Ubiquitin signalling in DNA replication and repair

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Abstract | Post-translational modification by ubiquitin is best known for its role in targeting its substrates for regulated degradation. However, non-proteolytic functions of the ubiquitin system, often involving either monoubiquitylation or polyubiquitylation through Lys63-linked chains, have emerged in various cell signalling pathways. These two forms of the ubiquitin signal contribute to three different pathways related to the maintenance of genome integrity that are responsible for the processing of DNA double-strand breaks, the repair of interstrand cross links and the bypass of lesions during DNA replication.

The integrity of a cell's genetic information is protected against harmful influences by three complementary, but cooperating, approaches: the sensing of DNA damage by cellular checkpoint systems, its removal by various specialized repair pathways and its bypass during DNA replication to avoid interference with DNA synthesis. Most of the basic mechanisms and factors involved in these genome maintenance systems are fairly well understood, so the challenges and most interesting questions concern the regulatory aspects by which the individual pathways are controlled. One of the main strategies that provide cells with adaptability and flexibility is the reversible, post-translational modification of proteins by members of the ubiquitin family. Hence, not surprisingly, protein ubiquitylation has emerged as an important factor in the control of genome stability. Although the ubiquitin system is best known for its function in regulated proteolysis, it is particularly the proteasome-independent aspects, traditionally called the non-conventional signalling functions of ubiquitylation, that seem to predominate in DNA damage processing.

This Review discusses the functional implications of ubiquitylation in the context of three systems for processing DNA damage that exemplify the different approaches of maintaining genome stability while highlighting the concepts of non-degradative ubiquitin signalling. These are: the damage response following a DNA double-strand break (DSB), the Fanconi anaemia pathway for the repair of interstrand cross links (ICLs) during DNA replication and the system of DNA damage tolerance for replicative lesion bypass. References to interesting parallels, crosstalk and overlaps in terms of the enzymes involved provide mutual connectivity from a mechanistic perspective. Although all examples focus on the contributions of the ubiquitin system to genome maintenance, they illustrate well the diversity of mechanisms by which ubiquitin exerts its biological functions.

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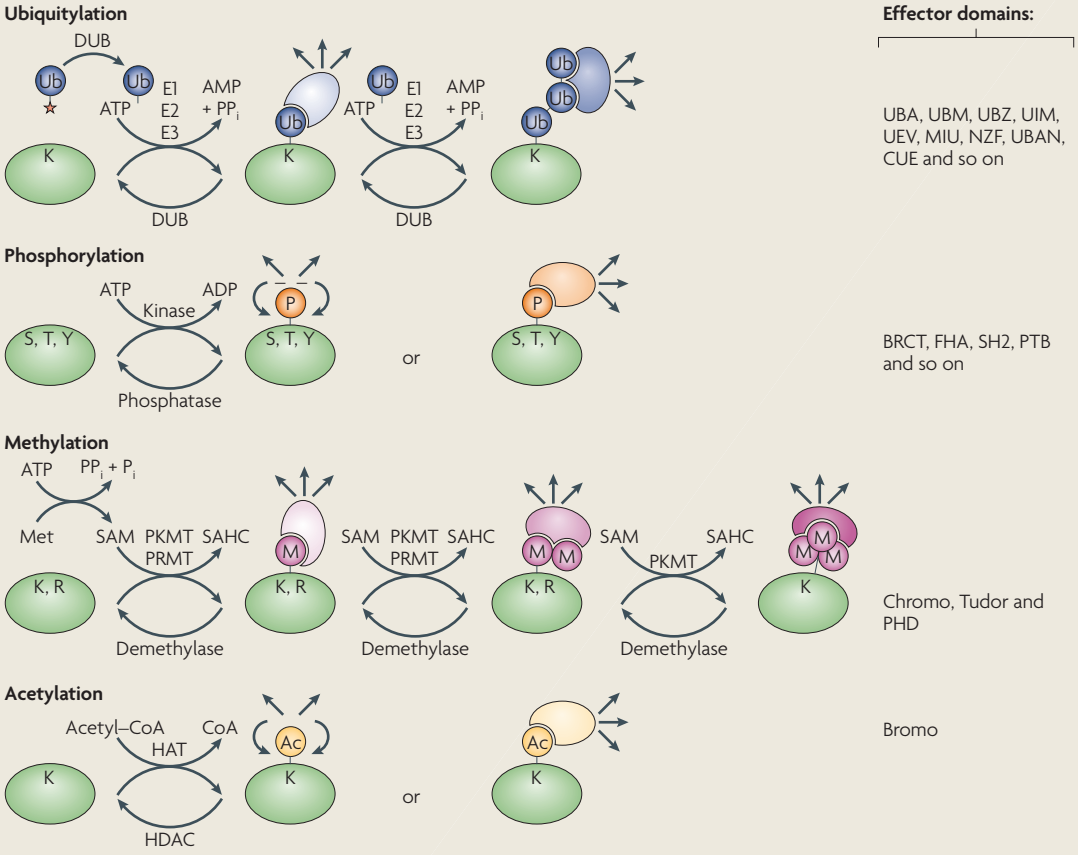
Non-degradative ubiquitin signalling

Ubiquitin and ubiquitin-like modifiers. Ubiquitin acts as a reversible post-translational modifier for other cellular proteins (BOX 1). The first protein recognized to be modified by ubiquitin was histone H2A, although at that time the importance of the modification remained unclear[1]. Soon after its discovery ubiquitin became best known as a signal for energy-dependent proteolysis[2]. Attachment of ubiquitin to a substrate is mediated by a cascade of enzymes (BOX 1), and repeated conjugation cycles can result in the formation of long polyubiquitin chains that mediate targeting to the 26S proteasome (reviewed in REF. 3). Following the discovery and mechanistic characterization of the ubiquitin system, several structurally related proteins were identified, most prominent among them the small ubiquitin-related modifier (SUMO), the mechanisms of conjugation and deconjugation of which closely resemble those of ubiquitin[4]. However, the function of ubiquitin-like modifiers was generally found to be unrelated to proteolysis, and even ubiquitin itself was discovered to have a range of non-degradative roles. Today, modification of a protein by ubiquitin or its relatives is therefore viewed much more broadly as a signal that is comparable to many other post-translational modifications (BOX 1).

Monoubiquitylation versus polyubiquitylation. Modification of a substrate by a single ubiquitin moiety can have several different consequences, ranging from the control of endocytosis and intracellular vesicle transport to the regulation of chromatin structure, transcription and DNA damage processing. However, the functional

Box 1 | **Post-translational protein modifications**



Covalent modification of cellular proteins by small chemical groups or entire proteins serves as an efficient means to modulate their properties. Attachment of ubiquitin (Ub), which is synthesized as an inactive precursor (indicated by a star) that requires carboxy-terminal processing, is mediated by a ubiquitin-activating enzyme (E1) in cooperation with a ubiquitin-conjugating enzyme (E2) and a ubiquitin protein ligase (E3) for substrate selection (reviewed in REF. 3). Many of the features of ubiquitylation resemble those of other post-translational modifications, such as phosphorylation (P), methylation (M) and acetylation (Ac) (see the figure; reviewed in REFS 118,119,120, respectively).

