Regulation of target proteins by small ubiquitin-like modifiers

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1 Post-translational modifications

1.1 Post-translational modifications, DNA damage repair and cancer

In 1953, James Watson and Francis Crick reported the twisted-ladder structure of deoxyribonucleic acid – DNA[1]. Since then, the mechanisms that cells have employed to preserve and transmit the genetic information encoded by DNA have been subject to extensive investigations[2]. DNA carries genetic information used in the development and reproduction of all cellular living organisms. However, the stability of DNA is constantly threatened by all kinds of damages (Figure 1). Unrepaired DNA damage leads to genomic instability, which is a characteristic of most cancers[3-6].

In order to preserve genome integrity, cells are obliged to detect different types of damages and to prevent injury to their genomes by building up an arsenal of repair factors and by employing a number of multi-step and interconnected mechanisms[7]. All those factors and mechanisms are strictly regulated to prevent unessential alterations in DNA structure in a timing controlled, distribution restricted and DNA damage specific manner, to initiate an appropriate repair pathway (Figure 1) and also in the case of irreparable damage, to induce cell cycle arrest or apoptosis[8].

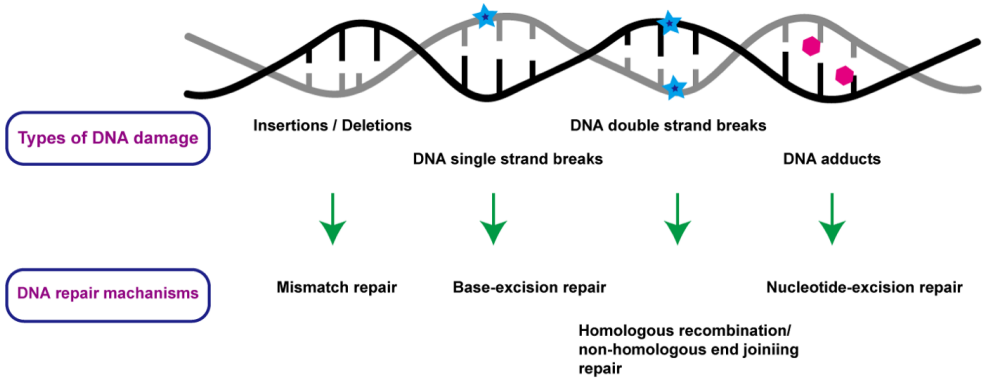

Figure 1. Different types of DNA damages. There are several types of DNA damages due to intrinsic and extrinsic factors and are repaired by damage-specific mechanisms, such as mismatch repair, base/nucleotide-excision repair, or homologous recombination (HR)/non-homologous end-joining (NHEJ).

Among numerous regulatory mechanisms that are available to the cell, rapid, reversible and flexible posttranslational protein modifications (PTMs) that covalently act during or after protein biosynthesis are markedly suited and selectively taken on to fine-tune these regulatory networks[9-11].

PTMs are chemical alterations to amino acids in proteins that reversibly regulate biochemical properties of proteins via specific domains by the addition of a modifying chemical group such as phosphate, acetyl and methyl or by an entire polypeptide including ubiquitin and SUMO (Small Ubiquitin Like Modifier) to one or more of its amino acid residues mainly through enzymatic activities (Figure 2).

