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Regulation of the Base Excision Repair Pathway by Ubiquitination

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Abstract

Genome integrity is under constant threat from cellular reactive oxygen species generated by endogenous and exogenous mutagens. The base excision repair (BER) pathway consequently plays a crucial role in the repair of DNA base damage, sites of base loss and DNA single strand breaks that can cause genome instability and ultimately the development of human diseases, including premature ageing, neurodegenerative disorders and cancer. Proteins within the base excision repair pathway are increasingly being found to be regulated and controlled by post-translational modifications, and indeed ubiquitination performs a key role in the maintenance of repair protein levels but may also impact on protein activity and cellular localisation. This process is therefore important in maintaining an efficient cellular DNA damage response, and if not accurately controlled, can cause DNA damage accumulation and promote mutagenesis and genomic instability. In this chapter, we will present up-to-date information on the evidence of ubiquitination of base excision repair proteins, the enzymes involved and the molecular and cellular consequences of this process.

Keywords: DNA repair, base excision repair, DNA damage, ubiquitin, ubiquitination

1. Introduction

Every human cell per day is thought to generate greater than 10,000 DNA base lesions and single strand breaks (SSBs) due to the instability of the DNA molecule [1]. These are largely created by cellular reactive oxygen species that are generated by hydrolysis, oxidative metabolism and environmental factors, including ionising radiation (IR). Typical sites of damage include sites of base loss (abasic sites), oxidised DNA bases (e.g. 8-oxoguanine and thymine glycol) and SSBs. If this DNA damage is left unrepaired, it can cause mutagenesis and genome instability which are contributors to the development of human diseases, including

premature ageing, neurodegenerative disorders and cancer. The base excision repair (BER) pathway was first identified in the 1970s by Tomas Lindahl (co-recipient of the 2015 Nobel Prize in Chemistry), who discovered the existence of a uracil DNA *N*-glycosylase that is able to excise uracil residues from DNA [2]. Lindahl then suggested that in order for repair to be completed, an endonuclease, a DNA polymerase and a DNA ligase would be required. This marked the establishment that a specific repair pathway exists in human cells to repair DNA base damage, and now nearly a half a century later, the major enzymes and mechanisms involved in BER are well known.

As Lindahl had shown, the first step of BER is recognition of the specific damaged DNA base by a DNA glycosylase. In fact 11 DNA glycosylases are now known to exist with each removing particular types of DNA base damage [3, 4]. Indeed there are three uracil DNA glycosylase enzymes that recognise uracil lesions (namely uracil DNA glycosylase, UNG; single-strand-selective monofunctional uracil DNA glycosylase, SMUG1; and thymine DNA glycosylase, TDG), one enzyme that recognises alkylated bases (*N*-methylpurine DNA glycosylase, MPG), two mismatch-specific glycosylases (methyl-CpG binding domain protein 4, MBD4 and MutY homologue, MUTYH) and five glycosylases that recognise oxidised bases (8-oxoguanine DNA glycosylase 1, OGG1; endonuclease III-like protein 1, NTH1; endonuclease VIII-like proteins 1, 2 and 3; NEIL1, NEIL2 and NEIL3). In general, DNA glycosylases utilise a base-flipping mechanism whereby the base is flipped 180° from the sugar phosphate backbone breaking the hydrogen bonds between the bases in the process, and placing the damaged base into the active site of the DNA glycosylase. However, there are two types of glycosylase enzyme as one type will only remove the damaged base (monofunctional enzyme) whereas another type will remove the base but also cleave the DNA backbone (bifunctional enzyme). The monofunctional DNA glycosylases (UNG, SMUG1, TDG, MPG, MUTYH and MBD4) that remove the damaged base will create an abasic site by cleaving the *N*-glycosidic bond linking the base to the phosphodiester backbone. The abasic site is then recognised by AP endonuclease 1 (APE1) that cleaves 5'- to the lesion, creating a one nucleotide gap containing a 3'-hydroxyl group on one end, and a 5'-deoxyribose phosphate (5'-dRP) group on the other [5, 6]. The 5'-dRP group is subsequently removed by the lyase activity of DNA polymerase β (Pol β) that also simultaneously inserts the correct, undamaged nucleotide [7, 8]. The remaining nick in the DNA backbone is then sealed by DNA ligase III α (Lig III α) that is in a stable complex with X-ray cross-complementing protein 1 (XRCC1), to restore the original DNA sequence (**Figure 1**) [9, 10]. In contrast, bifunctional DNA glycosylases create a single nucleotide gap that is flanked by different ends, depending on the glycosylase employed. OGG1 and NTH1 are known to catalyse β -elimination which creates a 5'-phosphate and a 3'- α,β -unsaturated aldehyde, however, these are thought to be low efficiency activities and therefore with the high cellular abundance of APE1, it is thought that APE1 can actually circumvent this product and cleave the abasic site itself [11]. The bifunctional DNA glycosylases NEIL1, NEIL2 and NEIL3 catalyse β,δ -elimination which create a phosphate moiety on both the 5'- and 3'-end of the single nucleotide gap. Since the 3'-phosphate is not the required end for Pol β activity, this requires removal by polynucleotide kinase phosphatase (PNKP) (**Figure 1**) [12]. Despite the dependence on either APE1 or PNKP, following bifunctional DNA glycosylase activity the end product is the same as monofunctional DNA glycosylase activity in that both Pol β and XRCC1-Lig III α are required to complete the repair process.