In addition to requiring dedicated enzymes that confer selectivity to the reaction, attachment of the modifier consumes energy, usually derived from ATP or, in the case of acetylation, acetyl–coenzyme A (acetyl–CoA). Similarly, reversal of the reaction is mediated by the action of a specific enzyme. Ubiquitin and acetyl groups are most often attached to Lys (K) residues, whereas methylation can also occur on Arg (R), and phosphorylation is targeted to Ser (S), Thr (T) and Tyr (Y). Phosphate and acetyl groups are usually attached as single moieties. By contrast, successive conjugation of ubiquitin can result in the formation of polyubiquitin chains of different linkages, and transfer of two or even three methyl groups to the same acceptor site also results in distinct methylation patterns. In some cases, such as acetylation and phosphorylation, the modifier directly affects the properties of the substrate protein by changing its surface charge. More often, however, the function of post-translational modifications is mediated by the recognition of the modified target by downstream effectors harbouring modular domains (referred to as effector domains) that specifically bind the relevant modification. BRCT, BRCA1 C-terminal domain; CUE, coupling of ubiquitin conjugation to ERAD; DUB, deubiquitylating enzyme; FHA, forkhead-associated domain; HAT, histone acetyl transferase; HDAC, histone deacetylase; Met, methionine; MIU, motif interacting with ubiquitin; NZF, NPL4 zinc finger; PHD, pleckstrin homology domain; PKMT, protein Lys methyl transferase; PRMT, protein Arg methyl transferase; PTP, phosphotyrosine-binding domain; SAHC, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SH2, Src homology 2; UBA, ubiquitin associated; UBAN, ubiquitin-binding in ABINs; UBM, ubiquitin-binding motif; UBZ, ubiquitin-binding zinc finger; UEV, ubiquitin-conjugating enzyme variant.

complexity of ubiquitin signalling is largely due to its ability to form polymeric chains. All of ubiquitin's seven Lys residues can be used for further ubiquitylation[5], and even its amino terminus has been shown to function as an acceptor for the formation of linear chains[6]. As the Lys residues are distributed over the surface of the molecule, chains of different linkage adopt distinct geometries. Therefore, linkage specificity of ubiquitin conjugation, mediated by either the E2 or the E3 enzyme, produces structurally discernable signals with unique consequences for the modified target[7]. For example, a Lys48-linked polyubiquitin chain directs the substrate to the 26S proteasome for degradation. By contrast, linear and Lys63-linked chains seem to mediate mainly proteasome-independent functions. Both chains have been implicated in inflammatory signalling by the nuclear factor-κB pathway, and

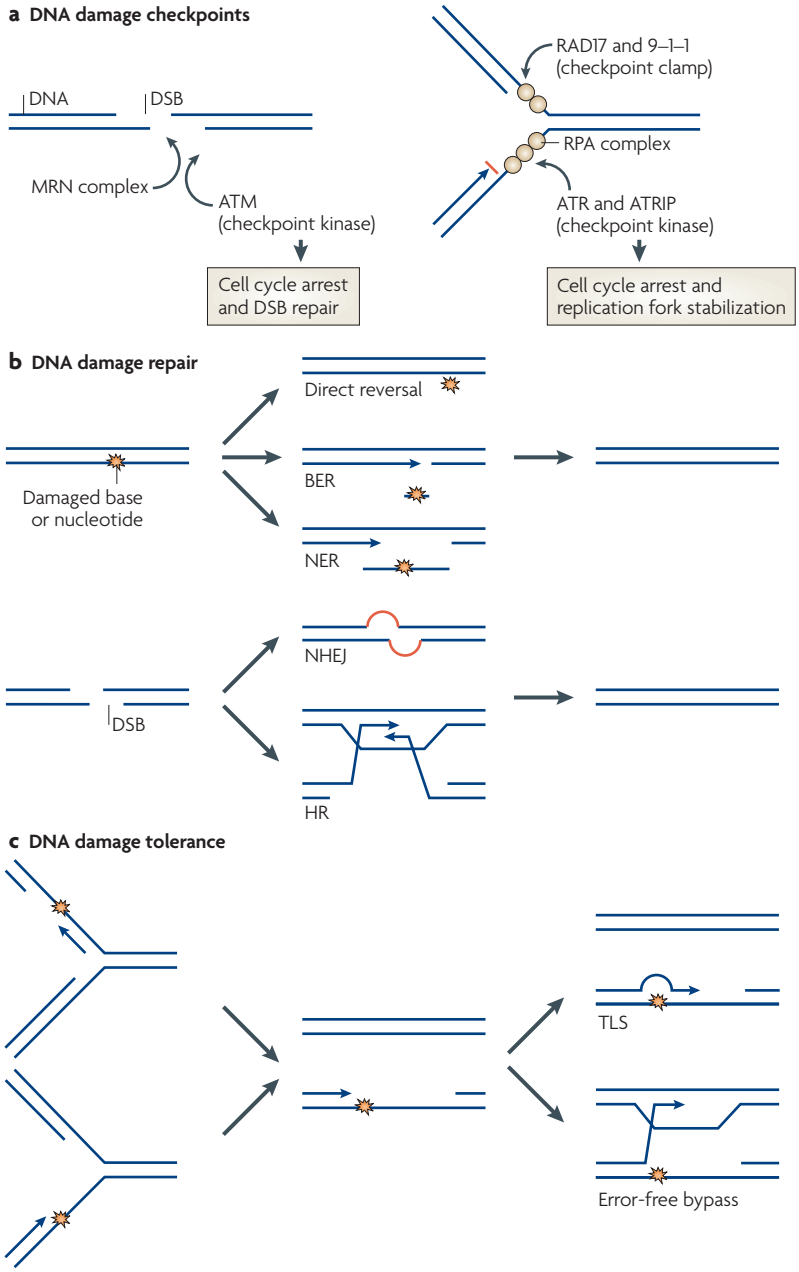


Figure 1 | **Processing of DNA damage. a** | DNA damage is sensed by checkpoint pathways. DNA double-strand breaks (DSBs) and replication problems (indicated by the red bar) activate the kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3-related (ATR), respectively, which initiate a phosphorylation-dependent signalling cascade leading to cell cycle arrest, stabilization of stalled replication intermediates and inhibition of late replication origins. Checkpoint signalling is also important for activating DNA repair systems. **b** | Simple lesions to bases or nucleotides can sometimes be directly reversed. More often, the damaged DNA is excised and the gap subsequently filled by a polymerase. Small base damage is processed by base excision repair (BER), whereas bulky adducts are removed by nucleotide excision repair (NER). DSBs can be repaired by non-homologous end-joining (NHEJ), which involves direct ligation of the ends, or homologous recombination (HR). **c** | If lesions remain in the DNA during replication and are encountered by the replication fork, they present an obstacle to replicative DNA polymerases. Bypass of the damage allows completion of replication, but does not remove lesions and is accomplished either by specialized, damage-tolerant but mutagenic DNA polymerases (through translesion synthesis (TLS)) or by an error-free mechanism possibly involving a template switch to the sister chromatid. Both pathways operate at least partially independently of replication fork progression[89,106,107]. ATRIP; ataxia telangiectasia and RAD3-related; RPA, replication protein A.

Lys63-linked chains are involved in endocytosis, ribosome function and several pathways related to genome stability (reviewed in REF. 8).