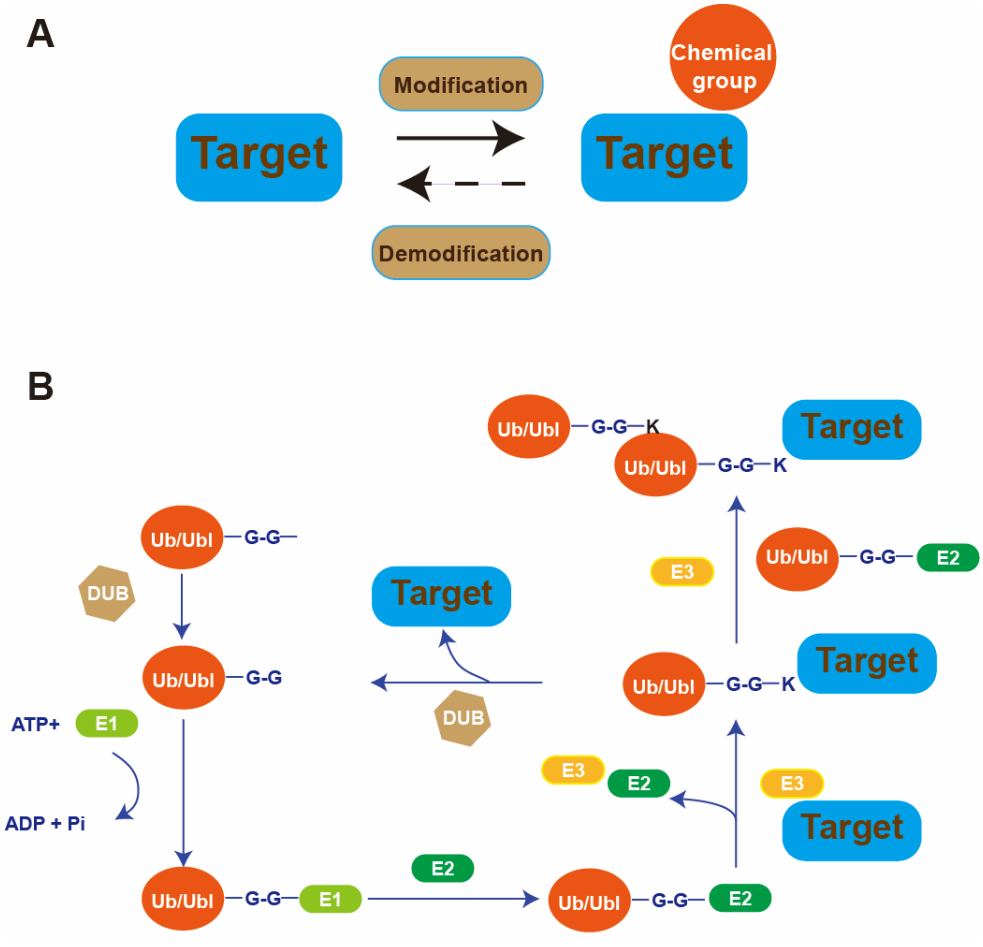

Figure 2. The process of chemical and ubiquitin like (UBL) modifications. A) The reversible process of chemical modifications is regulated via an enzymatic step to modify and de-modify target proteins. B) The reversible process of UBL modification is regulated through a three enzymatic step to modify and de-modify target proteins.

PTMs change the localization, conformation, interactions and stability of their targets proteins and bring functional diversity to them[11,12]. Some PTMs such as glycosylation are stable modifications of the protein to ensure proper folding and stability of newly synthesized proteins[13]. Others like phosphorylation, ubiquitination and ubiquitin-like modifications such as SUMOylation are more dynamic to quickly alter the function of proteins[10,14]. Moreover, different PTMs can act individually and/ or in concert to initiate, terminate and fine-tune different signalling pathways[10].

Alterations and disruption in PTMs pathways have been tightly connected to many types of diseases, especially cancer[12,15-18]. At the same time, regulators of PTMs serve as potential targets for drug development against those diseases. Global identification of protein posttranslational

modifications and intensive research in the biological function of PTMs in cellular processes will open new avenues to advance techniques for diagnosis and therapeutics and therefore are of great importance and an interesting field in basic scientific and drug development research[18-20].

1.2 phosphorylation, Ubiquitination, Ubiquitin-like proteins

1.2.1 Phosphorylation

Phosphorylation is one of the most extensively investigated and abundant PTMs[19,21]. It modifies activities of a large fraction of proteins by adding a phosphoryl group to a target protein in both prokaryotic and eukaryotic cells[22]. Protein phosphorylation is reversible, it requires protein kinases to deposit phosphates and protein phosphatases to remove phosphates from the target proteins[21]. In eukaryotes, phosphorylation normally take place on serine, threonine, tyrosine and histidine residues whereas in prokaryotes it also happens on the arginine and lysine residues[23,24]. Protein phosphorylation regulates key cellular functions such as cell growth, differentiation and apoptosis[25,26].

1.2.2 Ubiquitin and Ubiquitination

Ubiquitin was first identified in eukaryotic cells in 1975 as a widely expressed protein of unknown function[27]. It can be attached via the free carboxyl group of the glycine at its C-terminus to the ϵ-amino group of lysine in its target protein[14,28]. Furthermore, ubiquitin was reported to be able to bind to electron-rich nucleophiles in a protein known as non-canonical ubiquitination[29]. Furthermore, a N-terminal amino group in a protein could also be conjugated to ubiquitin in a similar manner as lysine. This kind of non-canonical ubiquitination was first identified in MyoD, and later on, characterized in ERK3 and p21 by mass spectrometry analysis[30-32]. N-terminal ubiquitination of proteins was further found to target proteins for degradation[32]. Cysteine can also be attached to ubiquitin. It was first reported as another non-canonical ubiquitination in the peroxisomal import factor (Pxp5). Beside thioester bonds, hydroxyester linkages can also be formed between ubiquitin and serines, threonines and tyrosines, which are more stable and suggested as part of the apoptotic pathways[33,34].

Ubiquitination is an enzymatic process that depends on ATP[14,33,35,36]. It involves the enzymatic cascade of three enzymes. They are ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). Ubiquitin is first activated by the ubiquitin-activating enzyme E1 and is then passed on to the active cysteine site of the E2s. The E2s act with E3 ubiquitin ligases to catalyse the finishing stage of the ubiquitination by adding ubiquitin to its targets. Ubiquitin E3 ligases contain either the Homologous to the E6-AP Carboxyl Terminus (HECT) domain or RING-betweenRING-RING (RBR) domain that both transiently bind ubiquitin or the Really Interesting New Gene (RING) domain that catalyses the straight transfer step to add ubiquitin to the substrates from E2 enzymes[28,36,37].