The pathway described above is commonly referred to as the short patch BER pathway, through which the majority of DNA repair events proceed [13]. However under certain conditions, particularly when the DNA ends are resistant to processing (e.g. if the 5'-dRP becomes reduced and thus cannot be cleaved by Pol β), then long-patch BER is employed (**Figure 1**). Following the addition of the first nucleotide by Pol β [14], there is a polymerase switch to DNA polymerases δ/ϵ (Pol δ/ϵ), which are principally involved in DNA replication. These polymerases typically add two to eight more nucleotides into the repair gap thus generating a 5'-flap structure. This structure is a substrate for flap endonuclease-1 (FEN-1), which acts in a proliferating cell nuclear antigen (PCNA)-dependent manner and then finally DNA ligase I (Lig I) completes the repair

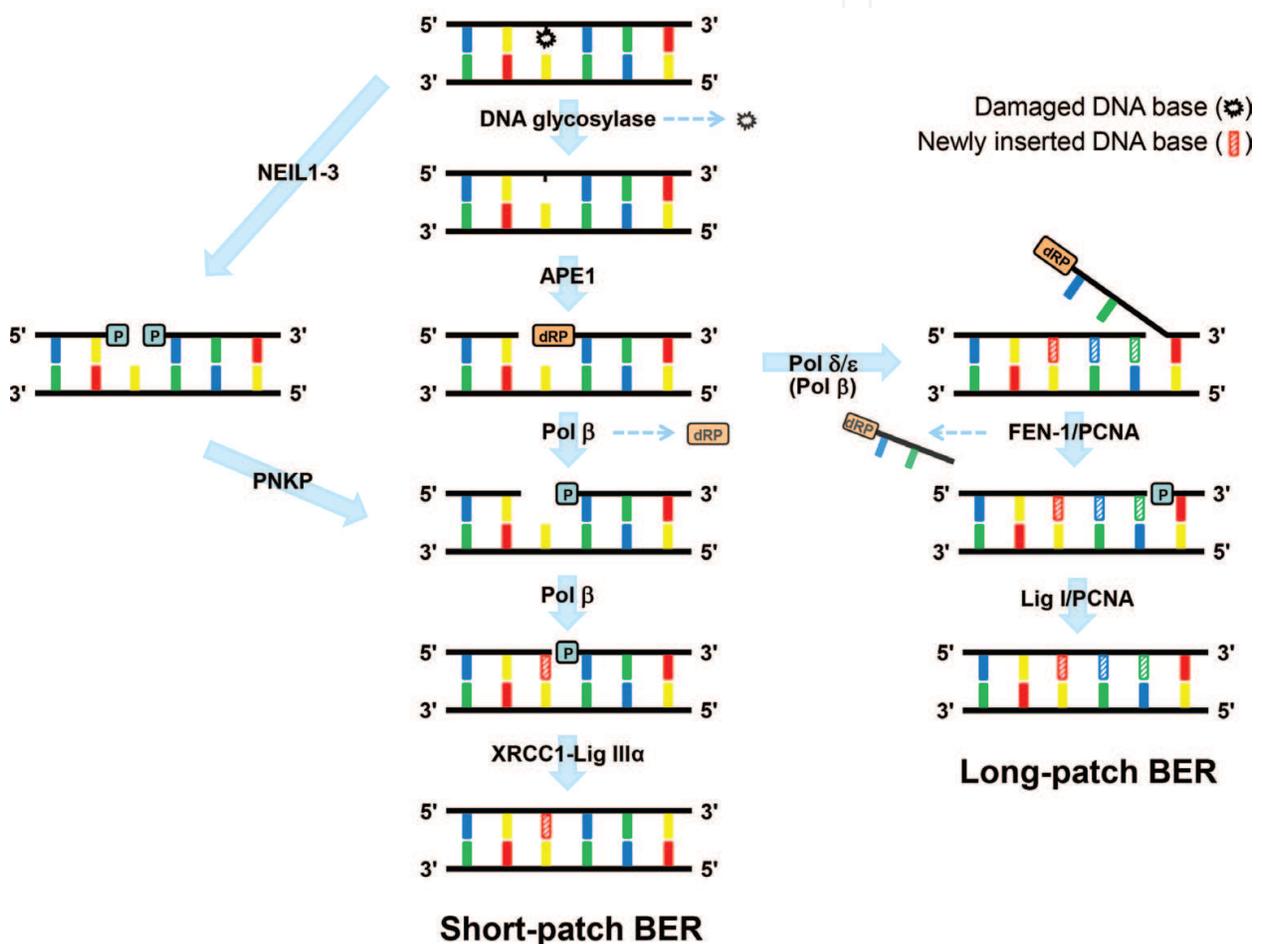


Figure 1. The mechanism of repair of DNA base damage by the BER pathway. The damaged DNA base is recognised and excised by damage-specific DNA glycosylases that catalyse cleavage of the N-glycosidic bond, creating an abasic site. APE1 recognises the abasic site and cleaves the DNA backbone, generating a single strand break containing a 5'-dRP moiety. The 5'-dRP is subsequently removed by the dRP lyase activity of Pol β that furthermore inserts a new undamaged nucleotide into the repair gap (central branch). If BER is initiated by the NEIL DNA glycosylases (NEIL1, NEIL2 and NEIL3), these enzymes generate a single nucleotide gap containing 3'- and 5'-phosphate ends through β,δ -elimination activity. The 3'-phosphate is subsequently removed by PNKP prior to the activity of Pol β that fills in the repair gap (left branch). Finally, the Lig III α -XRCC1 complex completes short patch BER by sealing the remaining nick in the phosphodiester backbone. If the 5'-dRP moiety is resistant to Pol β dRP lyase activity, a polymerase switch to Pol δ/ϵ occurs, which then stimulate the addition of two to eight more nucleotides into the single nucleotide gap. This generates a 5'-flap structure which is recognised and excised by FEN-1, in a PCNA-dependent manner (right branch). To complete long patch BER DNA ligase I, also in association with PCNA, seals the remaining nick in the DNA backbone.

process by sealing the remaining nick. It is important to note that with the constant, significant amount of DNA base damage and SSBs being induced in cells, that BER is a constitutively active process.