Recognition of the ubiquitin signal. In general, conjugation of ubiquitin does not seem to greatly alter the conformation of a target protein but instead to modulate its surface properties, mostly by providing an additional interaction site. Hence, the ubiquitin signal in both monomeric and polymeric form is often transmitted through dedicated ubiquitin receptors that recognize the modifier in the context of a specific target. To date, more than a dozen types of ubiquitin-binding domain (UBD) have been described (BOX 1; reviewed in REF. 9), many of which are non-discriminatory and gain their specificity for a particular ubiquitin signal only together with other domains that recognize the substrate protein. Some UBDs, however, are highly selective for a particular type of polyubiquitin linkage and can even discriminate between Lys63-linked and linear chains, which are structurally similar[10–12].

DNA damage signalling and processing

In light of its pervasive nature in cell metabolism, it is not surprising that the ubiquitin system affects nuclear functions that are associated with various DNA transactions. As a consequence, ubiquitin is an important mediator of genome stability and greatly influences the main lines of defence against DNA damage that are available to a cell.

DNA damage sensing. Perception of DNA damage is essential to counteracting it, and cells have developed intricate checkpoint mechanisms to recognize and mark sites of lesions to initiate an appropriate response (reviewed in REF. 13). DSBs trigger a phosphorylation cascade initiated by the checkpoint kinase ataxia telangiectasia mutated (ATM) that leads not only to a cell cycle arrest, but also to the recruitment of a series of factors that initiate repair of the break (FIG. 1a). As detailed below, ubiquitylation events, particularly those associated with Lys63-linked polyubiquitin chains, seem to be greatly important for amplifying the signal emanating from the break to induce a full-blown damage response. During DNA replication, damage is sensed primarily because of its inhibitory effect on DNA polymerases, which leads to the exposure of single-stranded DNA (ssDNA) at the sites of replication fork stalling and activation of the checkpoint kinase ataxia telangiectasia and RAD3-related (ATR). A subunit of the 9–1–1 complex, a heterotrimeric checkpoint sensor involved in the response to both DSBs and replication problems, was reported to be monoubiquitylated in response to DNA damage[14], although the importance of this modification for the checkpoint response has recently been called into question[15].

DNA repair. Following the sensing of damage, DNA repair mechanisms operate to remove a range of lesions from DNA, and as with the checkpoint response, the appropriate pathway is dictated by the type of lesion (FIG. 1b; reviewed in REF. 16). DSBs are repaired either by homologous recombination or by non-homologous end joining, and components of both pathways have been identified

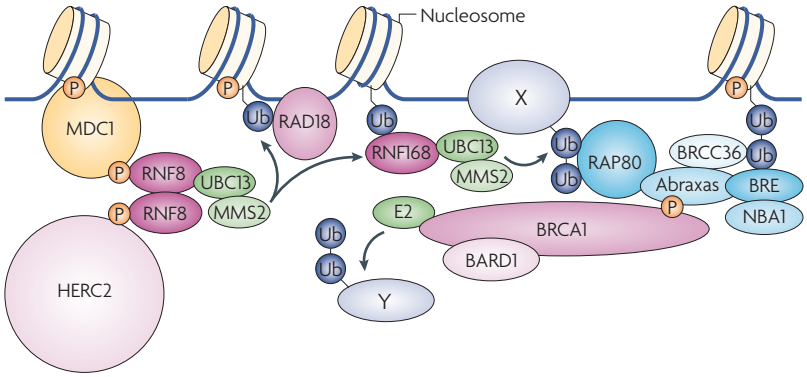


Figure 2 | **Ubiquitin signalling at DNA double-strand breaks.** The cascade of ubiquitylation and ubiquitin-binding events is shown schematically, indicating approximate size relationships. E3 ligases (RING finger protein 8 (RNF8), HECT domain and RCC1-like domain-containing protein 2 (HERC2), RNF168, RAD18, breast and ovarian cancer type 1 susceptibility protein (BRCA1) and BRCA1-associated RING domain protein 1 (BARD1)) are shown in shades of pink. E2 enzymes (ubiquitin-conjugating enzyme 13 (UBC13), MMS2 and an unspecified E2) are shown in shades of green. Unidentified ubiquitylation targets are labelled as X and Y. It should be noted that the polyubiquitin conjugates to which BRCA1/BRCA2-containing complex subunit 36 (BRCC36) and brain and reproductive organ-expressed (BRE) bind are unknown, even though the two proteins are shown here as binding to ubiquitylated histone H2A. In addition, although UBC13 is shown in complex with MMS2, participation of MMS2 in the ubiquitylation cascade is unconfirmed. A version of this figure with structural information is available as supplementary information (Supplementary information S1 (figure)). PDB codes for the structures used in Supplementary information S1 (figure) are listed in Supplementary information S2 (table). MDC1, mediator of DNA damage checkpoint protein 1, Ub, ubiquitin.

Ubiquitin-binding domain
(UBD). A domain that mediates non-covalent interactions with ubiquitin. UBDs are usually found in downstream effectors that selectively interact with ubiquitylated target proteins and come in various different types (reviewed in REF. 9), such as α-helical motifs in UBM, UIM and MIU, or zinc fingers, as in UBZ.

Replication fork
The part of replicating DNA in which the two strands are being separated and DNA synthesis is occurring to generate two copies of the parental DNA.

Homologous recombination
Genetic recombination process in which nucleotide sequences are exchanged between two strands of identical or similar DNA. This pathway is widely used in repairing DSBs.

Non-homologous end joining
A means to repair DSBs that is alternative to homologous recombination and involves direct ligation of the break ends without the need for a homologous template.

as ubiquitylation substrates (reviewed in REF. 17). Small adducts are recognized and excised by dedicated glycosylases in a process known as base excision repair. By contrast, bulky chemical adducts and lesions arising from ultraviolet radiation are removed by nucleotide excision repair. As discussed in several recent reviews, this pathway is heavily influenced by the ubiquitin system in both proteasome-dependent and proteasome-independent ways[17,18]. ICLs are particularly dangerous to the propagation and read-out of the genetic material, as they prevent the separation of strands needed for both replication and transcription. Their repair depends on the cooperation between several different repair systems, which in metazoans is controlled by a group of factors collectively known as the Fanconi anaemia pathway. As discussed in detail below, monoubiquitylation of two Fanconi anaemia components by other factors of the same pathway is the key event that activates ICL repair during DNA replication. Intriguingly, the Fanconi anaemia pathway is not only involved in the removal, but also in the sensing of ICLs, as it contributes to generating the signal that triggers an appropriate ATR-dependent checkpoint response[19].

DNA damage tolerance. When damage affects ssDNA arising during replication, repair by excision-based systems becomes inappropriate because strand incisions would cause replication forks to collapse. Therefore, repair is delayed, and lesions are first bypassed by the replication machinery in a process known as DNA damage tolerance (FIG. 1c). The pathway has also been termed post-replication repair, despite the notion that it largely operates during replication and does not mediate the actual removal of the damage[20]. Damage tolerance contributes to the overall resistance of cells to genotoxic agents. Moreover, it affects the fidelity with which damaged DNA is replicated, giving rise to a major portion of damage-induced mutagenesis. As described below, two forms of ubiquitylation, monoubiquitylation and Lys63-linked polyubiquitylation, are required for the full extent of DNA damage bypass in eukaryotic organisms.

The DNA double-strand break response

Orchestration of the damage response following a DSB involves a series of ubiquitylation enzymes cooperating in a cascade of modification events that has been discovered over the past few years. Although many open questions regarding its regulation, relevant ubiquitylation targets and the importance of ubiquitin chain linkage remain, a working model incorporating the main players and their interactions with checkpoint-mediated phosphorylation signalling has now emerged (FIG. 2).

