preclinical trials [113]. With the multitude of recent discoveries on the key roles of protein ubiquitylation in overcoming most fork-stalling insults, not least those induced by mainstay chemotherapeutic drugs, opportunities for future, rationally designed treatment strategies targeting replication stress-responsive ubiquitin signaling enzymes now seem within sight. The coming years thus hold promise for exciting advances on ubiquitin-mediated responses to replication stress on both a mechanistic level and from a clinical perspective.

Outstanding Questions

What is the precise division of labor, crosstalk, and coordination between different ubiquitylation platforms at stressed forks?

Relatively few substrates of replication stress-induced ubiquitylation are known; are there additional critical ubiquitylation targets at stressed forks?

How is the activity of replication stress-responsive ubiquitin ligases and DUBs, in particular those exhibiting target promiscuity, controlled?

Apart from E3 ligases and DUBs listed in Table 1, do other ubiquitin signaling enzymes known to associate with the replisome based on proteomic analyses have roles in the replication stress response?

To what extent does linkage specificity drive responses to fork-stalling insults, and do polyubiquitin chain conformations other than K48- and K63-linked chains have roles at stressed replication forks?

What is the identity of readers of the FANCI–FANCD2 and RPA ubiquitylation platforms?

How does SUMOylation, an abundant modification at the replisome, impact and regulate replication fork composition and responses to fork-stalling lesions, and does this involve crosstalk with ubiquitylation?

Can the growing mechanistic insights into the key roles of ubiquitylation in the replication stress response be harnessed therapeutically?

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Declaration of Interests

No interests are declared.

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