There are three types of ubiquitin modifications; mono-ubiquitination (adding one ubiquitin to one substrate residue), multi-ubiquitination (adding single ubiquitins to multiple substrate residues of one protein) and poly-ubiquitination (forming a ubiquitin chain on a single substrate residue by linking the

C-terminal glycine of a ubiquitin to an inner lysine of ubiquitin) (Figure 3)[38]. There are seven lysine residues in ubiquitin, which are K6 (lysine 6), K11 (lysine 11), K27 (lysine 27), K29 (lysine 29), K33 (lysine 33), K48 (lysine 48) and K63 (lysine 63). While K48-linked chains are the first identified chains that target proteins for proteolytic degradation, K63-linked chains have been well-characterized as not associated with proteasomal degradation. The function of other lysine chains and, mixed chains remain less clear[39]. Targeting proteins for ubiquitin-mediated 26S proteasome degradation is an irreversible choice which needs to be tightly regulated in a specific manner.

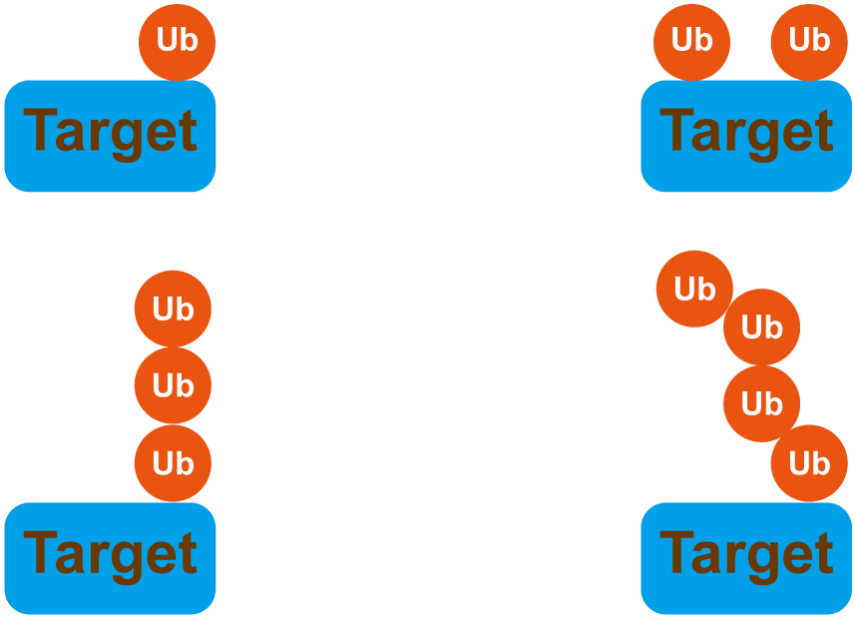

Figure 3. Four types of ubiquitination. mono-ubiquitination is to modify targets via adding one ubiquitin to one substrate residue, multi-ubiquitination is to modify targets via adding single ubiquitin to multiple substrate residues of one protein and poly-ubiquitination is to modify targets via forming a ubiquitin chain on a single substrate residue by linking the C-terminal glycine of a ubiquitin to an inner lysine of ubiquitin, there are two types of poly-ubiquitination, linear ubiquitination or branched ubiquitination.

Moreover, ubiquitin can also be linked linearly (the carboxyl group of glycine of one ubiquitin molecule linked to the amino group of methionine of another ubiquitin) (Figure 3). Linear ubiquitin chains are now known to be catalysed by two RING type E3 ligase HOIL1 and HOIP and are important for NF-κB activation and cellular responses to inflammatory cytokines[40,41].

1.2.3 Ubiquitin-like proteins

It is worth noting that although ubiquitin is the first identified and most famous and well-studied protein post-translational modifier, there are other families of proteins identified that are able to modify proteins in an ubiquitin like enzymatic cascade. They share a common β-grasp fold and are known as ubiquitin-like proteins (UBLs)[10,14,42-45].

UBLs identified so far are: Small Ubiquitin-like MOdifier (SUMO)[12,18], ubiquitin fold-modifier-1 (UFM-1)[46], autophagy-8 (ATG8) and -12 (ATG12)[47], ubiquitin-like protein FUBI (FUBI)[48], ubiquitin cross-reactive protein (UCRP or ISG15)[49], ubiquitin-related modifier-1 (URM1)[50], human leukocyte antigen F-associated (FAT10)[51], neuronal-precursor-cell-expressed developmentally downregulated protein-8 (NEDD8)[44], MUB (membrane-anchored UBL)[52] and ubiquitin-like protein-5 (UBL5)[43]. Whilst sequences of those proteins share only moderate homology with ubiquitin, they have similar three-dimensional folds[10,53,54].

1.2.4 UFM-1 and UFMylation

Ubiquitin-fold modifier 1 (UFM-1) is the most recently identified new member of the ubiquitin-like protein family. It was first described in 2004 and is a protein of 9.1 kDa[55]. Although UFM-1 shares little sequence identity to ubiquitin, they have a similar (α-helix and β-sheet) tertiary structure. Unlike ubiquitin functions that are extensively studied, the biological function of UFM-1 remains largely unknown[56].