Phosphorylation-mediated damage signalling. Among the earliest targets of ATM at a DSB is the histone variant H2AX[21]. Through a positive feedback loop involving mediator of DNA damage checkpoint protein 1 (MDC1), the signal spreads to the region surrounding the break[22]. Phosphorylated H2AX, also known as γ-H2AX, thus marks the affected chromatin domain for recruitment of various downstream effectors of the damage response, which recognize γ-H2AX by means of phosphate-binding domains, such as FHA (forkhead-associated domain) and BRCT (breast and ovarian cancer type 1 susceptibility protein (BRCA1) C-terminal domain) motifs. MDC1 itself binds γ-H2AX through tandem BRCT domains[23] and is responsible for initiating the ubiquitylation cascade.

Signal transduction through E3 ligases and UBDs. DNA repair and checkpoint signalling at DSBs crucially depends on the recruitment of the multifunctional E3 enzyme BRCA1 (BOX 2). Localization of BRCA1 to the break is in turn mediated by a series of ubiquitylation events that are initiated by RING finger protein 8 (RNF8) in complex with the E2 enzyme ubiquitin-conjugating enzyme 13 (UBC13)[24–26]. RNF8 binds to ATM-phosphorylated, chromatin-associated MDC1 through an N-terminal FHA domain, and the carboxy-terminal RING domain mediates recruitment of the E2 ligase. Fusion of UBC13 to a C-terminally truncated RNF8 lacking the RING domain overcomes the need for RNF8, indicating that it is the localization of UBC13 to the break that is responsible for damage signalling by RNF8 (REF. 27). Indeed, deletion of UBC13 in chicken cells causes a defect in DSB repair by homologous recombination[28]. RNF8 and UBC13 ubiquitylate H2A and H2AX, but other substrates probably exist.

These chromatin-associated ubiquitin marks (uH2A and uH2AX), which co-localize with radiation-induced γ-H2AX foci, are recognized by a second E3 ligase, RNF168, which harbours two motif interacting with ubiquitin (MIU) UBDs[29,30]. RNF168 also cooperates with UBC13 and seems to amplify the ubiquitin signal

Box 2 | **BRCA1 and DNA repair**

Breast and ovarian cancer type 1 susceptibility protein (BRCA1) is a multifunctional tumour suppressor with important roles in DNA double-strand break (DSB) repair and other DNA transactions (reviewed in REF. 48). It dimerizes with BRCA1-associated RING domain protein 1 (BARD1) to yield a functional E3 ligase. The heterodimer forms at least three independent complexes with additional factors involved in pathways that mediate three processes: the recruitment of BRCA1 to sites of DSBs (with abraxas, receptor-associated protein 80 (RAP80), BRCA1/BRCA2-containing complex subunit 36 (BRCC36), brain and reproductive organ-expressed (BRE; also known as BRCC45)and NBA1 (also known as MERIT40)); its localization to sites of replication problems, for example in response to interstrand cross links (ICLs) during S phase (with BRCA1-interacting protein C-terminal helicase 1 (BACH1; also known as FANCJ and BRIP1)); and BRCA1 function in the resection of DSBs (with CtBP-interacting protein (CtIP; also known as RBBP8)). The BRCA1–abraxas complex apparently contributes to the G2–M checkpoint. Depending on the E2 enzyme used for cooperation, BRCA1 can promote polyubiquitylation through several different linkages[121], and the mechanism by which its E3 ligase activity contributes to DNA repair is not entirely clear. Although ubiquitylation of CtIP has been implicated in the G2–M checkpoint response[122], other data suggest that BRCA1's ubiquitin ligase activity may not even be required for all aspects of its function[123]. A clear contribution to homologous recombination became evident when one of BRCA1's interaction partners, partner and localizer of BRCA2 (PALB2; also known as FANCN), was shown to act as a bridging factor mediating association with BRCA2 (also known as FANCD1)[124,125].

BRCA2 in turn is a second tumour suppressor protein responsible for the loading of the recombination factor RAD51 onto single-stranded DNA for the initiation of homology search and strand invasion. Thus, one of BRCA1's functions might be to act upstream of BRCA2 in the control of DSB repair by homologous recombination. How PALB2 and BRCA2 stand in relation to the three known BRCA1 complexes has not been fully characterized.

at the break for recognition by two ubiquitin interaction motif (UIM)-type UBDs in receptor-associated protein 80 (RAP80). Initially identified as an interactor of several nuclear receptors involved in transcriptional regulation, RAP80 is now known to mediate BRCA1 recruitment to damaged chromatin[31–34]. In this context, RAP80 forms a complex with the scaffold protein abraxas, the phosphorylated form of which is recognized by the tandem BRCT motifs of BRCA1. Three additional members of the complex — brain and reproductive organ-expressed (BRE; also known as BRCC45), NBA1 (also known as MERIT40) and the DUB BRCA1/BRCA2-containing complex subunit 36 (BRCC36) — are required for BRCA1 recruitment[31,33–37]. Interestingly, RNF8-mediated ubiquitylation also leads to the recruitment of the ubiquitin ligase RAD18 in a UBD-dependent manner[38]. RAD18 in turn seems to be responsible for the association of the recombination factor RAD51C; however, its E3 ligase activity is not required for this process, and it is unclear whether and how the RAD18-dependent events are connected to those initiated by RNF168.

Another putative E3 ligase was identified recently that contributes to the establishment of the ubiquitin signal at DSBs: HECT domain and RCC1-like domain-containing protein 2 (HERC2), which interacts with the FHA domain of RNF8 and promotes RNF8 interaction with UBC13 after damage-induced phosphorylation[39]. HERC2 deficiency is overcome by the same RING-deleted RNF8–UBC13 fusion that rescues an RNF8 deletion, suggesting that the function of HERC2 is to support ubiquitylation by stabilizing the RNF8–UBC13 interaction.

Ubiquitylation enzymes, substrates and chain linkage. Although the importance of the ubiquitylation cascade for the response to DSBs is undisputed, many mechanistic aspects of the pathway remain to be resolved, particularly the nature of the ubiquitin conjugates that contribute to signalling. Intriguingly, the importance of the HECT domain in HERC2 is still unclear, as it has not been resolved whether E3 ligase activity is required for its function[39]. UBC13, the partner of RNF8 and RNF168, usually cooperates with an E2-like protein, UEV1 (also known as UBE2V1) or MMS2 (also known as UBE2V2), for the synthesis of Lys63-linked polyubiquitin chains[40,41]. However, solid information on the contribution of these factors to DSB signalling is still missing. UEV1- and MMS2-independent activity has been postulated[27], although this would be difficult to reconcile with structural information showing that complex formation dictates linkage specificity of UBC13 (REF. 42) and with the notion that a mutant UBC13 that cannot interact with UEV1 or MMS2 fails to support DNA damage signalling[27]. The preference of the RAP80 UIMs for Lys63-linked chains is consistent with the importance of this linkage in the ubiquitylation cascade[31,33], and a structure in complex with Lys63-linked diubiquitin shows how selectivity of the tandem UIMs for a particular conformation of the ubiquitin dimer is achieved by means of the length of the peptide linking the two UIMs[12]. Accumulation of Lys63-linked as opposed to Lys48-linked polyubiquitin chains at DSB sites was indeed shown by the use of ubiquitin mutants and linkage-specific antibodies and has been attributed to the activity of RNF168 (REFS 29,30,33). However, RAP80 apparently also binds Lys6-linked chains[33]. Moreover, the MIU motifs of RNF168 were not found to exhibit any linkage preference, and both RNF168 and RAP80 also recognize ubiquitylated histones[29,30], for which Lys63-linked polyubiquitylation has not been shown. Instead, uH2A and uH2AX were found to be mostly monoubiquitylated, and the small amount of detectable diubiquitylated forms might result from modification at two different sites[24]. Finally, BRE and BRCC36, which contribute to BRCA1 recruitment and damage signalling, bind polyubiquitin conjugates but do not exhibit any Lys63 selectivity[37].

