ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 1, pp. 69-79. © Pleiades Publishing, Ltd., 2011.
Published in Russian in Biokhimiya, 2011, Vol. 76, No. 1, pp. 86-98.

REVIEW

Regulation of DNA Repair by Ubiquitylation

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Received August 24, 2010
Revision received October 8, 2010

Abstract—Cellular DNA repair is a frontline system that is responsible for maintaining genome integrity and thus preventing premature aging and cancer by repairing DNA lesions and strand breaks caused by endogenous and exogenous mutagens. However, it is also the principal cellular system in cancer cells that counteracts the killing effect of the major cancer treatments, e.g. chemotherapy and ionizing radiation. Although it is clear that an individual's DNA repair capacity varies, the mechanisms involved in the regulation of repair systems that are responsible for such variations are only just emerging. This knowledge gap is impeding the finding of new cancer therapy targets and the development of novel treatment strategies. In recent years the vital role of post-translational modifications of DNA repair proteins, including ubiquitylation and phosphorylation, has been uncovered. This review will cover recent progress in our understanding of the role of ubiquitylation in the regulation of DNA repair.

DOI: 10.1134/S0006297911010093

Key words: DNA repair pathways, regulation of DNA repair systems, post-translational protein modification, ubiquitylation

REGULATION OF BASE EXCISION REPAIR

Base excision repair (BER) is initiated by damage specific DNA glycosylases that release the corrupted base by hydrolysis of the N-glycosylic bond linking the DNA base to the sugar phosphate backbone (Scheme 1). The arising abasic site (AP-site) is further processed by AP-endonuclease 1 (APE1) that cleaves the phosphodiester bond 5′ to the AP-site, generating a DNA single strand break (SSB) with a 5′-sugar phosphate. This SSB is then repaired by a DNA repair complex that includes DNA polymerase β (Pol β), XRCC1, and DNA ligase IIIα (Lig III). Pol β possesses AP lyase activity that removes the 5′-sugar phosphate and also, functioning as a DNA polymerase, adds one nucleotide to the 3′-end of the arising single-nucleotide gap. Finally, Lig III seals the DNA ends, therefore accomplishing DNA repair (reviewed in [1]). This pathway is commonly referred to as the short patch BER pathway, through which cells are accomplishing the majority of repair (Scheme 1, left branch). However, if the 5′-sugar phosphate is resistant to cleavage by Pol β, then a switch to Pol δ/ε occurs that adds 2-8 more nucleotides into the repair gap, therefore generating a flap structure that is removed by flap endonuclease-1 (FEN-1) in a PCNA-dependent manner. DNA ligase I (Lig I) then seals the remaining nick in the DNA backbone, and this process is commonly referred to as long patch BER [2, 3] (Scheme 1, right branch).

To support error-free transcription and replication, BER proteins should be present in adequate amounts to be able to promptly repair DNA. Indeed, mutations affecting the amounts or enzymatic activities of these proteins increase genome instability and reduce cell viability in response to DNA damage [4-10]. On the other hand, the amount of BER enzymes should be tightly controlled since when overproduced, BER enzymes may affect other DNA transactions and also lead to genome instability and cancer [11, 12]. The number of DNA lesions in human cells originates from the chemical instability of the DNA molecule itself, but also depends on cellular metabolism and exposure to exogenous mutagens [13].

A combination of these factors leads to variations in DNA damage levels, and BER should be responsive to the changing environment and indeed, as it has been recently demonstrated, the amount of BER enzymes present within a cell at any time (the steady-state level of BER

Abbreviations: AP-site, abasic site (apurinic/apyrimidinic); BER, base excision repair; Lig III, DNA ligase III; NER, nucleotide excision repair.