UFM-1 covalently modifies the lysine residues of its substrates and is attached by an enzymatic cascade analogous to ubiquitination, including E1 (UBA5), E2 (UFC1), and E3(UFL1) enzymes and this process can be reversed by UFM-1 specific proteases[56]. UFM-1 is translated as an inactive precursor form (pro-UFM1) which has two additional amino acids beyond the single active conserved glycine. In human cells, so far the only active UFM-1 specific protease is UFSP2[57]. UFSP2 cleaves the UFM-1 C-terminal part to expose is C-terminal glycine. UFM-1 only possesses a single active glycine at the C-terminus, which is required for the covalent attachment to its target proteins. UFM-1, UBA5, and UFC1 are all conserved in metazoan and plants but not in yeast, suggesting its potential roles in various multi-cellular organisms.

Interestingly, UBA5 can also activate SUMO-2 and transfer SUMO-2 to the nucleus. However loss of UBA5 only affects UFM-1 conjugation[58,59].

UFM-1 and its system have been demonstrated to play a significant role in regulating protein interaction, localization and function[56,60]. It is also suggested that UFM-1 is involved in pathological conditions or diseases, like tumorigenesis[61], ischemic heart diseases[62] and diabetes[61]. However, to date, the biological function of UFM-1 remains poorly understood.

Although currently, several groups have reported their identification of UMF1-targets, such as ASC (activating signal co-integrator 1 or TRIP4)[15] and UFBP1[63] and recently, 494 UFMylated proteins

got identified, of which 82 passed the cut off[64]. None of them have been independently validated and no biological functional studies have been conducted to elucidate the role of protein UFMylation[64].

Previously, uS3, uS10, uL16, subunits of the ribosome have also been identified by mass spectrometry to be novel UFM-1 targets[65], thereby linking UFM-1 to a central biological process.

2 SUMO and SUMOylation

2.1 SUMO

Small Ubiquitin-related Modifier (SUMO) was first found as a Small Ubiquitin like MOdifier during the 1990s when several other ubiquitin-like proteins were identified[66,67]. It is a small protein of 12 kDa and is about 100 amino acids in length (depending on which organism the protein comes from and depending on the identity of the SUMO family member). Although SUMO strongly resembles ubiquitin with a similar structural protein fold and its ability to form chains, they share little sequence similarity with each other at the amino acid level. All SUMO proteins are translated into immature precursors whose C termini need to be removed by SUMO proteases (as described later in more detail) to expose the di-glycine (GG) motif. Mature SUMO is then ready for entering the SUMOylation cycle and conjugation to targets. SUMO often does not direct proteins for proteasomal degradation[68].

In budding yeast, a single form of SUMO protein exists, encoded by the SMT3 gene, which is essential [69]. In contrast there are three functional genes that can be translated to three SUMO proteins in mammalian cells, which are SUMO-1, 2, 3. SUMO-1 is a highly conserved small ubiquitin like protein that has been first identified as modifier of Ran GTPase-Activating Protein 1 (RanGAP1) at its lysine 526 residue[66,67]. Later on, two homologous coding sequences have been isolated and sequenced from mouse and human cDNA libraries and they are referred to as SUMO-2 (SMT3A) and SUMO-3 (SMT3B)[70].

SUMO-1 shares only about 50% amino acid sequence similarity with SUMO-2 and SUMO-3. SUMO-2 and SUMO-3 are almost indistinguishable[71]. They share a similarity of about 97% with each other in humans and cannot be distinguished by antibodies. Thus, they are grouped together into a subfamily named SUMO-2/3[68].

SUMO-1 and SUMO-2/3 were found to be conjugated to substrates under physiological conditions[72]. SUMO-2/3 conjugation can be further induced by cellular stresses[73]. The overall cellular levels of SUMO-1 is much lower than that of SUMO -2/3. Although mice are viable after SUMO-1 and SUMO-3 knockout, SUMO-2 deficient mice die during the embryonic period[71,72,74]. There is an internal SUMOylation site at lysine 11 of SUMO-2 and SUMO-3 and therefore they can form poly-SUMO chains, however, there is no internal SUMOylation site in SUMO-1. Although SUMO-1 cannot form poly-SUMO chains, the C-terminus of it can be linked to SUMO-2 or SUMO-3 which ends further chain elongation[75].

While identifying specific genes that are related to susceptibility to type 1 diabetes (T1D), the SUMO-4 gene was cloned[76]. SUMO-4 shows similarity to SUMO-2/3, but differs in having a proline instead of a glutamine at position 90. As a result, SUMO-4 can only be conjugated under stress-conditions like starvation. SUMO-4 was shown to be conjugated to IκBα and negatively regulated NFκB transcriptional activity[76]. However, no unique endogenous SUMO-4 peptides have been found, thus true evidence for SUMO-4 at the endogenous proteome level by mass spectrometry is missing and further evidence that SUMO-4 can be translated into protein needs to be provided.

SUMO-1, 2, and 3 are mainly localized in the nucleus and were shown to covalently attach to many kinds of proteins to regulate their interaction, stability, localization and function, not only under physiological conditions but also in response to cellular stresses[68]. However, our understanding of the role that SUMOs are playing in cells is quite limited due to the fact that the stoichiometry of SUMOylation is very low, which makes it very challenging to be detected[77].

