Role of ubiquitination in the DNA damage response: proteomic analysis to identify new DNA-damage-induced ubiquitinated proteins

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Abstract

The DDR (DNA damage response) is a signalling transduction cascade utilizing many forms of post-translation modification of proteins, including phosphorylation and ubiquitination. The well-known function of ubiquitination is to target proteins for proteasomal degradation; however, it is also involved in the regulation of protein function. The present review describes how ubiquitination regulates the function of certain proteins involved in DDR, in particular FANCD2 (Fanconi’s anaemia complementation group D2) and PCNA (proliferating-cell nuclear antigen). Also, the proteomic methods currently used to identify new ubiquitinated proteins in response to DNA damage, including the advantages of using the UBD (ubiquitin-binding domain) beads to purify the ubiquitinated proteins, are considered.

The DDR (DNA damage response)

In mammalian cells, DNA can be damaged through internal factors (e.g. oxidative stress) or through external factors (e.g. radiation). It is imperative to repair and pass the DNA code faithfully to the progeny cells as the un-repaired DNA may lead to mutations in key genes such as p53, resulting in the formation of cancer cells. Cells respond to DNA damage through sets of sensors, mediators and effectors orchestrating the cellular response, and may arrest cell cycle until damage has been repaired, or trigger apoptosis if the damage is too severe [1].

The DDR is a signal transduction cascade controlling many aspects of the cell and its main aim is to repair DNA and assist the progression of DNA replication [1]. The two key regulators of the DDR are the PI3K (phosphoinositide 3-kinase)-related protein kinases ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3-related). ATR is important for sensing ssDNA (single-stranded DNA) in replication fork blocks in order to cause cell arrest at the S-phase [2], whereas ATM is activated in response to double-stranded breaks at any point in the cell cycle [3].

Ubiquitination

Ubiquitin is a protein made of 76 amino acids and has a molecular mass of ~8 kDa. Ubiquitination is a post-translational modification involved in the regulation of various cellular processes including DNA repair and receptor trafficking [4]. Ubiquitination is a process of covalent bonding (isopeptide bond) between glycine at the C-terminus of ubiquitin and the lysine residue of the target protein. This reaction needs a cascade of enzymes: E1 activating enzyme, E2 conjugating enzyme and E3 ligase enzyme. The E3 enzyme usually works along with E2 but also confers specificity for the target protein. A computer analysis has estimated that there could be 16, 53 and 527 of E1, E2 and E3 enzymes respectively in humans [5]. Polyubiquitination can occur by the glycine-to-lysine linkage on any of the seven lysine residues on the previous ubiquitin. There are also deubiquitinating enzymes, of which there are estimated to be 184 in humans [5].

Ubiquitination can lead to the proteasomal degradation or modification of the function of the protein. Primarily, polyubiquitination through linkage between Gly76 of one ubiquitin to Lys48 of the previous ubiquitin acts to target the protein for degradation through the proteasome complex [6]. However, polyubiquitination via other lysine residues and mono-ubiquitination act to regulate the target protein in cellular functions such as DNA repair.

UBD (ubiquitin-binding domain)

Ubiquitination in signal transduction and regulation involves the non-covalent interaction between the attached ubiquitin and other proteins. These proteins are called ubiquitin-binding proteins, and they interact through the UBDs that they possess. The UBDs are small, between 20 and

Key words: DNA damage response (DDR), Fanconi’s anaemia complementation group D2 (FANCD2), proliferating-cell nuclear antigen (PCNA), proteomics, ubiquitin-binding domain (UBD), ubiquitination.

Abbreviations used: ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3-related; CIP, C-terminus-binding protein-interacting protein; DDR, DNA damage response; FA, Fanconi’s anaemia; FANCD2, FA complementation group D2; FANCI, FA complementation group I; FANCM, FA complementation group M; HEK, human embryonic kidney; HR, homologous recombination; Mdm2, murine double minute 2; PCNA, proliferating-cell nuclear antigen; ssDNA, single-stranded DNA; IUBA, ubiquitin-associated domain; IUBD, ubiquitin-binding domain; IUI, ubiquitin-like; UQ1, ubiquilin 1; TIS, translation synthesis.