2. Regulation of BER proteins through ubiquitination

Since BER is the major cellular mechanism for the repair of DNA base damage and SSBs, and thus for the maintenance of genome stability, it is important that this process is maintained and controlled. The most efficient way of achieving this, particularly in responding to fluctuations in the cellular DNA damage environment, is via controlling cellular protein activity, localisation and overall protein levels. Indeed there is a growing list of the various protein post-translational modifications (PTMs) of BER proteins that have been reported to achieve this [15]. However a role for ubiquitination in controlling BER protein levels, and thus cellular BER activity, has been highlighted particularly in the last decade. Polyubiquitination of BER proteins catalysed by specific E3 ubiquitin ligases has been shown to largely control cellular protein levels via degradation by the 26S proteasome, but additionally monoubiquitination has been observed in some instances that can act by compartmentalising BER proteins or controlling BER protein activity. There are also instances of crosstalk between ubiquitination and other PTMs in controlling cellular BER. Below we aim to summarise all of the available evidence highlighting the enzymes and mechanisms involved in the control of BER proteins through ubiquitination.

2.1. Ubiquitination of DNA glycosylases

2.1.1. Uracil DNA glycosylases: UNG, SMUG1, MBD4, TDG

Of the four members of the uracil DNA glycosylases, only UNG, SMUG1 and TDG have been shown to be ubiquitinated by specific E3 ubiquitin ligases. Binding of the human immunodeficiency virus type 1 (HIV-1) accessory protein Vpr to UNG and SMUG1 was shown to induce their ubiquitination-dependent proteasomal degradation following expression in 293T cells. This was thought to be promoted through the E3 ubiquitin ligase scaffold proteins, Cullin 1 (Cul1) and Cullin 4 (Cul4), as Vpr interacts with these ligases along with UNG and SMUG1 following overexpression and immunoprecipitation from 293T cells [16]. Vpr was subsequently shown to bind to damage-specific DNA-binding protein 1 (DDB1), which is another component of Cul4A E3 ubiquitin ligases, that mediates the degradation of UNG in 293T cells [17]. This is thought to be a specific mechanism that allows the HIV virus to regulate the levels of abasic sites in viral reverse transcripts and thus promotes viral replication. Therefore whether UNG and SMUG1 are targeted for ubiquitination during normal cellular processing and for BER is not yet known. The third member of the uracil DNA glycosylase family, TDG, is largely known for being regulated by the small ubiquitin-like modifier (SUMO). TDG was shown to be modified by SUMO-1 and SUMO-2/3 on lysine 330 following immunoprecipitation from HeLa cells, and this reduces the abasic site affinity of TDG [18]. TDG SUMOylation induces a conformational change in the protein which overcomes product inhibition and is thus a mechanism for increasing enzymatic turnover [19, 20]. More recently, and similar to UNG and

SMUG1, TDG has been shown to be a target for ubiquitination-dependent degradation by a Cul4-DDB1 associated E3 ubiquitin ligase complex [21, 22]. Specifically, TDG degradation was catalysed by Cul4A-DDB1 in association with the RING finger protein ROC1/RBX1 and Cdt2 (collectively called CRL4^{Cdt2}), in a PCNA-dependent manner. This was discovered both in a *Xenopus* extract system during DNA repair but also in HeLa cells during S phase of the cell cycle where TDG interacts with PCNA, and is thought to provide a mechanism for the regulated control of TDG protein levels. The fourth uracil DNA glycosylase member, MBD4 which actually removes mismatches opposite guanine, has not been reported to be ubiquitinated. However MBD4 has been shown to interact with both the E3 ubiquitin ligase, ubiquitin-like with PHD and RING finger domains 1 (UHRF1) and with the deubiquitinating enzyme, ubiquitin specific protease 7 (USP7) following overexpression of the protein in HEK293T cells [23]. Given these interactions, MBD4 could potentially be a target for regulation by ubiquitination.

2.1.2. Helix-hairpin-helix (HhH) DNA glycosylases: OGG1, NTH1, MUTYH

The HhH DNA glycosylases are named after the DNA-binding motif which is present in all three members of the family and are OGG1, NTH1 and MUTYH. OGG1 is the major DNA glycosylase targeting 8-oxoguanine DNA base damage and only one report has suggested that it is a target for ubiquitination. Specifically OGG1 was found to be degraded following mild hyperthermia by the E3 ubiquitin ligase C-terminus of Hsc70-interacting protein (CHIP) [24]. CHIP is well known to be involved in protein quality control, by targeting damaged or misfolded proteins for ubiquitination-dependent degradation via interaction with the molecular chaperones Hsc70 and Hsp90 [25], and as will become clear later in this chapter, can target multiple BER proteins for degradation via the proteasome. Degradation of OGG1 by CHIP through heat inactivation in HeLa cells was shown to cause a reduction in the efficiency of repair of oxidised DNA base damage and cell growth inhibition following treatment with a photosensitiser. NTH1 is the second member of the HhH DNA glycosylases that excises oxidised pyrimidines from DNA, including 5-hydroxycytosine and thymine glycol, although there are no current reports that it is directly targeted for ubiquitination. However there is evidence that MUTYH, the third member of the family that specifically removes adenine residues incorrectly incorporated opposite 8-oxoguanine residues during DNA transcription or replication, is ubiquitinated both *in vitro* and *in vivo*. Ubiquitination between residues 475 and 500 within the MUTYH protein was shown to be catalysed by the Mcl1 ubiquitin ligase E3/ARF binding protein 1 (Mule/ARF-BP1) that subsequently stimulates proteasomal degradation [26]. This was evidenced *in vitro* using recombinant proteins, and also *in vivo* ubiquitination of MUTYH decreased in HEK293T cells following Mule siRNA and led to increased protein stability. A ubiquitination-deficient mutant of MUTYH lacking five critical lysine residues (477, 478, 495, 506 and 507) that were mutated to arginines, was found to be more stable in HEK293T cells than the wild type protein and preferentially bound to chromatin [26]. Mule overexpression in A2780 cells led to an increased mutation frequency following potassium bromate treatment that was predicted to be a consequence of the lack of MUTYH through Mule-dependent degradation. Mule has also been demonstrated to regulate the protein levels of Pol β and Pol λ (see below), and therefore appears to be a critical regulator of BER at both the base excision and gap filling stages. How the activity of Mule is co-ordinated towards these specific proteins and stages is not currently clear.