SUMOs are mainly attached to nuclear proteins in a redundant manner. Consequently, mutating one single SUMO acceptor lysine in a SUMO target protein often has no clear physiologic effect. Many studies support the concept of protein group modification, thus a pre-enrichment and a proteomic wide analysis of SUMO targets and a group modification view of them are key to understand SUMO signaling transduction[73,78-83].

2.2 SUMOylation

Resembling ubiquitination, SUMOs can also be attached to targets through sequential activity of three well conserved enzymes, involving a SUMO E1 activating enzyme, a SUMO E2 conjugating enzyme and SUMO E3 ligases[10]. SUMOylation is an energy consuming process that depends on ATP[68,77,84,85].

All eukaryotic SUMO proteins are initially synthesized in an immature form that cannot be conjugated to targets and need to be further processed by SUMO proteases[86,87]. SUMO proteases remove some C-terminal residues to expose two glycine residues that can be subsequently conjugated to a lysine residue on the target protein. Mature SUMO is activated by forming a thioester bond with the catalytic cysteine in the E1 enzyme. Consequently, SUMO is relocated from E1 to the catalytic cysteine of a single E2 conjugating enzyme-UBC9. SUMO E3 ligases catalyse the final step efficiently by transferring SUMO to the acceptor lysine residues of target proteins. The isopeptide bond between SUMO and its target proteins can be cleaved by SUMO proteases. SUMOylation modifies a single or multiple lysines within target proteins (mono-SUMOylation or multi-SUMOylation) and SUMO can also modify itself on its lysines (poly-SUMOylation). SUMO proteases balance the free and conjugated SUMO pool and regulate transient, dynamic and reversible SUMOylation in the cell[68,88].

2.3 SUMO machinery

2.3.1 SUMO E1 activating enzymes

The SUMO activating enzyme (E1) was first identified in 1997 as activator of Smt3p in budding yeast to induce its conjugation to other proteins. The E1 is found in the form of a heterodimeric enzyme consisting of Aos1p (activation of Smt2p)/Uba2p (Ubiquitin Activating enzyme E1-like)[69,89]. Later on, the SUMO-1 activating enzyme was purified from human cells and shown to be a heterodimer consisting of SAE1/SAE2 (SUMO activating enzyme), homologous to Aos1p/Uba2p. SAE1 was able to catalyse the thioester formation between SUMO-1 and SAE2, in an ATP-dependent manner[90].

2.3.2 SUMO E2 conjugating enzyme

In contrast to many E2 conjugating enzymes that regulate ubiquitination, in human cells, there is one unique SUMO E2 conjugating enzyme characterized – UBC9, that plays an essential role in SUMOylation[89,91].

Activated SUMOs can be transferred from a reactive cysteine residue in SAE2 to UBC9 on its active cysteine to form a SUMO-UBC9 thioester complex[92]. It is believed that UBC9 directly selects SUMO target proteins. Many of these targets carry a SUMOylation consensus site (ψKxE, ψ: hydrophobic amino acid and x: any amino acid) to which UBC9 can bind directly, although with low affinity. In vitro, UBC9 binds to the SUMOylation consensus site and this is adequate to conjugate SUMO to this lysine residue of the substrate. However in vivo, this process might be facilitated by SUMO E3 ligases[93,94].

Mouse embryos deficient for UBC9 die at the early post-implantation stage[95]. UBC9 deficient blastocysts are still viable and can be further cultured for at most two days. Without UBC9, inner cell mass (ICM) regression happens due to increased apoptosis, supporting the pivotal role of SUMOylation for the ICM development during the post-implantation phase of embryogenesis[95].

2.3.3 SUMO E3 ligases

SUMO E3 ligases catalytically transfer SUMOs from the E2 conjugating ligase UBC9 to lysine residues within target proteins and determine the efficiency of SUMOylation and specificity[96]. SUMO E3 ligase activity has been demonstrated for several types of proteins. The most convincing and conserved SUMO E3 ligases are Siz/PIAS (SP) proteins. These SP E3 ligases contain only one catalytically C3HC4 domain that resembles the RING finger domain of ubiquitin E3 ligases and an adjacent SP C-terminus domain that does not coordinate a Zn^{2+} ion[97-99]. Siz1/2 was first characterized as an E3-like factor in the SUMO pathway by attachment of SUMO to septins in vivo in *S. cerevisiae*. Pli1 is another SP E3 ligase in fission yeast[100-103].

In mammals, protein inhibitor of activated STAT PIASy (PIAS4) was first reported as a novel SUMO E3 ligase that remarkably stimulates LEF1 SUMOylation[97]. In total, there are four PIAS family members, PIAS1, PIAS2 (PIASx), PIAS3 and PIAS4 (PIASy). They were identified as inhibitors of the

JAK-STAT signalling pathways. In addition, the Mms21 (the methyl methanesulphonate-sensitivity protein) (NSMCE2 in humans and Nse2 in yeast) also bears an SP-RING domain and contributes to SUMOylation of proteins such as Scc1[104].

The Ran binding protein 2 (RanBP2), which is a nuclear pore complex protein is regarded as another type of SUMO E3 ligase. It interacts with SUMOylated RanGAP1 and forms a stable complex that localized at kinetochores and the mitotic spindle. It can also tightly bind UBC9 and mediates SUMOylation of Topoisomerase 2α (Top2α) and is required for Top2α localization at mitotic chromosomes[105-107].