These observations call into question the exclusive role of Lys63-linked polyubiquitylation and suggest that monoubiquitylation and potentially other linkages are also important, particularly for the early events leading to the recruitment of RNF168. At the same time, additional, as-yet-unidentified ubiquitylation targets, modified preferentially by Lys63-linked chains, are likely to emerge. Further downstream in the pathway, when BRCA1 enters the picture, the situation becomes even more complex, as neither the linkage type nor the modification targets that are relevant for repair are known (BOX 2). Another intriguing observation concerns the checkpoint signalling factor p53-binding protein 1 (53BP1), the recruitment of which strictly depends on RNF8- and RNF168-related ubiquitylation events; however, 53BP1 is not known to interact with ubiquitin and instead binds to methylated histone H4 (REF. 43).

Base excision repair
A DNA repair pathway that is primarily responsible for removing small, non-helix-distorting base lesions that affect only one strand, such as alkylation or oxidative damage.

Nucleotide excision repair
A pathway responsible for the removal of bulky, helix-distorting lesions that affect only one strand, involving the excision of a lesion-containing oligonucleotide and the resynthesis of the affected region.

FHA
A 65–100-residue phosphorylation-specific protein–protein interaction motif that was first identified in forkhead transcription factors. It is often found in proteins that also contain BRCT repeats.

BRCT
A ~90-residue phosphate-binding tandem domain that interacts with specific motifs in their phosphorylated form, such as in γ-H2AX.

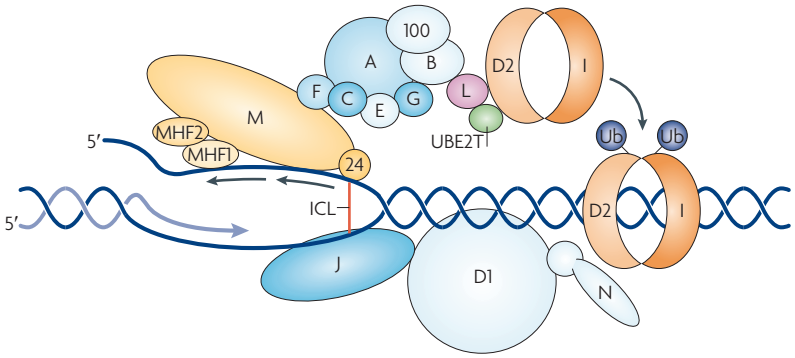


Figure 3 | **The Fanconi anaemia pathway.** The recognition complex (comprising Fanconi anaemia group M protein (FANCM) and Fanconi anaemia-associated protein 24 (FAAP24)) in association with the DNA-binding histone-fold proteins MHF1 and MHF2 is bound at a DNA interstrand cross link and recruits the core complex (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FAAP100) through interaction with FANCG. FANCL binding to the FANCD2–FANCI complex is shown with a subsequent ubiquitylation event leading to the recruitment of FANCD2–FANCI to chromatin. The downstream repair complex (comprising FANCJ, FANCD1 and FANCN) is shown beneath the DNA. A version of this figure with structural information is available as supplementary information (Supplementary information S3 (figure)). PDB codes for the structures used in Supplementary information S3 (figure) are listed in Supplementary information S2 (table). ICL, interstrand cross link; Ub, ubiquitin.

Complementary to the ubiquitylation cascade, removal of ubiquitin by dedicated isopeptidases is expected to play a regulatory part in the damage response, and several DUBs have been suggested as potential candidates: BRCC36, a subunit of the BRCA1–abraxas complex, preferentially cleaves Lys63 linkages[33], and numerous DUBs were found to act on uH2A *in vivo* (reviewed in REF. 44). Among them, USP3 is particularly likely to process the targets of RNF8 and RNF168, as its overexpression abolishes the formation of damage-induced uH2A foci, whereas knockdown causes them to be more persistent[30,45]. Finally, even SUMO has emerged as a new participant in the process, as several SUMO-specific E3 enzymes localize to damage-induced foci and exert positive effects on damage signalling. Sumoylation of BRCA1 itself was reported to stimulate its ubiquitin E3 ligase activity, but evidence that this effect is responsible for the observed phenomenon is still lacking[46,47].

Implications of ubiquitin-dependent signalling at DSBs. Although the mechanism by which BRCA1 recruitment contributes to damage signalling is by no means clear, the importance of the pathway for genome stability is corroborated by the consequences of defects in the relevant factors. BRCA1 itself is a major tumour suppressor protein with many functions (BOX 2; reviewed in REF. 48). Defects in RNF168 cause RIDDLE syndrome, which is associated with radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties[49], and RNF8-dependent histone ubiquitylation and BRCA1 recruitment have even been implicated in the nucleotide excision repair pathway[50]. The observation that herpes simplex virus downregulates ubiquitin-dependent damage signalling by targeting RNF8 and RNF168 for degradation to avoid entry into the lytic cycle further supports the central role of E3 ligases in the control of genome stability[51].

RING
A zinc-binding protein–protein interaction motif found in RING-type E3 enzymes that scaffolds two zinc ions and forms the hallmark of the largest class of E3 ligases.

HECT
A domain with a catalytic Cys residue (found in a class of E3 enzymes) that forms a thioester intermediate during ubiquitin transfer to the substrate protein.

The Fanconi anaemia pathway

An example of the importance of the monoubiquitin signal in DNA repair is the Fanconi anaemia pathway. Fanconi anaemia is a rare, recessive or X-linked disorder that is characterized by high frequency of chromosomal abnormalities and susceptibility to ICL-inducing agents, such as mitomycin C. Therefore, the Fanconi anaemia pathway is thought to function in the sensing and repair of ICLs. Additional functions are likely, as defects in ICL repair do not account for some of the characteristics associated with the disease, such as skeletal abnormalities, defects in growth hormone signalling and bone marrow failure. Indeed, interactions of the Fanconi anaemia core complex (see below) with components of the developmental Notch signalling pathway and a possible contribution to transcriptional regulation have been suggested[52,53].

The Fanconi anaemia DNA repair network involves many factors, but specifically comprises 13 distinct genes, mutations in which lead to a Fanconi anaemia phenotype. The 13 proteins participate in what can be categorized as four complexes (FIG. 3). These include the recognition complex, which detects the ICL and comprises Fanconi anaemia group M protein (FANCM) and the related protein Fanconi anaemia-associated protein 24 (FAAP24), and the core complex, comprising FANCA, FANCB, FANCC, FANCE, FANCG, FAAP100 and the catalytic subunit, FANCL. The core complex is responsible for the monoubiquitylation of the FANCI–FANCD2 complex (also known as the I–D2 complex; for recent reviews, see REFS 54,55); monoubiquitylation of FANCD2 and FANCI results in their retention in chromatin foci[56–57]. Finally, the repair complex comprises FANCD1, FANCN and FANCJ, which contribute to the BRCA pathway that controls homologous recombination (BOX 2).