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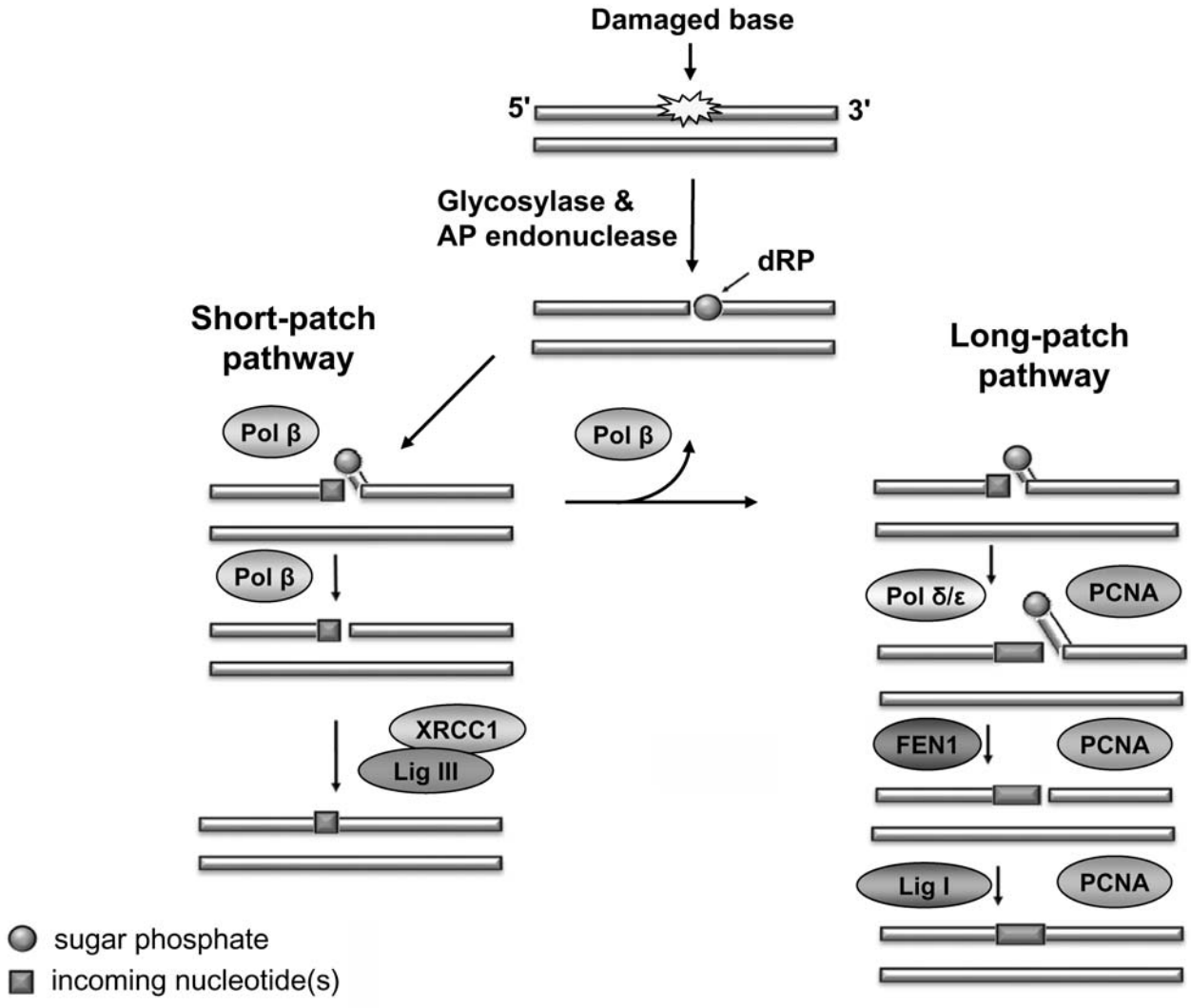
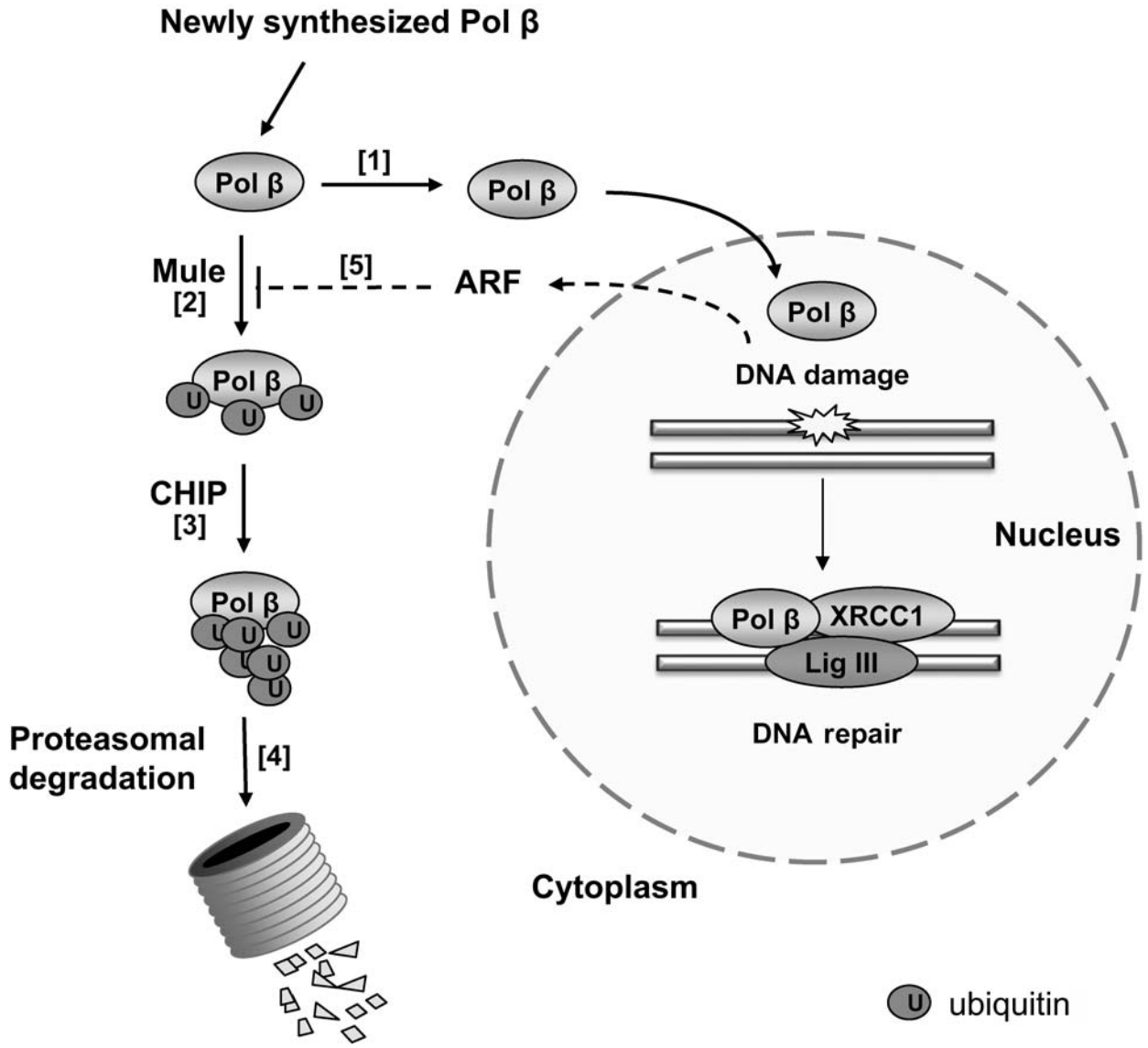