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150 amino acids, and are independently folded to interact with mono- and poly-ubiquitin. They show varied affinity towards ubiquitin, with \( K_d \) values from 3 \( \mu \)M to 2 mM, and although there is no particular similarity between the UBDs, they all recognize the part of the ubiquitin that overlaps Ile44 [4,7,8].

The first UBD, UIM (ubiquitin-interacting domain), was characterized in the proteasome subunit S5a/RPN10 [9]. There are many structurally diverse UBDs found so far including the UBA (ubiquitin-associated domain). The ubiquitin-binding proteins contain one or more UBDs of the same or different class, reflecting the functional diversity of the protein. UBDs can be found on ubiquitinating or deubiquitinating enzymes or ubiquitin receptors that transduce the signalling message [8].

Different types of UBDs show varying binding properties and preferences for mono- and poly-ubiquitin chains. Raasi et al. [10] studied the interaction of 30 UBA domains with three topologies of polyubiquitin chains, which were sorted into four classes. The first class consisted of UBAs that preferentially bind to Lys48-linked chains, the second class binds to Lys63-linked chains, whereas the third class binds to none of these chains and the fourth class binds to all three types of chains. UQ1 (ubiquitin 1)-UBA and Dsk2-UBA belong to the fourth class and they also bind to mono-ubiquitin.

Owing to the commercial availability of some of the UBDs attached to agarose beads, they have been used in studies to purify ubiquitinated proteins instead of conventional methods such as using immunoprecipitation.

**Ubiquitination in the DDR**

Ubiquitination has an important role in the DDR, both to degrade key proteins and as a signalling mechanism. Indeed, several proteins involved in ubiquitination and degradation are substrates of ATM/ATR in response to DNA damage [11]. Furthermore, many proteins involved in the DDR are regulated by the ubiquitin–proteasome pathway, such as Mdm2 (murine double minute 2) and Cdc25A (cell division cycle 25A) [12,13].

However, only a small number of the DNA-damage-dependent ubiquitinated proteins that participate in signal transduction have been identified. Two of the well-studied proteins modified by ubiquitination in the DDR are FANCd2 [FA (Fanconi’s anemia) complementation group D2] and PCNA (proliferating-cell nuclear antigen). There are also two recently reported proteins, CtIP (C-terminus-binding protein-interacting protein) and Rad17, which have an ubiquitination-dependent role in the DDR [14,15].

**FA pathway**

FA is a rare recessive genetic disease characterized by bone-marrow failure and an increase in cancer predisposition [16] due to the mutations of genes involved in the FA pathway proteins. The FA pathway results in the ubiquitination of FANCd2 in order to repair cross-linked DNA [17].

There are at least 13 FA complementation group proteins that co-operate in the same cellular pathway (FA pathway) to load proteins on chromatin and repair DNA cross-linking [18]. This FA pathway is activated by mitomycin C, hydroxyurea, UV light and ionizing radiation, which results in the mono-ubiquitination of FANCd2 at Lys64 [17].

Eight of the FA proteins (A, B, C, E, F, G, L and M) form a nuclear complex. A fraction of the FANCd (FA complementation group M) protein is constitutively attached to the chromatin via FAAP24 (FANC-associated polypeptide 24), and the rest of the complex (ABCEFGL) associates with FANCd during S-phase of the cell cycle and in response to DNA damage [19]. The subunit L of the FA core complex is an E3 ligase, which, in response to DNA damage or replication fork arrest, mono-ubiquitinates the downstream target FANCd2 [20] and the recently identified target, FANCi (FA complementation group I) [21]. Interestingly, ubiquitination of one of these two targets is important for the ubiquitination of the other. A fraction of mono-ubiquitinated FANCd2 and FANCi form heterodimers (ID complex) and are loaded on to the chromatin perhaps through interaction with a protein containing UBD [19].