The recognition complex. The FANCM–FAAP24 heterodimer has DNA translocase activity and is thought to recruit the core complex to sites of DNA damage[58–59]. Although FANCM is not part of the core complex, its depletion through small interfering RNA (siRNA) results in loss of FANCD2 monoubiquitylation and focus formation[60,61], and a recent overexpression study has revealed that FANCM binds directly to FANCF, supporting this recruitment role of FANCM–FAAP24 (REF. 62). The FANCM domain that is necessary for FANCF binding, MM1, is also required for FANCD2 monoubiquitylation *in vivo*. This suggests that, although assembly of the core complex *in vivo* does not require FANCM[59,62], its function is FANCM dependent. It has yet to be established whether FANCM is also required for FANCI monoubiquitylation. Several recent reports have also indicated a role for FANCM that is independent of its role in the core complex and has to do with replication fork remodelling, DNA chain elongation and ATR activation *in vivo*[63,64]. Two DNA-binding histone-fold proteins, MHF1 (also known as CENPS and FAAP16) and MHF2 (also known as CENPX and FAAP10), were found in tight association with FANCM and are required both for its fork remodelling activity and for normal FANCD2 ubiquitylation[65,66]. The fact that they are present in organisms lacking the

core complex, such as yeast, suggests a conserved role in replication fork maintenance.

The core complex. Complementation analyses with cells from patients with Fanconi anaemia have revealed that mutations in any member of the core complex lead to a reduction or loss of FANCD2 monoubiquitylation. This has led to the notion that the complex acts as a multisubunit E3 enzyme. However, this idea has been challenged recently by the finding that, *in vitro*, FANCL[67], UBE2T (the E2 enzyme for the pathway[68]) and FANCI are sufficient for the Lys561-specific monoubiquitylation of FANCD2 (REF. 69). Moreover, many lower eukaryotes apparently lack a complete core complex. It is unclear whether all the core complex components are required for *in vivo* monoubiquitylation of FANCI, but the modification is not supported in FANCD2-, FANCC- or FANCL-deficient cells in the chicken DT40 system[70]. *In vitro*, the UBE2T–FANCL E2–E3 pairing is sufficient for FANCI monoubiquitylation[71]. Thus, it is still unclear how the core complex ultimately affects the modification of FANCI–FANCD2.

Recently, the structure of the catalytic subunit FANCL was reported[72]. Contrary to secondary structure predictions of a protein containing WD40 repeats[67], the crystal structure of *Drosophila melanogaster* FANCL reveals a repeating unit of E2-like folds, giving rise to a three-domain protein that contains a catalytic RING domain, an E2-like fold with no known function and a central double Arg-Trp-Asp (RWD) domain (also a fold belonging to the E2 superfamily), apparently required for substrate interaction. Although the full-length structure of the catalytic heart of the complex is now available, the relevance of the other core subunits remains unclear, particularly as *D. melanogaster* apparently has no other core complex homologues.

Other questions remaining include how the modification of FANCI and FANCD2 is restricted to monoubiquitylation. A recent study reveals that UBE2T can apparently support polyubiquitylation in cooperation with BRCA1 *in vivo*[73], so the restriction may not be at the level of the E2 enzyme. It is possible that the E2-like fold interacts with the monoubiquitylated substrate, thus capping the modification. Alternatively, a member of the core complex may have a similar role, with an as-yet-unidentified homologue in the invertebrates. It is clear, however, that further structural characterization of the core complex and substrates is required for a complete understanding of the modification mechanism.

Monoubiquitylation and deubiquitylation of FANCI–FANCD2. Monoubiquitylation of FANCD2 is the central event in the Fanconi anaemia repair pathway[57] and is absolutely required for the repair of ICLs. Monoubiquitin fused to a non-ubiquitylatable FANCD2 Lys561Arg mutant partially rescues cellular defects even when FANCI cannot be monoubiquitylated[70,74]. A recent breakthrough has finally confirmed Fanconi anaemia as a genuine DNA repair disorder[75]. Using a *Xenopus laevis* egg extract cell-free system and depleting FANCD2, one study showed that FANCI–FANCD2 is required for nucleotide insertion opposite the ICL and for the incisions that allow unhooking of the cross link in replication-coupled ICL repair. Addition of the recombinant FANCI–FANCD2 fully rescues the depletion defects, but the complex containing the FANCD2 Lys561Arg mutant does not. In this system, ubiquitylation occurs when the replication fork stalls one nucleotide upstream of the cross link, consistent with a need for FANCI–FANCD2 during the insertion and incision steps[76].

Deubiquitylation of FANCD2 by USP1 (also known as WDR48) is required for ICL repair[77,78], indicating that the monoubiquitylation of FANCD2 is not the sole driving force for repair and that regulated deubiquitylation is also important. This may partially explain why the monoubiquitylated FANCD2 Lys561Arg mutant does not completely rescue cellular defects. Indeed, a mouse USP1 knockout reveals increased chromatin localization of monoubiquitylated FANCD2, but impaired assembly of FANCD2 repair foci, sensitivity to mitomycin C and defects in homologous recombination[79]. siRNA knockdown studies have also shown that USP1 deubiquitylates FANCI[56], but it is unclear whether this is required for efficient ICL repair.

Future directions. A major question remaining is to define the mechanism by which the monoubiquitylation signal is relayed into repair focus formation. Targeting of FANCI–FANCD2 to repair foci requires monoubiquitylation in human cells. This suggests the presence of a chromatin-associated ubiquitin receptor, but the identity of such a receptor has remained elusive. Reversionless 1 (REV1), a polymerase harbouring two UBDs, has been suggested as a potential candidate[75,80]. It has also been proposed that there are further substrates for the pathway[54,55]. This is a possibility, given the multisubunit nature of the core complex, not dissimilar to the anaphase-promoting complex, a large E3 ligase required for cell cycle control[81]. A recent study suggests an additional role for FANCC in the regulation of tumour necrosis factor[82]. How the ubiquitylation events are rendered site specific and restricted to single ubiquitin moieties are also intriguing mechanistic questions.

DNA damage tolerance

Key to the regulation of replicative lesion bypass is the modification of the sliding clamp protein proliferating cell nuclear antigen (PCNA) (FIG. 4; reviewed in REF. 83). This modification system has turned out to be one of the most intriguing examples for the interplay between monoubiquitylation, polyubiquitylation and sumoylation, and its functional implications are by no means fully understood.

PCNA modification in DNA replication and damage bypass. In budding yeast, PCNA is subject to monoubiquitylation, Lys63-linked polyubiquitylation and sumoylation at a highly conserved Lys164 residue[84]. Ubiquitylation is induced by DNA damage and has been observed in all the eukaryotic species examined, whereas sumoylation is constitutive during S phase and seems to be less widespread. Each of the modifications labels PCNA for an alternative

WD40 repeat
Repeat of ~40 amino acids with a characteristic central Trp–Asp motif.