BER pathways

Scheme 1

enzymes) is tightly regulated and is linked to the number of DNA lesions [14]. This is achieved by controlling the nuclear pool of three major BER enzymes (XRCC1, Lig III, and Pol β) through targeted proteasomal degradation. Proteins targeted for degradation are marked with a chain of ubiquitin (Ub, small 76 amino acid protein) molecules. Ubiquitylation consists of conjugation of Ub, via its C terminus, onto the ε-amino group of a lysine residue of the substrate protein and is achieved by a cascade of ubiquitin-activating enzymes. First, Ub is activated through an ATP-dependent reaction by an ubiquitin-activating enzyme (E1) to form an E1–Ub thioester. Second, the activated Ub is delivered to a ubiquitin-conjugating enzyme (E2) and finally, a complex is formed between the E2–Ub thioester, the target protein, and a ubiquitin ligase (E3) that conjugates Ub to the protein. Polyubiquitylated proteins are recognized by the 26S proteasome that unfolds the protein, removes the polyubiquitin chains, and degrades the protein (for review see [15]).

If the levels of BER proteins exceed the level of DNA lesions, then the excessive BER enzymes are ubiquitylated and thus labeled for proteasomal degradation. For example, sequential ubiquitylation of Pol β by the E3 ubiquitin ligases Mule (ARF-BP1) and CHIP leads to Pol β degradation and down regulates its steady state level [16]. However, when more Pol β is required for DNA repair, Mule activity is down regulated by ARF protein [17, 18], whose release is modulated in response to DNA damage [19]. Release of ARF and Mule inhibition leads to accumulation of Pol β and increased DNA repair (Scheme 2). However, as well as inhibiting E3 ubiquitin ligase activities, in many cases the steady state level of proteins is also controlled by an opposing activity. In this scenario, ubiquitylation leading to protein degradation is counteracted by deubiquitylation, primarily by ubiquitin



Regulation of base excision repair by Mule, CHIP, and USP47. Depending on the amount of Mule, newly synthesized Pol β is either directly transferred to the nucleus [1] or ubiquitylated by Mule [2]. Ubiquitylated Pol β is then a target for CHIP-mediated polyubiquitylation [3] and subsequent degradation by the proteasome [4]. However, if DNA damage is detected, the activity of Mule is inhibited by ARF [5], which then generates more active Pol β that is able to enter the nucleus to participate in DNA repair

Scheme 2

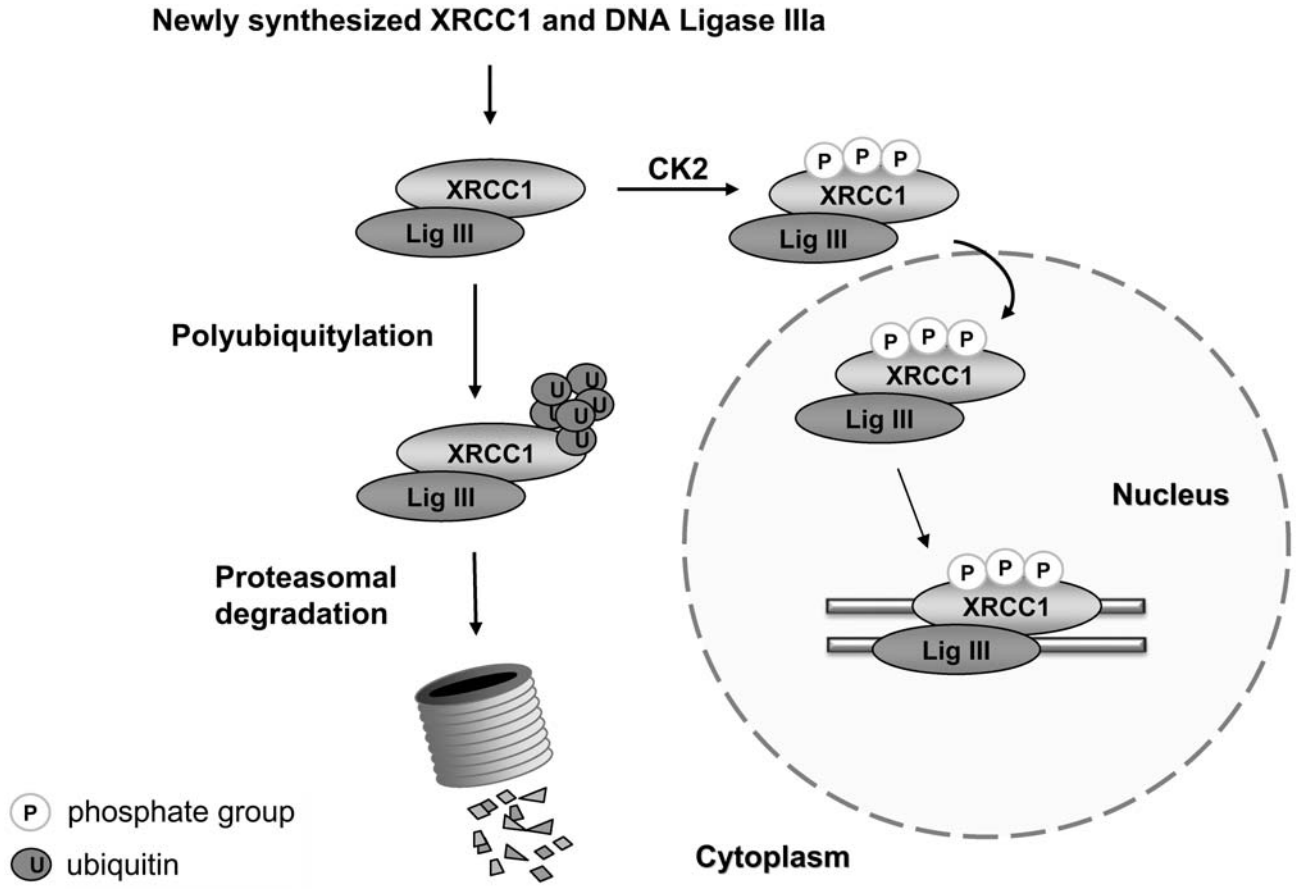
specific proteases (USPs) that are the major class of deubiquitylation enzymes. There are several examples of the role of USPs in the DNA damage response (for review see [20, 21]).