2.1.3. Endonuclease VIII-like glycosylases: NEIL1, NEIL2, NEIL3

NEIL1 and NEIL2 both have a broad range substrate specificity that largely covers those of the HhH enzymes OGG1 and NTH1. However, these DNA glycosylases appear more active on single stranded DNA and bubble structures and so may be more important during replication and transcription where these structures are formed [27, 28]. There is also evidence that NEIL1 is active at complex DNA damage sites, where two or more DNA base lesions are formed in close proximity [29, 30], and on telomeric DNA [31]. NEIL3 substrate activity has also proven to be elusive but can similarly act on telomeric DNA [31, 32] and more recent data has described a role for NEIL3 in unhooking of DNA interstrand crosslinks [33]. The only evidence of ubiquitination-dependent regulation of the NEIL DNA glycosylases is through very recent data involving NEIL1. Using an *in vitro* ubiquitination system in combination with fractionated HeLa cell extracts, NEIL1 was demonstrated to be targeted by two E3 ubiquitin ligases, namely Mule and tripartite motif 26 (TRIM26) [34]. Both enzymes appear to promote ubiquitination of NEIL1 on the same C-terminal lysine residues (319, 333, 356, 357, 361, 374 and 376) within the protein, and an siRNA knockdown of either Mule or TRIM26 in U2OS cells caused an increase in cellular NEIL1 protein levels demonstrating that they both target the protein for ubiquitination-dependent degradation. Interestingly in response to IR-induced DNA damage, there was an accumulation of NEIL1 protein which occurred in a Mule-dependent, but not a TRIM26-dependent, manner. This demonstrated a requirement for both Mule and TRIM26 in controlling the cellular steady state levels of NEIL1, in addition to those required in response to DNA damage.

2.1.4. N-methyl purine glycosylase (MPG)

MPG is a DNA glycosylase that excises alkylated DNA base damage, including 3-methyladenine and 7-methylguanine. There is no current evidence that this enzyme is regulated directly by ubiquitination, although MPG has been reported to interact with the E3 ubiquitin ligases UHRF1 and UHRF2 following overexpression in HEK293 cells, and interacts with UHRF1 in a number of cancer cell lines, including MCF7, HeLa and H1299 [35].

2.2. Ubiquitination of DNA strand break binders/processors

2.2.1. Poly(ADP-ribose) polymerase 1 (PARP-1)

PARP-1 functions in binding to SSBs created during BER, where it mediates poly(ADP-ribose)ylation of itself and other proteins involved in the repair process and thus promotes the assembly of repair protein complexes, chromatin remodelling and its own eventual dissociation from the DNA. Inhibitors targeting PARP-1 activity have recently been approved for the treatment of BRCA-deficient cancers, through which they cause synthetic lethality. This therapeutic exploitation provides an added incentive to enhance our understanding of the regulation of cellular PARP-1, particularly through ubiquitination.

The first report to show that PARP-1 is ubiquitinated was in mouse embryonic fibroblasts following treatment with the proteasome inhibitor ALLN [36]. PARP-1 ubiquitination was

further examined *in vitro* and shown to involve specifically lysine 48-linked chains, suggesting that this would likely target the enzyme for proteasomal degradation. PARP-1 modification by SUMOylation, specifically SUMO-2 at lysine 203 and 486 induced by the PIASy SUMO E3 ligase, has been demonstrated following overexpression of the enzymes in HeLa cells in response to heat shock stress, which also rendered PARP-1 as a target for ubiquitination by the poly-SUMO-specific E3 ubiquitin ligase ring finger protein 4 (RNF4) [37]. PARP-1 ubiquitination resulted in degradation of the protein, and provides evidence of crosstalk between two PTMs thought to be involved in regulating PARP-1 transcriptional activation, rather than playing a role during BER. Another incidence of crosstalk has been revealed between PARP-1 ubiquitination and poly(ADP-ribosylation). The E3 ubiquitin ligase ring finger protein 146 (RNF146), also known as Iduna, ubiquitinates PARP-1 *in vitro* and in MCF7 cells overexpressing Iduna leading to its proteasomal degradation providing that PARP-1 itself is poly(ADP-ribosylated) [38]. This phenomenon was more pronounced following N-methyl-N-nitro-N-nitrosoguanidine (MNNG)-induced alkylated base damage, suggesting a DNA damage inducible response. Furthermore, an shRNA-induced knockdown of Iduna caused an accumulation of abasic sites following MNNG treatment, and an accumulation of SSBs following IR in MCF7 cells. This is difficult to comprehend given the abundance of cellular APE1 and its ability to cleave any abasic sites following MNNG treatment, and that PARP-1 should still be able to dissociate from IR-induced SSBs following poly(ADP-ribosylation) and allow for subsequent repair protein recruitment. However since Iduna has also been shown to polyubiquitinate other BER proteins, including XRCC1 and Lig III, the effects of Iduna on the cellular DNA damage response are not clear cut.