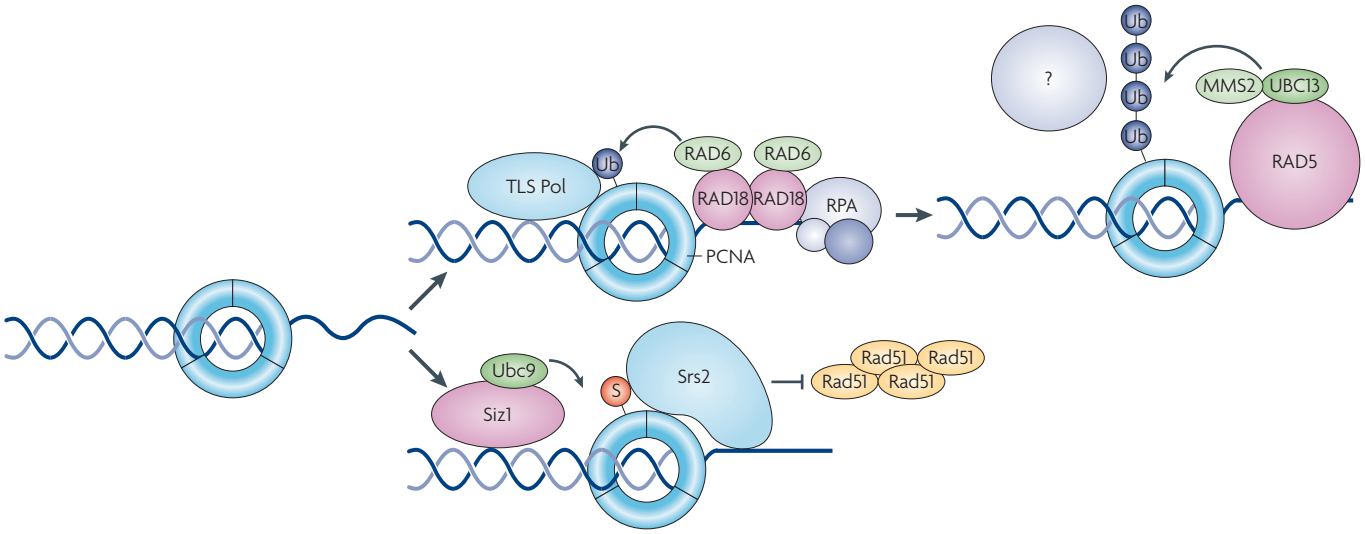


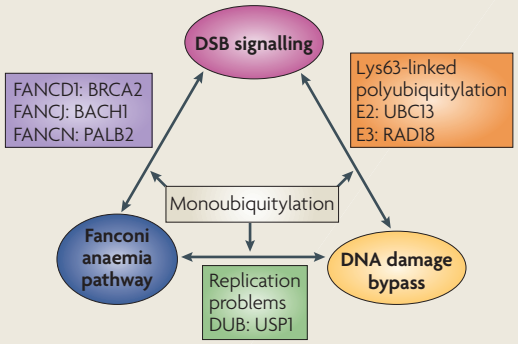
Figure 4 | **Ubiquitin-dependent DNA damage bypass.** The modifications on proliferating cell nuclear antigen (PCNA) and their downstream effects are shown schematically. The branch indicating sumoylation is specific for the situation in budding yeast. PCNA is represented as a ring-shaped molecule encircling the DNA. Monoubiquitylated PCNA recruits damage-tolerant DNA polymerases (translesion synthesis (TLS) Pol) for TLS, whereas sumoylated PCNA interacts preferentially with the antirecombinogenic helicase Srs2. The downstream effectors through which polyubiquitylation-dependent error-free damage bypass is initiated are unknown. A version of this figure with structural information is available as supplementary information (Supplementary information S4 (figure)). PDB codes for the structures used in Supplementary information S4 (figure) are listed in Supplementary information S2 (table). RPA, replication protein A; Siz1, SAP and Miz-finger domain-containing protein 1; S, SUMO; Ub, ubiquitin; Ubc9, ubiquitin like-conjugating 9; UBC13, ubiquitin-conjugating 13.

function. Monoubiquitylation of PCNA at a stalled replication intermediate leads to the recruitment of several damage-tolerant DNA polymerases[85–87]. These enzymes can use damaged DNA as a template for translesion synthesis (TLS), and their action on a range of lesions is a major cause of damage-induced mutagenesis. Although ubiquitin-independent activity has been reported for a portion of TLS[88–90], defects in PCNA monoubiquitylation cause not only a severe damage sensitivity, but also defects in immunoglobulin diversification[91]. The polymerases have a basal affinity for unmodified PCNA, but a preference for the modified clamp is achieved through UBDs of the ubiquitin-binding zinc finger (UBZ) or ubiquitin-binding motif (UBM) type[92]. Polyubiquitylation of PCNA at the same Lys residue activates an alternative, error-free pathway of damage bypass that relies on the information encoded by the undamaged sister chromatid, which is particularly important for the resistance to chronic low doses of ultraviolet radiation[93]. It may involve a template switch of the stalled primer terminus, but the mechanism by which the pathway operates, or how it is triggered by Lys63-linked polyubiquitin chains, remains unknown. It is clear, however, that the Lys63-linked polyubiquitin chains on PCNA do not act as proteasomal targeting signals[94]. Sumoylation of PCNA in budding yeast targets predominantly Lys164 and to a lesser extent Lys127 (REF. 84). The modification leads to the recruitment of the helicase Srs2, which prevents the accumulation of recombination factors at replication forks[95,96]. When replication problems are encountered, this promotes ubiquitin-dependent damage bypass at the expense of homologous recombination. Analogous to the situation with the damage-tolerant polymerases, preferential interaction of Srs2 with the sumoylated form of PCNA is achieved by a SUMO interaction motif at the C-terminus of the helicase. In addition, SUMO seems to negatively affect the association of establishment of cohesion 1 (Eco1), which is involved in the establishment of cohesion with PCNA[97]. The functions of PCNA sumoylation in other systems in which the modification can be observed, such as *X. laevis* egg extracts or chicken B cells, have not been elucidated and are unlikely to involve a similar mechanism, as no convincing homologues of Srs2 have been identified.

Mechanism of PCNA polyubiquitylation. *In vitro* reconstitution of PCNA modification has been instructive, as it has revealed that the loading of the clamp onto DNA is crucial for both sumoylation and ubiquitylation[98,99]. Moreover, it has given insight into the mechanism of cooperation between the conjugation factors responsible for monoubiquitylation and polyubiquitylation. PCNA is one of only two known polyubiquitylation targets the modification of which is mediated by the successive action of two E2–E3 enzyme pairs. In the case of PCNA, the two steps occur independently: a complex of the E2 RAD6 and the E3 RAD18 attaches a single ubiquitin moiety exclusively to Lys164. The UBC13–MMS2 heterodimer in cooperation with the E3 RAD5 (or its homologues SHPRH or HLTF in higher eukaryotes) then extends this to a Lys63-linked chain, based on the unique linkage specificity of the E2 enzyme[100,101]. RAD5 has been shown to stimulate the discharge of ubiquitin from the UBC13 while at the same time — through physical interaction with PCNA — directing E2 activity towards the appropriate substrate[100].

Translesion synthesis
The processing of a lesion-containing replication template by a damage-tolerant polymerase that inserts either correct or inappropriate nucleotides opposite the lesion, thus potentially contributing to damage-induced mutagenesis.