Cellular signaling at the molecular level (that involves regulating protein activity and/or affinity to their substrates, protein–protein interactions, and cellular localization) is accomplished by different post-translational modifications and, in addition to ubiquitylation, phosphorylation plays an important role [22]. Phosphorylation of proteins by one of an estimated 500 protein kinases existing in mammalian cells involves the transfer of the γ-phosphate of an ATP molecule onto the target protein. Phosphorylation plays an important role in regulating a wide range of different cellular processes, including the DNA damage response and DNA repair, but also phosphorylation can promote or inhibit ubiquitylation that can also effect protein degradation and protein trafficking. Phosphorylation of XRCC1 by casein kinase 2 (CK2) is one of the best examples of the role of phosphorylation in BER. XRCC1 is central to the BER process since it operates as a scaffold protein supporting multiple protein–protein interactions required for DNA repair. XRCC1 is a 69 kDa protein comprising several domains – an N-terminal domain (NTD) that binds both nicked DNA [23] and Pol β [24], a central breast cancer gene 1

C-terminal domain (BRCT I) that interacts with poly(ADP-ribose) polymerases (PARP-1 and PARP-2 [25, 26]), and a C-terminal BRCT II domain that binds Lig III [27].

In addition to this, XRCC1 is also known to interact with several other proteins including APE1 [28], polynucleotide kinase (PNK) [29, 30], and proliferating cell nuclear antigen (PCNA) [31]. The cellular importance of XRCC1 is highlighted by the fact that a deficiency in mice is embryonic lethal, and cell lines lacking XRCC1 are defective in the repair of SSBs and sensitive to alkylating agents such as methylmethanesulfonate, as well as other DNA-damaging agents [32]. XRCC1 has various phosphorylation sites within the protein. DNA damage-dependent phosphorylation of XRCC1 by DNA-dependent protein kinase at serine 371 [33] and, more recently, Chk2-dependent phosphorylation of XRCC1 at threonine 284 in response to DNA damage have been observed [34]. However, the biological role of these phosphorylations is unclear. Additionally, between the two BRCT domains is a region (amino acids 403-538) that is known to be phosphorylated *in vitro* by CK2 [29, 35, 36].

It was recently demonstrated that the cytoplasmic form of CK2 is the major protein kinase activity involved in phosphorylation of XRCC1 in human cell extracts and that XRCC1 phosphorylation is required for XRCC1–Lig III complex stability [37]. It was also shown that XRCC1–Lig III complex containing mutant XRCC1, in which CK2 phosphorylation sites have been mutated, is unstable and subject to proteasomal degradation. Accordingly, a knockdown of CK2 by siRNA results in both reduced XRCC1 phosphorylation and stability, which also leads to a reduced amount of Lig III and accumulation of DNA strand breaks [37] (Scheme 3). Most probably, the majority of BER proteins are regulated by phosphorylation and ubiquitylation. There are some indications that APE1 [38] and PARP-1 [39] are regulated by ubiquitylation in addition to uracil DNA glycosylase [40]. However, more research is required to identify the entire mechanism and proteins involved in the regulation of BER.



Model for regulation of XRCC1–Lig III complex stability. The majority of newly synthesized XRCC1 is phosphorylated by CK2 before or after formation of a complex with Lig III. The XRCC1–Lig III complex stabilized by phosphorylation is then translocated into the nucleus and can participate in DNA repair. Alternatively, if XRCC1 is in excess, the newly synthesized protein is either not phosphorylated or is dephosphorylated, which then makes it susceptible to ubiquitylation and is subsequently degraded by the proteasome