A third E3 ubiquitin ligase has been identified for PARP-1, namely the checkpoint with forkhead-associated and RING finger domain protein (CHFR), which was shown to polyubiquitinate PARP-1 *in vitro* and following overexpression of CHFR in HEK293T and HCT116 cells *in vivo* [39]. CHFR regulates the mitotic checkpoint and following mitotic stress PARP-1 was shown to undergo poly(ADP-ribosylation), and similarly to the case for Iduna, this facilitated polyubiquitination-dependent degradation of the protein. CHFR-knockout mouse embryonic fibroblasts displayed elevated PARP-1 levels and did not undergo cell cycle arrest in response to mitotic stress. An additional report similarly described poly(ADP-ribosylated) PARP-1 as a CHFR target [40]. CHFR was recruited to laser-induced DNA damage sites in a poly(ADP-ribosylation)-dependent manner in U2OS cells as revealed by PARP inhibition and PARP-1 siRNA. Interestingly, lysine 48-linked ubiquitin chains were conjugated to poly(ADP-ribosylated), but not unmodified, PARP-1 by CHFR *in vitro* in the presence of the E2 conjugating enzyme UbcH5C, but lysine-63 linked chains were added in the presence of Ubc13/Uev1a. Both lysine 48 and 63-linked chains were then found attached to PARP-1 following irradiation of CHFR-overexpressing HCT116 cells. Potentially more important than its eventual degradation, CHFR-mediated ubiquitination prompted the displacement of PARP-1 from chromatin, and CHFR-knockout mouse embryonic fibroblasts were demonstrated to display delayed SSB repair kinetics and increased sensitivity to IR.

As multiple E3 ubiquitin ligases have been implicated as effectors of PARP-1 ubiquitination, more research is required to determine which of these are crucially involved in the regulation of steady state PARP-1 levels and which function specifically during BER. It is apparent that

PARP-1 regulation is multifaceted, with the added complexity of crosstalk between ubiquitination and other PTMs such as SUMOylation and poly(ADP-ribosyl)ation, therefore it may be some time before the intricacies of this regulation are elucidated.

2.2.2. AP endonuclease I (APE1)

APE1 is the major enzyme targeting abasic sites for incision in human cells, and both an overabundance and lack of this protein can cause genome instability, so the protein levels must be tightly regulated. APE1 was first shown to be monoubiquitinated within the N-terminus of the protein in HCT116 cells by overexpression of the E3 ubiquitin ligase mouse double minute homologue 2 (MDM2), the major enzyme regulating the p53 tumour suppressor protein [41]. Depletion of MDM2 consequently increased APE1 protein levels, thought to be as a result of reduced ubiquitination-dependent degradation. The same authors then reported that phosphorylation of APE1 at threonine 233 by cyclin-dependent kinase 5 (Cdk5)-enhanced MDM2-dependent ubiquitination of APE1 [42]. Indeed, a phosphomimetic mutant (T233E) of APE1 exhibited augmented ubiquitination following expression in HCT116, SW480 and A549 cells. However MDM2 knockout mouse embryonic fibroblasts expressing the phosphomimetic mutant of APE1 still displayed significant APE1 ubiquitination, demonstrating the existence of other E3 ubiquitin ligases for the protein. In fact utilising an *in vitro* ubiquitination assay incorporating APE1 as a substrate and fractionated proteins from HeLa whole cell lysates has revealed that the major E3 ubiquitin ligase targeting APE1 for ubiquitination was ubiquitin protein ligase E3 component N-recognin 3 (UBR3) [43]. *In vitro* ubiquitination of APE1 by UBR3 was localised within the N-terminus on multiple lysine residues (6, 7, 24, 25, 27, 31, 32 and 35). UBR3 knockout mouse embryonic fibroblasts displayed significantly higher APE1 protein levels, suggesting that ubiquitination targeted APE1 for proteasomal degradation, and consequently led to an increase in endogenously formed DNA double strand breaks and genomic instability. A third E3 ubiquitin ligase has recently been identified for APE1, namely the Parkinson's disease-associated protein, Parkin [44]. Overexpression of Parkin was found to ubiquitinate APE1 in A549 cells, and in an engineered mouse embryonic fibroblast cell line containing low APE1 protein overexpression of both Parkin and APE1 caused a decrease in protein stability. However, inducible expression of Parkin in 293-PaPi cells did not alter endogenous APE1 protein levels and a combination of Parkin and hydrogen peroxide treatment only caused an ~30% reduction in the protein, suggesting that Parkin may only have a minor role in APE1 regulation.

2.2.3. Polynucleotide kinase phosphatase (PNKP)

PNKP acts to remove the 3'-phosphate group remaining from the actions of NEIL1–3 during BER, but also displays kinase activity for 5'-DNA ends and thus plays a role in the repair of SSBs and double strand breaks. A crosstalk between phosphorylation and ubiquitination has been revealed to be important in the regulation of PNKP protein levels. Phosphorylation catalysed by the ataxia telangiectasia mutated (ATM) protein kinase on serines 114 and 126 of PNKP was shown to stabilise the protein in response to oxidative stress in HCT116 cells, which was mediated through inhibition of ubiquitination, and which was required for efficient SSB