Furthermore, there are some unexplored SUMO E3 ligases such as ZNF451 family members and Polycomb group (PcG) Chromobox Protein Homolog 4 (CBX4) that show high specificity for SUMO, but their function needs to be further studied[108].

2.3.4 SUMO proteases

SUMO proteases precisely cleave between the SUMO C-terminal glycine and the SUMO substrate lysine or depolymerize poly-SUMO chains. Some SUMO proteases also process SUMO precursors by recognizing SUMO precursors and cleaving C-terminal residues to expose the di-glycine motif and therefore affect SUMO conjugation indirectly[87].

Ulp1 (UBL-specific protease 1) was the first identified SUMO protease in *S. cerevisiae*. Later on through comparing the catalytic domain sequence of Ulp1 to sequence databases, Ulp2 was also identified in *S. cerevisiae* together with many other putative SUMO proteases in other species[109-111]. In human cells, the first confirmed SUMO protease was sentrin-specific protease 1 (SENP1). Through extensive database searches, other putative human SENPs were identified. They are SENP1, 2, 3 and SENP5, 6, 7[ref. 112].

Further investigation showed that SUMO proteases possess substrate specificity. In yeast Ulp1 is not only responsible for SUMO precursor maturation, it is also involved in removing SUMOs from protein substrates. Ulp2 cleave isopeptide linkages between SUMO and substrates but it also possesses high activity to cleave SUMO–SUMO linkages in poly-SUMO chains[110,113]. In human cells, although not well understood, SENP1 shows a preference for deconjugating SUMO-1, SENP2 could efficiently processes SUMO-2 and SUMO-3 precursors, SENP5 has significant activity to maturate SUMO-3 precursor but has no or limited activity towards SUMO-1. SENP6, 7 are not responsible for SUMO precursor maturation, but have a preference to process SUMO chains[87].

DESI1 is another known SUMO protease that has been identified in yeast two-hybrid screening while searching for partners of BZEL. BZEL is a transcriptional repressor that is expressed in effector lymphocytes and binds to the promoter of target genes like blimp-1. DESI1 has SUMO-1, 2, and 3 deconjugation activity from BZEL as well as deconjugation of poly-SUMO-2/3 chains[86,114].

USPL1 (Ubiquitin-specific protease-like 1) was identified as a protein that can cleave SUMO from its targets. Overexpression of USPL1 caused loss of SUMO-2/3 conjugates but not SUMO-1, which

identifies it as a SUMO protease specifically for SUMO-2/3. Knock down of USPL1 does not cause accumulation of SUMO conjugates, but does show impaired cell growth which can be attenuated by the non-catalytic function of USPL1[ref. 115].

3 Cellular roles of SUMOylation in DNA damage repair

Factors that cause alteration of the chemical structure of DNA can be called DNA damaging agents. DNA damage can be produced by exogenous agents like alkylating agents including methyl methanesulfonate (MMS), crosslinking agents such as mitomycin C, cisplatin and deoxyribonucleotide pool depleting agents including hydroxyurea (HU) or physical factors such as ionizing radiation and ultraviolet light[116-118]. Endogenous processes during DNA metabolism can also generate DNA damage, for example DNA base loss during hydrolysis caused by spontaneous DNA depurination, oxidized DNA bases caused by reactive oxygen species (ROS), a natural byproduct derived from normal metabolism of oxygen, dNTP mis-incorporation during DNA replication and replication fork stalling or collapse caused by DNA replication stress. All these endogenous processes let cells experience huge numbers of spontaneous DNA lesions per day[119].

To counteract DNA damage, eukaryotic cells have developed well-coordinated defence mechanisms to recruit and activate specific factors in the right place throughout different cell cycle phases[120]. Repair systems for specific types of lesion have been adopted (Figure 1)[116,121]. DNA mismatch repair is employed to recognize and replace mis-paired DNA bases[122,123]. Base excision repair is used to remove specific non-bulky chemical alterations of DNA bases[124]. Nucleotide excision repair is introduced to cut a short single-stranded DNA segment that contains a lesion[125,126]. With the assistance of protein networks involved in Fanconi Anemia protein clusters and many endonucleases, intrastrand crosslink repair pathway is used to excise intrastrand crosslinks, non-homologous end joining (NHEJ) as well as homologous recombination (HR) can work together in different cell phases to promote the repair of the most hazardous damage - DNA double strand breaks (DSBs)[127-129].

3.1 DNA Replication stress derived damage and repair

The progress of replication forks is strongly impeded by different types of DNA damage such as DNA lesions, DNA-protein complexes, secondary DNA structures, an RNA–DNA hybrid and DNA-DNA crosslinks which are usually considered as side products produced during DNA replication progression. This kind of DNA damage can be repaired by fine-tuned reactions mentioned above. The response to replication stress derived DNA damage is an interesting area for further research because the instability of DNA replication is one of the hallmarks of cancer and confers genetic diversity during tumorigenesis.