FANCd2 [22] and FANCi [21] are important components of HR (homologous recombination) repair. Other members of the FA pathway are also implicated in HR repair and TLS (translesion synthesis) [18]. Mono-ubiquitinated FANCd2, at the chromatin site of DNA damage, interacts with other proteins to promote repair of cross-linked DNA. After the repair, FANCd2 is deubiquitinated by the USP1 (ubiquitin-specific peptidase 1)–UAF1 (USP1-associated factor 1) enzyme complex, which causes the DNA repair complex to dissociate, thereby completing repair [23,24].

**PCNA**

PCNA is a processivity factor for replication polymerases. It forms into a trimeric ring to encircle the DNA and interacts with numerous other factors during replication and repair [25]. The ubiquitination status of PCNA determines the repair pathway in replication fork blocks and this is well understood in the budding yeast *Saccharomyces cerevisiae*. A fraction of PCNA in normal S-phase is SUMOylated at two sites; one of these sites is Lys164. In response to DNA damage, the Rad18–Rad6 complex mono-ubiquitinates PCNA at the same residue, Lys164, where Rad6 is an E2 enzyme and Rad18 is an E3 enzyme that also binds to ssDNA [25]. The mono-ubiquitinated PCNA activates polymerase \( \eta \) and rev1 [26] to repair DNA damage by the error-prone DNA damage repair mechanism TLS. TLS is a process where specialized polymerases work on damaged DNA with low fidelity [27]. The UBDs UBM (ubiquitin-binding motif) and UBZ (ubiquitin-binding zinc finger) are conserved among all Y-family TLS polymerases and these UBDs are required for their interaction with mono-ubiquitinated PCNA [28].

PCNA can be further polyubiquitinated at the same residue, Lys164, to form a Lys64-linked chain. This is carried
out by the E2 enzyme Ubc13-Mms2 and the E3 ligase Rad5. Polyubiquitinated PCNA is essential for the error-free repair at the stalled replication through the HR repair pathway [25].

Interestingly, sumoylation at Lys164 acts as an antagonist of PCNA mono-/poly-ubiquitination [29,30].

In mammalian cells, DNA-damage-induced mono-ubiquitination at Lys164 is also dependent on Rad6/Rad18 and the mono-ubiquitinated PCNA interacts with polymerase η [31,32]. Polyubiquitination through Lys63 linkage also occurs leading to error-free DNA repair [33]. In humans, there are two Rad5 homologues, SHPRH (SNF2 histone linker PHD RING helicase) and HLTF (helicase-like transcription factor), which interact with Ubc13 to cause Lys63-linked ubiquitination of PCNA.

### Ubiquitination of CtIP and Rad17 in response to DNA damage

CtIP and Rad17 are two newly reported proteins that are ubiquitinated in response to DNA damage. CtIP is ubiquitinated by the E3 ligase BRCA1 (breast-cancer susceptibility gene 1), which is dependent on the phosphorylated form of CtIP interacting with the ligase and is also dependent on DNA damage. After this ubiquitination, CtIP associates with chromatin and is involved in the regulation of G2/M checkpoint [14].

The 9-1-1 complex is composed of Rad9–Hus1–Rad1 in humans and Dde1–Mec3–Rad17 in *S. cerevisiae*. The 9-1-1 complex is a sliding clamp structurally similar to PCNA and acts as a sensor for replication fork stalls. A study carried out in yeast revealed that the Rad17 component of the 9-1-1 complex is mono-ubiquitinated in response to DNA damage, which is involved in transcriptional regulation checkpoint functions.

### Proteomic approach to identify ubiquitinated proteins involved in the DDR

Generally, in order to assess the regulation by ubiquitination, the protein of interest that may already have a role in DDR can be analysed by biochemical methods to first show that it is ubiquitinated and then how this affects its functional role. Another way is the proteomic approach, where global representation of ubiquitinated proteins is pulled down and identified by MS to see how many of these have a known role in the DDR. However, the latter approach may not be able to identify proteins that are ubiquitinated only after DNA damage.

Ubiquitinated protein levels in cells are usually very low due to their high turnover rate (readily deubiquitinated); therefore, it is essential to enrich them before any analysis can take place. Many affinity approaches have been used to purify ubiquitinated proteins in yeast and human cells, including epitope-tagged ubiquitin (e.g. FLAG, polyhistidine tag, HA (haemagglutinin) tag, Myc tag and biotin), ubiquitin antibodies and UBD [8,34]. Previous identification of UBDs has led to their use in large-scale proteomics [35]. Different types of UBDs show varying binding properties and preferences for mono- and poly-ubiquitin chains [10].