repair [45]. An *in vitro* ubiquitination assay, using PNKP as a substrate in combination with fractionated proteins from HeLa whole cell lysates, revealed that Cul4A-DDB1 in association with the WD-40 repeat protein serine-threonine kinase receptor-associated protein (STRAP) was the major E3 ubiquitin ligase complex that was targeting PNKP for ubiquitination, specifically on lysines 414, 417 and 484. A phosphomimetic mutant (serine 114 and 126 to glutamic acid) was more stable than the wild type protein following expression in HCT116 cells, and the protein itself was resistant to *in vitro* ubiquitination by Cul4A-DDB1-STRAP. Mouse embryonic fibroblasts from STRAP knockout cells also had significantly elevated PNKP protein levels due to reduced ubiquitination-dependent degradation, and displayed increased resistance to oxidative stress. In addition, an interaction between PNKP and the deubiquitination enzyme ataxin-3 has been demonstrated *in vivo*, and which promotes the 3'-phosphatase activity of PNKP *in vitro* [46]. However whether this enzyme contributes to regulating PNKP protein levels, particularly in opposition to Cul4A-DDB1-STRAP-mediated ubiquitination, has yet to be investigated.

2.2.4. Flap endonuclease-1 (FEN-1)

FEN-1 acts to remove the flap structures created by Pol δ/ϵ during long-patch BER. Ubiquitinated FEN-1 has been observed at the end of a sequence of PTMs initiated in late S phase of the cell cycle [47]. It was observed in HeLa cells that phosphorylation of FEN-1 at serine 187, promotes SUMOylation at lysine 168 with SUMO-3, which in turn stimulates polyubiquitination at lysine 354 by the E3 ligase pre-mRNA processing factor 19 (PRP19) to stimulate proteasomal degradation. This was largely discovered through overexpression of individual components within the pathway in HeLa cells, rather than examining endogenous proteins. Furthermore, PRP19 was only characterised in ubiquitinating FEN-1 *in vitro* using partially purified HeLa cell extracts and a complete suppression of ubiquitination was not observed following immunodepletion of PRP19, suggesting the existence of alternative E3 ubiquitin ligases for FEN-1. Nevertheless, this sequence of events beginning in late S phase is thought to regulate FEN-1 protein levels at the end of DNA replication, rather than being required for long-patch BER. Therefore further work is required to determine whether this, or an alternate mechanism for FEN-1 ubiquitination, plays a role in the regulation of this protein during BER.

2.3. Ubiquitination of DNA polymerases

2.3.1. DNA polymerase β (Pol β)

Pol β is the principal polymerase employed within the BER pathway, and primarily acts to insert the correct nucleotide into the repair gap, but also acts as a dRP lyase activity. The stability of Pol β was found to be significantly reduced in XRCC1 deficient EM-C11 cells and in HeLa cells following XRCC1 siRNA treatment, suggesting that Pol β and XRCC1-Lig III α form a stable complex that protects Pol β from degradation [48]. The major E3 ubiquitin ligase for Pol β was then revealed through the utilisation of *in vitro* ubiquitination assays in combination with fractionated HeLa cell extracts to be CHIP. Ubiquitination was localised to the 8 kDa N-terminal domain, which contains the dRP lyase activity, and CHIP depletion by

siRNA in HeLa cells led to increased protein levels of Pol β whereas overexpression of CHIP reduced cellular Pol β . Interestingly, this investigation highlighted that CHIP also appeared to be involved in the ubiquitination-dependent degradation of XRCC1 and Lig III α , in addition to Pol β . This study was followed by the identification of a second E3 ubiquitin ligase that specifically catalysed monoubiquitination of Pol β [49]. Monoubiquitination was shown to occur in the same 8 kDa N-terminal region as that targeted by CHIP, but was catalysed by Mule. The precise ubiquitinated residues were identified as lysine 41, 61 and 81, and a lysine to arginine mutant Pol β protein was more stable than the wild type protein following expression in HeLa cells. Monoubiquitinated Pol β was observed exclusively within the cytoplasm in HeLa cells and was therefore deemed a specific target for ubiquitination-dependent degradation mediated by CHIP. Indeed, an siRNA knockdown of Mule decreased the levels of monoubiquitinated Pol β , increased total cellular Pol β levels and caused an increase in the rate of repair of SSBs and alkali-labile sites induced by hydrogen peroxide. Conversely a knockdown of ARF, which inhibits the activity of Mule, caused an increase in monoubiquitinated Pol β and a decrease in the rate of repair of hydrogen peroxide-induced SSBs and alkali-labile sites. A later study agreed that Pol β stability was dependent on XRCC1 and was regulated by ubiquitination-dependent degradation, but reported that this ubiquitination occurred on lysines 206 and 244 and was not reliant on Mule or CHIP [50]. However, the experimental system employed was very artificial, utilising an unusual cell line containing deletions of, amongst others, the ARF protein and modified to stably overexpress Pol β rather than examining endogenous protein levels. The identification of the deubiquitination enzyme that is able to reverse Mule- and CHIP-dependent ubiquitination of Pol β , and in fact the only such enzyme characterised in regulating BER, has been identified as ubiquitin specific protease 47 (USP47). USP47 was purified and identified from fractionated HeLa cell extracts in combination with an *in vitro* deubiquitination assay using ubiquitinated Pol β as a substrate, and was demonstrated to be capable of removing both CHIP-dependent polyubiquitin chains and Mule-dependent monoubiquitin moieties from Pol β [51]. An siRNA knockdown of USP47 in HeLa cells resulted in an increase in Mule-dependent monoubiquitinated Pol β , a reduction in cytoplasmic and therefore overall Pol β protein levels, and ultimately led to reduced efficiency of repair of SSBs and alkali-labile sites created through oxidative and alkylated DNA base damage. This study led to a complete picture of how Pol β protein levels are regulated in the cellular response to DNA damage, which involves an interplay between Mule, CHIP, ARF and USP47 activities that control a specific cytoplasmic pool of Pol β that acts as a source of protein that can be utilised in the nucleus in the event of any increase in DNA damage. The above studies together establish that Pol β protein levels are tightly regulated by the promotion or reversal of ubiquitination-dependent proteasomal degradation.