Accurate and complete DNA replication is essential for accurate transmission of genetic information and the maintenance of genomic stability, it requires numerous factors including an ample pool of nucleotides (dNTPs), complete components of the replication machinery, adequate active replication origins, histones and histone chaperones[127,130].

As mentioned, faithful completion of DNA replication is under stress caused by spontaneous and exogenous insults[131]. A wide variety of obstacles can hamper DNA replication and lead to replication stress. These obstacles include DNA lesions, DNA-protein complexes and secondary DNA structures. Some of these obstacles occur during S phase and will consequently slow down or stop replication fork progression by impeding the capacity of replication polymerases and replication helicases. If those obstacles are not removed, mutations arising from DNA damage will be transmitted during mitosis and consequently can lead to developmental abnormalities and cancer[132].

Once faced with obstacles that specifically associate with DNA replication, the progression of the DNA polymerase is affected and consequently uncoupled from DNA templates. However DNA replicative helicases sometimes continue to unwind the parental DNA and lead to the formation of single-strand DNA (ssDNA)[133,134]. In addition, due to cyclin dependent kinase (CDK) controlled resection at S phase and G2 phase, single-stranded DNA (ssDNA) intermediates also form through exonuclease resection of damaged DNA structures such as DSB[135]. The persistence of ssDNA is recognized by the cell cycle checkpoint machinery as a signal of DNA damage that needs to be repaired (Figure 4).

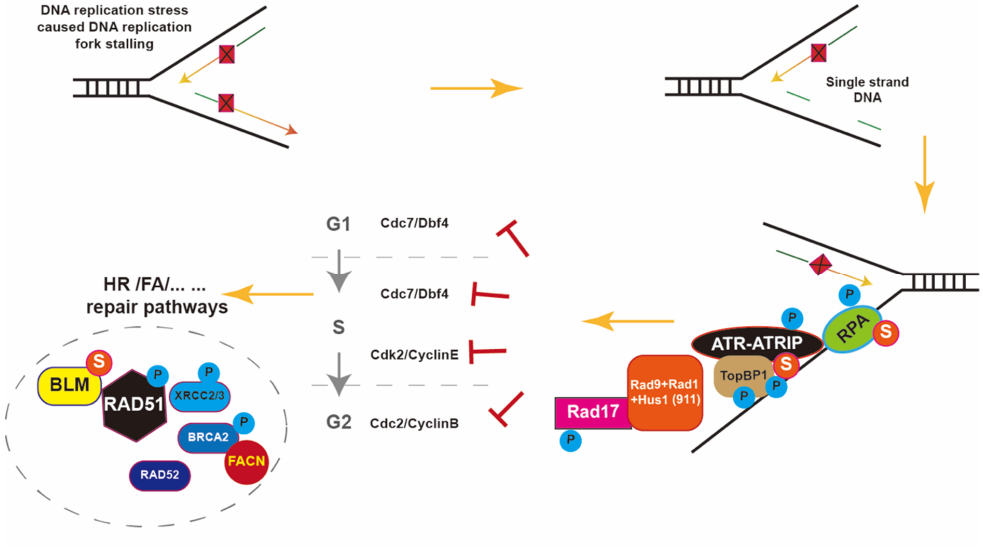

Figure 4. DNA replication fork stalling caused DNA damage and repair. General scheme depicting phosphorylated components that cells have selected to fine-tune regulatory networks during DNA replication fork stalling, to mediate cell-cycle arrest, to slowdown the replication progression and save time for proper DNA damage repair.

Prolonged ssDNA will be coated by replication protein A (RPA) consisting of RPA14, 32 and 70[136]. RPA coated ssDNA not only prevent ssDNA from degradation or DNA secondary structures

formation but serves as a signaling platform to recruit and activate downstream response factors, such as the protein kinase ataxia-telangiectasia mutated and Rad3 related (ATR) and its interaction partner ATRIP (ATR interacting protein). ATR is a phosphatidylinositol 3 kinase like kinase which is defect in ataxia telangiectasia, it acts in many forms of DNA damage including stalled DNA replication forks[134,137,138].

Cells will employ a host of mechanisms to mediate cell-cycle arrest, to slowdown the replication progression, to prevent cells from going into mitosis and to save time to repair damaged DNA. Once damage has been repaired, arrested cells re-enter into cell-cycle progression, otherwise cells are stuck in a permanent cell-cycle arrest or undergo apoptosis.

3.1.1 Checkpoint signaling pathways

Checkpoints are cellular surveillance mechanisms. Upon DNA replication stalling, RPA coated ssDNA activates the check point kinase ATR. Activated ATR serves as a central replication stress response kinase to phosphorylate a large number of downstream targets such as the effector kinase CHK1 (checkpoint kinase 1), with the help of several replication checkpoint proteins such as RAD9, RAD17, TOPBP1, BRCA1 and claspin[139,140].

Activated CHK1 phosphorylates downstream cell cycle phosphatases, including CDC25-A, -B and -C. Phosphorylated CDC25-A triggers the signal for its degradation by the proteasome and results in inactivated cyclin E/A-CDK2 and cyclin B-CDK1 and arrest of cell cycle progression. Chk1 activation in response to DNA replication fork stalling is thought to be sufficient for Cdc25A degradation, to suppress late origin firing, to save time for the DNA damage repair and consequently to ensure the completion of DNA replication under stress or induce apoptosis[141-143].