Scheme 3

NUCLEOTIDE EXCISION REPAIR

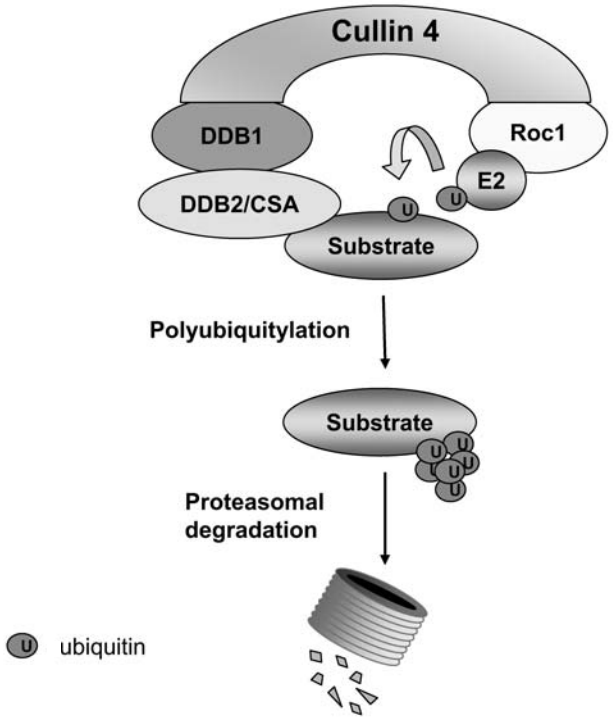
Nucleotide excision repair (NER) is a DNA repair pathway for the removal of bulky DNA adducts, such as cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (64PPs), induced by UV irradiation and chemical mutagens. There are at least two distinct pathways of NER, namely transcription coupled repair (TCR) and global genome repair (GGR). TCR operates predominantly in the repair of lesions occurring on actively transcribed regions of the DNA, whilst GGR can repair lesions throughout the genome. These pathways also differ by the way in which the DNA damage is sensed. TCR occurs as a result of a stalled RNA polymerase due to blockage of transcription elongation by the DNA lesion, and damage recognition is further aided by the Cockayne syndrome proteins, CSA and CSB. In contrast, GGR is initiated by the binding of the xeroderma pigmentosum C/HR23B (XPC/HR23B) complex and the heterodimer of UV-damaged DNA binding proteins 1 and 2 (DDB1 and DDB2) that play essential roles in DNA damage sensing.

Following DNA damage recognition, both pathways of NER are thought to involve the same proteins and mechanism of repair, which involves the multiprotein complex transcription factor IIH (TFIIH), XPA and RPA that are involved in damage verification, the endonucleases ERCC1-XPF and XPG that incise the damaged strand, and a DNA polymerase and DNA ligase that fill and seal the gap, respectively. Abnormalities in the NER pathway give rise to the human disorders xeroderma pigmentosum and Cockayne syndrome that are characterized by severe UV sensitivity and neurological defects [41].

Interestingly, certain proteins required for both TCR and GGR are also components of E3 ubiquitin ligase complexes that are involved in protein ubiquitylation. There are two known protein complexes involved in GGR and TCR, and both of them contain a scaffold protein cullin-4 (Cul4A or Cul4B) and also DNA damage binding protein 1 (DDB1) (Scheme 4). The crystal structure of the Cul4A–DDB1 complex revealed that the N terminus of Cul4 interacts with DDB1, whereas the C terminus interacts with two other proteins, regulator of cullins-1 (Roc1) and an E2 conjugating enzyme [42]. To target this ubiquitin ligase complex to specific substrates, it also includes one of a number of WD-40 repeat proteins, providing specificity for ubiquitylation [43]. Examples of these WD-40 repeat protein adaptors are DDB2 and CSA that are involved in GGR and TCR, respectively [44]. The role of DDB1 in this complex is to mediate interaction between the ubiquitin ligase scaffold protein Cul4 and WD-40 repeat proteins that provide interaction with various substrates. Not surprisingly, a knockout of DDB1 in mice is embryonic lethal [45]. However, Cul4A conditional knockout mice have recently been found to be viable and healthy although Cul4B, which is a close homolog of Cul4A, may be compensating for the loss of Cul4A [46].

During TCR, RNA polymerase II is stalled by the presence of DNA damage, which then results in the recruitment of CSA and CSB proteins, which in turn recruit other NER proteins. It was recently proposed that the role of the Cul4A–Roc1–DDB1–CSA ubiquitin ligase complex in TCR is ubiquitylation of CSB that stimulates its proteasomal degradation and removal from DNA damage when recruitment of NER factors is accomplished [47, 48]. However, it is not clear whether this degradation is important for TCR. Interestingly, it was recently found that the CSB protein contains a ubiquitin binding domain, and it was proposed that the ubiquitylation activity of the Cul4A–Roc1–DDB1–CSA complex may be required for ubiquitylation of some TCR proteins and thus promote CSB recruitment [49]. Therefore, more studies are required to further establish the role of the CSA-containing ubiquitin ligase, particularly in TCR.

A similar role is assigned to the ubiquitylation activity of the Cul4A–Roc1–DDB1–DDB2 ubiquitin ligase complex. It has been proposed that after binding to the UV induced DNA lesions and initiation of GGR by recruitment of the required NER factors to DNA damage sites, the complex is subsequently removed from the DNA by self-ubiquitylation and subsequent degradation [50-52]. There may be other targets of the Cul4A–Roc1–



Structure of Cullin 4 containing ubiquitin ligases

Scheme 4

DDB1–DDB2 ubiquitin ligase complex, so therefore the DDB heterodimer may also promote the ubiquitylation of cellular proteins in association with Cul4A, which may have other consequences apart from protein degradation. Indeed, the DDB2-containing ubiquitin ligase complex has also been shown to ubiquitylate histones that may be involved in chromatin remodeling to allow access of NER repair factors to the DNA lesion [53, 54]. Specifically, ubiquitylation of H3 and H4 has been shown to occur by the Cul4A–Roc1–DDB1 ubiquitin ligase complex in response to UV irradiation, and it is thought that this causes a disruption of the interaction of histones with DNA and enhances the recruitment of XPC to the UV DNA damaged site, therefore providing a link between chromatin remodeling and DNA repair in response to UV irradiation [54].