2.3.2. DNA polymerase λ (Pol λ)

Although Pol β is the chief polymerase in the BER pathway, Pol λ is thought to have a back-up role, specifically in the bypass of 8-oxoguanine lesions and thus avoiding the tendency for the misincorporation of the wrong adenine base opposite the lesion. Initial evidence that Pol λ is regulated by ubiquitination was demonstrated by the observation that a Cdk2/cyclin

A phosphorylation defective mutant of Pol λ at threonine 553, was less stable than the wild type protein following expression in either 293T or U2OS cells, and that this was mediated via increased ubiquitination [52]. Phosphorylation of Pol λ was observed most notably in late S and G2 phases of the cell cycle and was thought to stabilise the protein via inhibition of ubiquitination and to allow Pol λ to repair any DNA damage incurred at this stage. The major E3 ubiquitin ligase responsible for Pol λ ubiquitination was subsequently identified using the protein as a substrate in *in vitro* ubiquitination assays containing fractionated HeLa whole cell lysates and shown to be Mule [53]. Mule was found to ubiquitinate Pol λ on lysines 27 and 273 *in vitro*, and an siRNA-mediated depletion of Mule in HEK293T cells significantly increased Pol λ protein levels. Cdk2/cyclin A-dependent phosphorylation of Pol λ was found to inhibit Mule-dependent ubiquitination, and promote binding of the protein to chromatin via interaction with MutYH. The E3 ubiquitin ligase CHIP has also been shown to ubiquitinate Pol λ *in vitro*, although there is no evidence that CHIP plays a role in the regulation of cellular Pol λ *in vivo* [54]. Since Pol λ and Pol β are both regulated by Mule, this suggests that Mule plays a vital role in controlling the polymerase stage of BER, in addition to the base excision stage described above, and thus is a central player in coordinating the cellular DNA damage response.

2.3.3. DNA polymerase δ/ϵ (Pol δ/ϵ)

Pol δ and ϵ participate in long-patch BER by adding multiple complimentary nucleotides into the repair gap vacated by Pol β , thus creating a 5'-flap structure which is a substrate for FEN-1. Pol δ is synthesised in human cells as a heterotetramer of subunits p125, p68, p50 and p12. Using an *in vitro* ubiquitination assay with fractionated HeLa cell lysates, the p12 subunit has been shown to be a target for ubiquitination by the E3 ubiquitin ligase RNF8 [55]. An siRNA knockdown of RNF8 in A549 cells led to an increased stability of p12, particularly following UVC irradiation but also following MNNG treatment. However the precise contribution of this mechanism to BER efficiency is currently unknown. Additionally there is no evidence suggesting that Pol ϵ is regulated by ubiquitination.

2.4. Ubiquitination of DNA ligases

2.4.1. X-ray cross-complementing protein 1 and DNA ligase III α (XRCC1-Lig III α)

Lig III α functions in a stable complex with the scaffold protein XRCC1 to seal the nick in the DNA backbone to complete the BER process. Lig III α itself been shown to undergo ubiquitination in two separate reports. In the first, CHIP was demonstrated as an E3 ubiquitin ligase for Lig III α *in vitro*, but also an siRNA knockdown of CHIP in HeLa cells caused an accumulation of Lig III α protein *in vivo* as a consequence of a lack of ubiquitination-dependent degradation [48]. A second E3 ubiquitin ligase for Lig III α *in vitro* has been identified as Iduna/RNF146, which ubiquitinates the protein but only when modified by poly(ADP-ribose)ylation [38]. However in this study, Iduna was shown to interact with and ubiquitinate a number of DNA repair proteins, including both Lig III α and XRCC1 but also PARP1 (as discussed previously) which was dependent on protein poly(ADP-ribose)ylation. Therefore the

particular significance of Iduna-mediated ubiquitination of Lig III α and XRCC1 specifically on BER regulation remains unclear. XRCC1 has been shown to be phosphorylated *in vitro* and *in vivo* by casein kinase 2 (CK2) and this prevents the ubiquitination-dependent degradation of the protein. This was demonstrated by reduced stability of XRCC1 following CK2 siRNA in HeLa cells and that a CK2 phosphorylation deficient mutant of XRCC1 expressed in EM9 cells was less stable than the wild type protein as a direct consequence of increased ubiquitination [56]. A separate study also demonstrated ubiquitination of XRCC1, although conversely a phosphorylation deficient mutant of XRCC1 expressed in U2OS cells appeared not to be stabilised following proteasomal inhibition [57]. Similar to Lig III α , XRCC1 has been found to be ubiquitinated *in vitro* by the E3 ubiquitin ligase CHIP, and an siRNA-mediated depletion of CHIP in HeLa cells resulted in an increase in XRCC1 protein levels owing to a reduction in ubiquitin-dependent protein degradation [48]. Overexpression of CHIP in HeLa cells was also found to cause a reduced stability of XRCC1 protein. These findings were supported by a separate study that demonstrated that CHIP-dependent ubiquitination of XRCC1 occurs, but only when the protein is not bound to Pol β or heat shock protein 90 (HSP90) [50]. Regarding the site of ubiquitination within XRCC1, this has been identified within the BRCA1 C-terminus (BRCT II) motif after it was demonstrated that truncated XRCC1 lacking this domain was considerably more stable than the full length protein when expressed in either HeLa or EM-C11 cells [48]. This ubiquitination site within the BRCT II motif of XRCC1 was also verified in an independent study [57].