The UQ1-UBA and Dsk2-UBA are two interesting UBDs as they bind to mono- and poly-ubiquitin with high affinity and also in a linkage-independent manner [10,36].

The UBL (ubiquitin-like)-UBA family of proteins are ubiquitin receptors of the proteasome, as the receptors simultaneously bind to ubiquitin protein via the UBA domain, and proteasome via the UBL domain. One such protein is the human presenilin-interacting protein, UQ1, which contains a UBL domain at the N-terminus and UBA domain at the C-terminus. The UBA domain of the UQ1 protein and its interaction with ubiquitin have been studied by using NMR [36]. The interaction between ubiquitin and UQ1-UBA are primarily hydrophobic. UQ1-UBA forms a compact three-helix bundle structure and has a *K*ₐ of ~20 μM, making it one of the tight binders of ubiquitin. This domain binds to individual ubiquitins and therefore has slightly higher affinity for polyubiquitin chains than for mono-ubiquitin. Such characteristics of this UBD mean that they can be used to pull down representative ubiquitinated proteins. The advantages of using UBD beads over the conventional methods of purification are that endogenous ubiquitinated proteins are purified and the appearance of non-ubiquitinated proteins in the purification is reduced. This method is also less expensive than the other methods. In addition, the availability of different UBD peptides showing varying binding capacities towards the types of ubiquitination makes it possible to enrich and purify certain low-abundant proteins that may not be found through conventional methods such as His-tagged purification.

As mentioned previously, proteomic studies have looked at ubiquitinated proteins from cells and tissues without causing DNA damage. This means proteins that are ubiquitinated after DNA damage may not be found. Therefore, to our knowledge, an approach to globally identify ubiquitinated proteins in response to DNA damage has not been attempted. However, there is one related study that identified ubiquitinated proteins in response to stress through proteomics [37]. In that study, His₆-ubiquitin–GFP (green fluorescent protein) plasmid was expressed in HEK (human embryonic kidney) -293 cells and the ubiquitinated proteins were purified by nickel affinity chromatography, from both untreated and arsenite- (which induces cell stress) treated cells. Subsequently, these proteins were subjected to in-solution tryptic digestion and the mixture of peptides was separated and identified by LC-MS/MS (liquid chromatography with tandem MS). Interestingly, PCNA was found in the arsenite-treated cells.

Our aim was to identify new ubiquitinated proteins in response to DNA damage through proteomic analysis (Figure 1). We decided to use the UQ1-UBA beads for the purification of ubiquitinated proteins from both untreated and etoposide- (which causes double-stranded DNA breaks) treated HEK-293 cells, which were identified by MS. The proteomic approach has indeed identified many previously unknown ubiquitinated proteins after DNA damage.
Purification of ubiquitinated proteins from DNA-damaged cells with UQ1-UBA beads and identification by MS

DNA damage

Cell lysis

Ubiquitinated protein purification

With UQ1-UBA beads

SDS-PAGE

1D-GE

Mass spectrometry

Protein Identification

Role in DNA damage response

(J. Selvarajah and A. Moumen, unpublished work). As a validation for our analysis we were able to find H2A and H4 proteins in the etoposide-treated sample, and these which are known to be ubiquitinated after DNA damage. Also of interest is that we found many proteins that were found only in the untreated sample, which suggests that the proteins are stabilized by DNA damage and therefore have a role in the DDR.

In conclusion, it is clear from the proteins such as Mdm2 and Rad17 that ubiquitination has an important role in DDR. The availability of UBD peptide beads and the use of proteomic methods in response to various DNA damages and Rad17 that ubiquitination has an important role in DDR suggests that the proteins are stabilized by DNA damage. Also of interest is that we found many proteins and H4 proteins in the etoposide-treated sample, and validation for our analysis we were able to find H2A (J. Selvarajah and A. Moumen, unpublished work). As a method for the purification of ubiquitinated proteins, Proteomics 7, 1016–1022.

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