DOUBLE STRAND BREAK REPAIR AND CHROMATIN REMODELING

In mammalian cells, genomic DNA is wrapped around a histone octamer, consisting of histones H2A, H2B, H3, and H4, to form nucleosomes that are further packaged to form chromatin. Thus, damaged DNA is masked and is not readily available for DNA repair. Remodeling (opening and closing) of chromatin is thought to occur by post-translational modifications on the N-terminal tails of the histones, which is required for several cellular processes including DNA repair. Histone ubiquitylation is one such modification, and although its precise role in chromatin remodeling and the effect on different DNA repair pathways remains to be studied in detail, increasing information is becoming available regarding the role of histone ubiquitylation during double strand break repair.

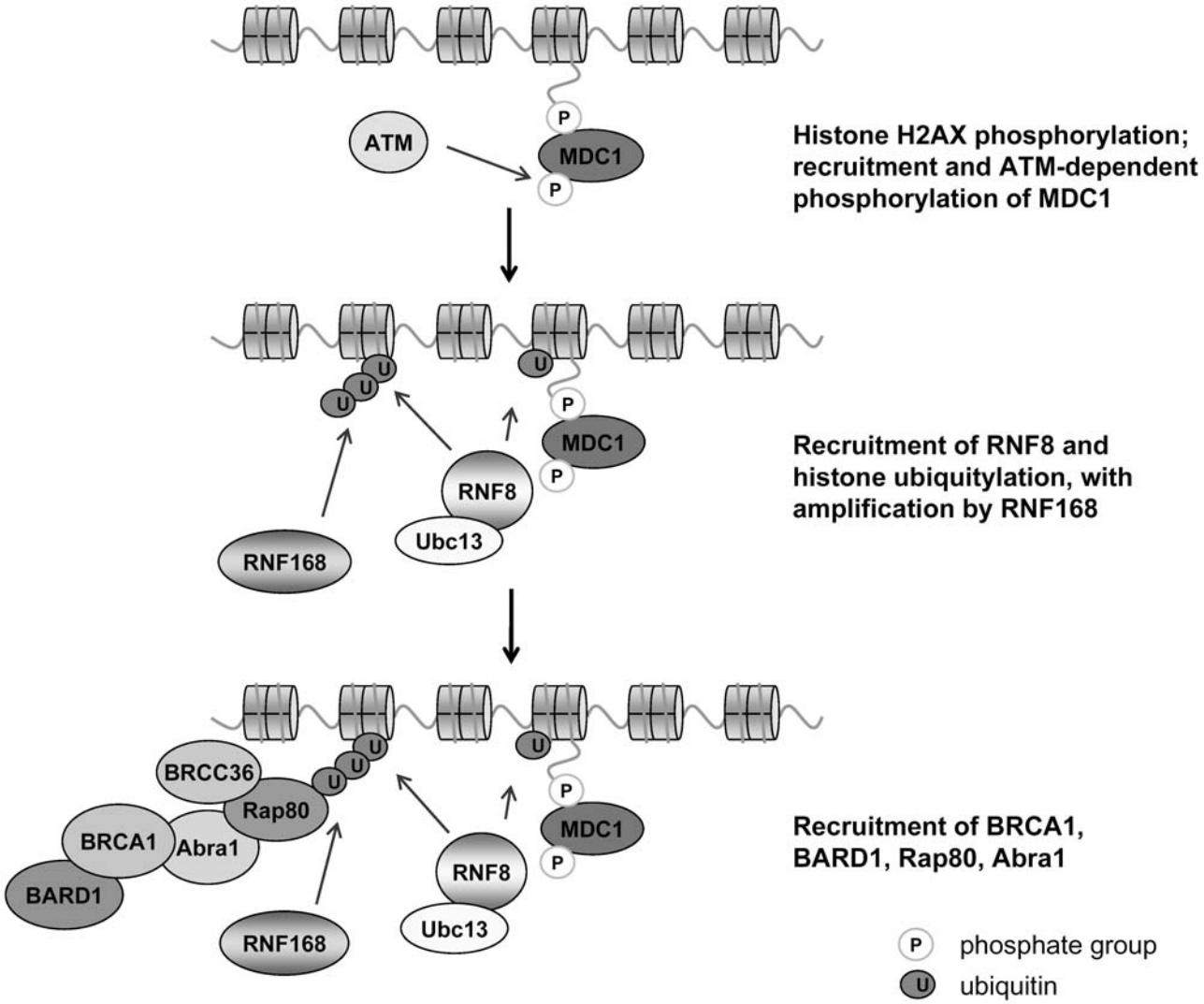
DNA double strand breaks (DSBs) are potentially toxic lesions that can arise from various endogenous and exogenous sources, such as collapsed replication forks and ionizing radiation, and the cellular response to DSBs involves a battery of proteins that sense the DNA damage, cause cell cycle arrest through activation of checkpoint proteins, and also recruit DNA repair enzymes.