Box 3 | **Crosstalk between damage-processing pathways**



The ubiquitin system provides a striking number of connections between the three pathways discussed in this Review. Not only does monoubiquitylation (of histone H2A, Fanconi-anaemia group D2 protein (FANCD2)–FANCI and proliferating cell nuclear antigen (PCNA)) constitute a method of damage signalling that is common to all three systems, several enzymes involved in ubiquitin conjugation and deconjugation shared between the pathways provide even closer links (see the figure).

The signalling events at a DNA double-strand break (DSB) and the Fanconi anaemia pathway both seem to converge on homologous recombination factors, jointly controlled by the E3 enzyme breast and ovarian cancer type 1 susceptibility protein (BRCA1) (reviewed in REF. 126). This is reflected by the dual nomenclature representing, on the one hand, the BRCA1–BRCA2 tumour suppressor system and, on the other hand, the Fanconi anaemia phenotype associated with defects in the respective genes: BRCA2 is FANCD1, BRCA1-interacting protein C-terminal helicase 1 (BACH1; also known as BRIP1) is FANCJ and partner and localizer of BRCA2 (PALB2) is FANCN. DSB repair by homologous recombination is independent of DNA replication, whereas the Fanconi anaemia pathway deals specifically with lesions arising during S phase. This feature is shared by PCNA-dependent damage tolerance, the only system among the three pathways that is conserved in lower eukaryotes. In higher organisms, PCNA modification and the Fanconi anaemia pathway seem to be coordinately regulated by the deubiquitylating enzyme (DUB) USP1 (also known as WDR48), which is responsible for the deubiquitylation of both PCNA and FANCD2, and both events likely affect the fidelity with which damaged DNA is replicated[79,109]. Finally, damage bypass and the DSB response use several common signalling strategies and components, first and foremost Lys63-linked polyubiquitylation mediated by ubiquitin-conjugating enzyme 13 (UBC13). Although the downstream effectors for polyubiquitylated PCNA are unknown, the notion that Lys63-linked chains initiate a recombination-based mechanism in both error-free damage bypass and the DSB response is particularly intriguing, considering that BRCA1 is not conserved in yeast. Finally, even the E3 ligase responsible for PCNA monoubiquitylation, RAD18, seems to contribute to DSB repair in both yeast and vertebrates, and is recruited to DSBs in a manner that depends on RING finger protein 8 (RNF8)[38,127,128].

Notably, the attachment site of the initial ubiquitin moiety on PCNA is not crucial for RAD5-mediated polyubiquitylation[94,101]. In contrast to some E2 enzymes known for the en bloc transfer of pre-assembled polyubiquitin units, UBC13–MMS2 seems to act largely in a stepwise manner by conjugating single ubiquitin moieties[101].

Regulation of PCNA modifications. As suggested by the *in vitro* analysis, DNA has a major influence on the modification of PCNA. Loading onto the chromatin seems both necessary and sufficient for sumoylation, thus providing an effective mechanism to couple modification to S phase[99]. Ubiquitylation is strongly induced by a range of agents causing an uncoupling of the replicative helicase from the DNA polymerase[102–105]. In this situation, stretches of ssDNA, covered by the replication protein A (RPA) complex, mediate the recruitment of RAD18 to sites of replication problems in a manner analogous to the activation of the replication checkpoint[102,103]. An important question concerns the timing of damage bypass. Although it has been shown that the damage bypass pathway operates during S phase and prevents checkpoint activation in response to low levels of ultraviolet irradiation[106], PCNA ubiquitylation and the events that follow are apparently uncoupled from replication fork progression in both yeast and vertebrate cells and can be delayed until after bulk genome replication without any adverse effects[89,106,107]. This suggests that ubiquitin-dependent damage bypass occurs at daughter strand gaps rather than directly at replication forks. A pronounced peak of expression of yeast *REV1*, encoding one of the TLS enzymes, suggests that post-replicative processing may be physiologically relevant[108], but it remains to be determined whether this is true for other organisms and for all types of damage. In higher eukaryotes, additional factors such as the tumour suppressor protein p53, the cell cycle inhibitor p21 and PAX-interacting protein 1 (PTIP; also known as Swift in *X. laevis*), an adaptor for checkpoint kinases, have been reported to exert a regulatory influence on damage bypass (reviewed in REF. 83). Finally, ubiquitylated PCNA is subject to deconjugation. In mammalian cells, this is achieved in a regulated manner by USP1, the same DUB that is responsible for the deubiquitylation of FANCD2 (REF. 109).

Unresolved questions. Numerous intriguing observations have recently indicated unanticipated complexities in the PCNA modification system. Although in yeast and *X. laevis* egg extracts PCNA ubiquitylation occurs independently of checkpoint signalling[102,104,105], controversial results have been obtained in higher eukaryotes[103,110,111]. In the chicken DT40 cell line, a low level of RAD18-independent PCNA monoubiquitylation by as-yet-unidentified E2 and E3 enzymes was observed[112]. Possible candidate E3 ligases in this context are RNF8 and the cullin RING ligase 4 ($CRL4^{Cdt2}$), which were both recently implicated in PCNA modification in higher eukaryotes[113,114]. Even more striking, modification of budding yeast PCNA at Lys107 with a Lys29-linked polyubiquitin chain was reported to occur in *cdc9* mutants, which are deficient in DNA ligase I[115]. Intriguingly, the modification seemed to require RAD5, UBC4 and MMS2, but not UBC13, RAD6 or RAD18. As MMS2 is not known to associate with E2 enzymes other than UBC13 or to promote polyubiquitylation by linkages other than Lys63, these observations clearly contradict several established concepts, and follow-up experiments will be necessary to validate their importance. Perhaps the main unresolved question, however, concerns the mechanism by which Lys63-linked chains trigger error-free damage bypass. Obtaining insight into this pathway will require the isolation of a relevant downstream effector that recognizes the polyubiquitylated form of PCNA. Synthetic mimics of ubiquitylated PCNA, such as chemically ubiquitylated PCNA[116] and linear fusions involving either the N- or C-termini[94] of PCNA or even a splitting of the backbone[117], will undoubtedly be useful for further mechanistic investigations.

Conclusion

The pathways discussed in this Review illustrate the marked influence of the ubiquitin system on various aspects of genome integrity, ranging from the sensing of

26S proteasome
A large multisubunit protease complex that selectively degrades polyubiquitylated proteins.

DNA damage over its repair to its replicative bypass during S phase. Hardly any of the functions described here involve the traditional activity for which ubiquitin is best known, the targeting of its substrate to the 26S proteasome. Instead, both monoubiquitylation and polyubiquitylation are emerging as versatile strategies to modulate any kind of protein–protein interaction the specificity of which is largely determined by highly selective UBDs. Particularly noteworthy in the context of genome stability is the large degree of crosstalk between the different pathways through shared components and regulatory strategies (BOX 3). Given that the only system conserved throughout eukaryotes is the damage tolerance system mediated by monoubiquitylation and polyubiquitylation of PCNA, it is likely that ubiquitylation as a means to control DNA damage processing has undergone a substantial diversification in the course of evolution, possibly by co-opting and further developing existing factors for new tasks.

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Competing interests statement
The authors declare no competing financial interests.

DATABASES

UniProtKB: http://www.uniprot.org
BRCA1 | BRCC36 | BRE | FANCD2 | FANCI | HERC2 | MDC1 | NBA1 | RAP80 | RNF8 | RNF168 | UBC13

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