2.4.2. DNA ligase I (Lig I)

Lig I is employed during long-patch BER, but is also involved in DNA replication. The only reported evidence of Lig I ubiquitination is through the Cul4A-DDB1 E3 ubiquitin ligase complex [58]. Overexpression and immunoprecipitation of Lig I from 293T cells revealed that lysine 376, and possibly lysine 79 and 192, were potential ubiquitination sites and that a lysine to arginine mutant of Lig I at four sites (79, 192, 226 and 376) was more stable than the wild type protein to degradation through serum starvation. Lig I was then demonstrated to interact with and to be ubiquitinated *in vitro* by a Cul4A-DDB1 complex with the associated factor DCAF7. An siRNA knockdown of DCAF7 in GM00847 cells was shown to suppress the degradation of Lig I following serum starvation. This study would suggest that Lig I protein levels are controlled during DNA replication, however the impact of this mechanism for the efficiency of long-patch BER, is still unknown.

2.5. Summary and future outlook

An increasing amount of evidence is strengthening the fact that BER is carefully regulated and controlled by ubiquitination. This largely appears to be a mechanism for controlling cellular BER protein levels via the 26S proteasome and therefore plays an important role in suppressing DNA damage accumulation and coordinating an efficient cellular DNA damage response. In this Chapter we have presented evidence that the majority of BER proteins have been shown *in vitro* and *in vivo* to be targeted for ubiquitination by specific E3 ubiquitin ligases. However there are other proteins (e.g. the DNA glycosylases MBD4, MTH1, NEIL2 and NEIL3) which have not yet been demonstrated to be ubiquitinated directly (**Table 1**). It is interesting to note

E3 ubiquitin ligase	BER protein	Reference
CHIP	Lig III	[48]
	OGG1	[24]
	Pol β	[48]
	Pol λ	[54]
	XRCC1	[48]
Cul1	SMUG1	[16]
	UNG	[16]
Cul4	Lig I	[58]
	PNKP	[45]
	SMUG1	[16]
	TDG	[21, 22]
	UNG	[16]
Iduna/RNF146	Lig III	[38]
	PARP-1	[38]
	XRCC1	[38]
MDM2	APE1	[41]
Mule	MUTYH	[26]
	NEIL1	[34]
	Pol β	[49]
	Pol λ	[53]
RNF8	Pol δ	[55]
TRIM26	NEIL1	[34]
UBR3	APE1	[43]
Unknown	MBD4	
	NEIL2	
	NEIL3	
	NTH1	
	Pol ϵ	

Table 1. Summary of the known E3 ubiquitin ligases targeting BER proteins for ubiquitination.

that some of the identified E3 ubiquitin ligases appear to target more than one BER protein for ubiquitination. For example, Mule can ubiquitinate both the DNA glycosylases NEIL1 and MUYTH, and the DNA polymerases Pol β and Pol λ for ubiquitination-dependent degradation which would suggest that this E3 ubiquitin ligase, and others targeting multiple BER proteins, can control BER at several different points in the repair pathway. The Cullins, Cul1 and

Cul4, also appear to regulate several BER members although this is unsurprising given that they represent the largest family of E3 ubiquitin ligases with several hundred members. In fact the most important element of these complexes is the adaptor proteins that provide specificity of ubiquitination to their target protein. Indeed Cdt2, DCAF7 and STRAP have already been identified as adaptors of the Cul4A-DDB1 complexes that target TDG, Lig I and PNKP, respectively for ubiquitination. Nevertheless, we currently do not have a clear understanding of how these ubiquitination events are controlled and co-ordinated to ensure an efficient BER response to DNA damage. In particular there is insufficient knowledge on how the identified E3 ubiquitin ligase enzymes are activated and directed to their specific enzyme targets. This could be achieved by either compartmentalisation of the enzymes or targets within the cell, or by activation of ubiquitination enzymatic activity by PTMs. Secondly, with ubiquitination being a reversible process, the identities of deubiquitination enzymes that work in concert with E3 ubiquitin ligases in the regulated control of BER proteins have not yet been fully elucidated. In fact the only evidence for this is by USP47, which has been demonstrated to be actively involved in the deubiquitination of Pol β . Thirdly, in addition to ubiquitination, it is clear that BER proteins are also regulated by other PTMs, including acetylation, methylation, phosphorylation and SUMOylation. There is some evidence of crosstalk between these modifications and ubiquitination in regulating BER protein levels and activities, particularly for PARP-1, FEN-1 and PNKP, although this is not yet fully understood. However these regulatory “switches” are undoubtedly an efficient way of controlling the cellular BER response to DNA damage. Ultimately further research is necessary in order to fully identify and understand the specific E3 ubiquitin ligase and deubiquitination enzymes for individual BER proteins, and the precise mechanisms that are co-ordinated in association with other PTMs, which act to ensure an efficient repair process.

In addition to discovering the molecular details for regulating cellular BER, research into the associations of these and the development of human diseases, including premature ageing, neurodegenerative diseases and cancer is essential. It is understood that BER protein levels are frequently misregulated in these diseases although whether defective ubiquitination is contributory to this effect is largely unknown and understudied. This information may also uncover novel therapeutic strategies for the treatment of specific diseases. Indeed the BER pathway is known to be an attractive therapeutic target, which is exemplified by the success of PARP inhibitors in the treatment of BRCA-deficient breast cancers which block BER and cause synthetic lethality due to the inability of these cells to process DNA double strand breaks. It is therefore entirely possible that the discovery of E3 ubiquitin ligases or deubiquitination enzymes targeting BER proteins may provide novel cellular targets for drugs or small molecule inhibitors which can be used in combination with radiotherapy and/or chemotherapy for treatment of diseases, such as cancer.

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