An early event in the repair of DSBs is the ATM-dependent phosphorylation of the histone H2A variant, H2AX, and the subsequent recruitment of the mediator of DNA damage checkpoint 1 (MDC1). ATM subsequently phosphorylates MDC1, which is consequently recognized by a complex consisting of the E3 ubiquitin ligase RNF8 and the E2 conjugating enzyme Ubc13, which then monoubiquitylates γ-H2AX and H2A. Ubiquitylation of γ-H2AX by RNF8/Ubc13 has also been shown to be amplified by another ubiquitin ligase, RNF168, a protein that is mutated in RIDDLE syndrome patients who are immunodeficient and radiosensitive due to defective DSB repair [55, 56]. Histone γ-H2AX polyubiquitylation stimulates recruitment of Rap80, Abra1, 53BP1, BRCA1, and other proteins required for DSB repair [57-60] (Scheme 5). Since ubiquitylation of γ-H2AX and H2A is involved in recruitment of cell cycle checkpoint and DNA repair proteins in response to DSBs, this event should be reversible following the repair of the DNA damage and should be catalyzed by deubiquitylation enzymes. Indeed, Rap80 is also known to form a complex with BRCC36, a deubiquitylation enzyme that is thought to reverse the ubiquitylation caused by RNF8/Ubc13, and therefore these two protein complexes possibly modulate the levels of ubiquitylation events occurring at DSBs [61]. However, USP3 has also been shown to deubiquitylate H2A, although a direct role for USP3 following the repair of DSBs requires further investigation [62].

While thought to be specific for double strand break repair in response to ionizing radiation, H2A ubiquitylation has also been observed in response to UV irradiation. Using cells stably expressing green fluorescing protein (GFP)-tagged ubiquitin, monoubiquitylation of H2A following UV irradiation by the ubiquitin ligase Ring2 has been observed and was dependent on both the presence of a functional NER process and on the DNA damage signaling protein kinase ATR [63]. However, it is thought that Ring2 actually regulates the nuclear ubiquitin pool, and therefore the observed decrease in H2A ubiquitylation following Ring2 knockdown was thought to be an indirect effect. Subsequently, the same authors demonstrated that H2A ubiquitylation following UV irradiation was actually performed by the Ubc13/RNF8 ubiquitin ligase complex [64]. The recruitment of Ubc13/RNF8 following UV irradiation, similarly to ionizing radiation, also occurs via interaction with MDC1. This appears to suggest that the cellular response to UV and ionizing radiation may involve the same pathway of H2A ubiquitylation that provides a universal signal of DNA damage detection. This proposed mechanism would in fact recruit a plethora of DNA repair enzymes, and following DNA damage detection only a subset of these enzymes would actually be used to repair the DNA lesion. Ubiquitin ligase activity on histones within chromatin has also been demonstrated for Mule [65], Cul4–DDB–Roc1 [54], Mdm2 [66], and Bre1 [67] E3 ubiquitin ligases, although the biological role of these ubiquitylation events is unclear.

UBIQUITYLATION IN THE FANCONI ANEMIA PATHWAY

The diverse roles for ubiquitylation in the regulation of DNA repair certainly come to light in the example of the Fanconi anemia (FA) pathway. This pathway is involved in the coordination of the repair of DNA interstrand cross-links (ICLs), a toxic lesion that locks together the two strands of DNA, blocking both DNA replica-

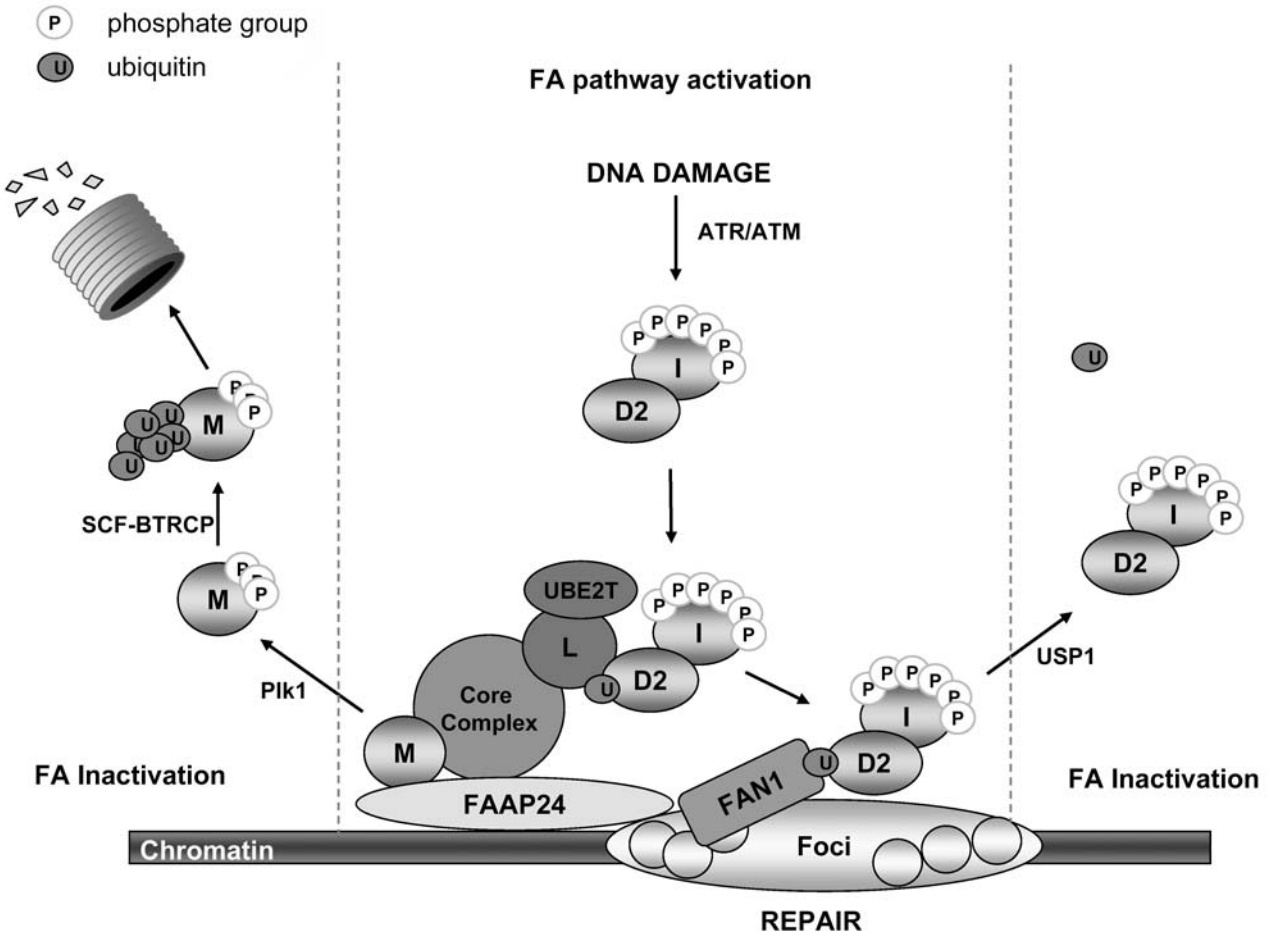


Model for regulation of DNA double strand break repair

Scheme 5

tion and transcription. These lesions are repaired through multiple repair pathways, primarily the NER, translesion synthesis (TLS), and homologous recombination (HR) pathways, and it is well accepted that the FA pathway is necessary for the coordination of these repair events [68-72]. Indeed, a loss of normal function in any of the 13 FA pathway genes (FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, and FANCN) manifests clinically as Fanconi anemia, a disease highlighted by the patient's hypersensitivity to ICLs and susceptibility to the development of blood cancer and solid tumors [73-76]. The FA pathway is activated only during the S-phase of the cell cycle in response to the presence of ICLs, and is thought to incur deleterious effects if active at other points in the cell cycle. It is therefore not surprising that it appears to be a highly regulated pathway with multiple mechanisms allowing for its transient activity. These include phosphorylation-dependent ubiquitylation affecting protein localization and pathway activation, as well as deubiquitylation- and ubiquitylation-dependent degradation allowing for pathway inactivation.

FA pathway activation in response to the presence of ICLs is ultimately dependent on the monoubiquitylation status of the FANCD2–FANCI complex, more importantly that of FANCD2. This monoubiquitylation is triggered by ATR-mediated phosphorylation of FANCI at six sites in response to DNA damage [77]. FANCI phosphorylation appears to stimulate FANCD2 monoubiquitylation, through FANCD2–FANCI recruitment to the E3 ligase complex that consists of the PHD domain E3 ligase, FANCL, the E2 conjugating enzyme, UBE2T, and a multisubunit core complex of eight FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL,



Mechanisms of Fanconi anemia pathway regulation

Scheme 6

FANCM) and two FA-associated proteins FAAP24 and FAA100 [78-81]. Upon FANCD2 monoubiquitylation at residue K561, the FANCD2– FANCI complex localizes to repair foci on DNA containing HR and TLS factors such as Rad51, BRCA1, PCNA, and γ-H2AX [82-85] (Scheme 6).

Recently it was shown that monoubiquitylated FANCD2 at repair foci is necessary for the recruitment of the newly identified FAN1 nuclease through direct interaction with the N-terminal ubiquitin binding zinc finger of FAN1 (UBZ domain) [86-89]. Furthermore, FAN1 exonuclease/endonuclease activity was implicated in the HR repair step of ICL repair and deemed necessary for repair completion. The monoubiquitin moiety on FANCD2 is therefore essential for FA pathway functionality through its direct interaction with ubiquitin binding domains on repair factors in the repair of the toxic ICL lesion.

The availability of the core complex factors provides a level of regulation for the FA pathway, as a loss of any of the ten core complex factors reduces either the level of FANCD2–FANCI monoubiquitylation or repair through recruitment to sites of damage. FANCM for instance dimerizes with FAAP24, and despite being associated with the core complex is not necessary for core complex contribution to FANCD2 monoubiquitylation [90, 91]. The importance of the FANCM–FAAP24 complex rather lies in its ability to recognize sites of DNA damage, consequently recruiting the core complex to where it is needed on chromatin [91, 92]. Interestingly, post-replication and repair, FANCM is hyperphosphorylated by polo-like kinase, Plk1, and this modification results in the formation of a phosphodegron, consequently leading to FANCM degradation by the β-TRCP component of the SCF ubiquitin E3 ligase [93]. In the absence of FANCM, the core complex is no longer recruited to DNA, and this ensures that the pathway is active only during S phase.

Another major source of negative regulation for the FA pathway comes with the discovery that monoubiquitylation of FANCD2 can be reversed leading to FANCD2–FANCI complex dissociation from DNA and

consequent inactivation of the FA pathway [20]. This is carried out by the deubiquitylation enzyme, USP1, complexed to UAF1 (USP1 associated factor 1) [20, 94]. This complex has been shown to deubiquitylate FANCD2–FANCI in the absence of DNA damage, thereby preventing FA pathway activity. To complement this regulatory role, in the presence of DNA damage USP1 transcriptional expression is switched off, and it has also been shown to induce its own degradation through endo-proteolytic cleavage at a conserved internal diglycine motif (Gly-Gly), thus ensuring that monoubiquitylated FANCD2 remains available for repair [94, 95].

It is clear that ubiquitylation and deubiquitylation are critical events involved in the cellular responses to DNA damage through different DNA repair pathways. However, further studies are required to advance our understanding of the orchestration of the events occurring at the sites of DNA damage.

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