Although the ATM checkpoint kinase (ataxia-telangiectasia mutated) is regarded as less imperative for the response to replication blocks than ATR, it can still slow DNA replication progression in response to DNA damage caused DNA replication fork stalling[144]. The MRN (MRE11 (meiotic recombination 11)–RAD50–NBS1) complex together with 53BP1, MDC1 and other mediator proteins could sufficiently activate ATM. Activated ATM phosphorylates its effector kinase CHK2. CHK2 can also phosphorylates serine 123 in Cdc25A. Phosphorylation of Cdc25A serves as a signal for the ubiquitin machinery and targets Cdc25A for degradation, inactivating cyclin A-CDK2 and inhibiting further firing of early origins of replication during S phase[145,146].

3.1.2 Action of the ATR pathway

ATR plays a vital role in protecting replication forks from stalling. ATR activation needs interactions between the ATR/ATRIP complex and other proteins that contain AADs (ATR-activating domains).

So far in humans, TopBP1 is the only AAD containing protein that is found to be responsible for ATR/ATRIP activation[147]. The 9-1-1 (Rad9-Rad1-Hus1) complex is independently recruited to ssDNA. TopBP1 binds the C-term of Rad9 and RHINO and stimulates ATR kinase activity through its AAD.

Additionally, Rad17, RAD9 and the MRN (MRE11-RAD50-NBS1) complex also act to recruit TopBP1 to RPA-ssDNA[148,149].

Upon ATR auto-phosphorylation and activation, it phosphorylates a variety of substrates. One substrate of ATR that is being phosphorylated upon DNA damage is SMARCAL1. With the help of SLX4 associated nucleases, phosphorylated SMARCAL1 prevents aberrant fork structures to result in double strand break formation. Other ATR substrates include Chk1, FANCI and Polη, RPA, MCMs, WRN and some others[150-152].

ATR phosphorylates Chk1 at S317 and S345. Chk1 not only controls cell cycle arrest as described before but also regulates replication fork progression and replication related DNA damage. ATR phosphorylation of FANCI plays a vital role in the FANCD2-FANCI complex location to the DNA damage sites and restarting DNA replication forks at intrastrand cross-links[153]. Polη is also an important ATR substrate that plays its role in translesion synthesis repair[154].

3.1.3 Homologous Recombination repair during DNA replication.

HR (Homologous Recombination) not only mediates genetic recombination in meiosis, HR can also repair DNA double strand breaks, interstrand crosslinks and stalled or damaged replication forks[155]. Its activity is strictly controlled by CDKs during S and G2 phase of the cell cycle and uses sister chromatids in the genome produced by DNA replication as repair templates.

Homologous recombination is initiated by the binding of the MRN (MRE11-RAD50-NBS1) complex in humans in the case of DNA double strand breaks, subsequently initiating the nucleolytic processing at the DNA break site, with the help of other endonuclease such as BLM, human EXO1 (exonuclease 1) resects DNA ends and generates ssDNA[156,157].

Once DNA ends are resected, RPA binds ssDNA efficiently to generated RPA coated ssDNA. This melted DNA secondary structure facilitates the loading of recombinase RAD51, which is mediated by a large number of RAD51 interacting proteins like XRCC2, XRCC3, BRCA2 and RAD52 (Figure 4)[155,158].

BRCA2 interacts with the FA protein FANCN and promote RAD51-mediated D-loop formation and the second DNA end is used to generate a Holliday junction. During the last step of DNA repair synthesis, the remaining HJ structures are dissolved by a complex of BLM (Bloom syndrome, RecQ helicase-like), TOPIIIa and hRMI1 to form a non-crossover[155,159].

3.2 Cellular roles of SUMO in DNA damage response pathways.

SUMOylation regulates almost all kinds of fundamental nuclear functions, ranging from protein degradation, DNA damage response, DNA replication, transcription, cell cycle checkpoint control, signal transduction to chromatin organization, ribosome biogenesis, nuclear trafficking, and pre-mRNA splicing. This thesis focusses on the role of SUMO in DNA damage response pathways.

3.2.1 SUMOylation of RPA

Replication protein A (RPA) is a protein that binds to single-stranded DNA (ssDNA). It was shown to be regulated by SUMO in DNA damage response pathways both in yeast and in human cells. In yeast through SUMO pull down under denaturing conditions, SUMOylation of Rfa1 (RPA1 homolog) can be detected upon treatment with the alkylating agent methyl-methanesulfonate (MMS)[78].

In human cells, RPA consists of three subunits, RPA1, 2, 3. The largest subunit RPA1 was shown to stably associate with SENP6. After Hydroxyurea treatment or UV irradiation, which efficiently caused replication fork stoppage, RPA1 did not dissociate from SENP6[160]. However, SENP6 depletion caused the accumulation of SUMOylated RPA and SUMOylation of RPA increased its interaction with RAD51, promoted RAD51 foci formation and promoted homologous repair. Further study revealed that there are two SUMO sites within RPA, lysine 449 and lysine 577. Lysine 449 was modified by a poly-SUMO chain and lysine 577 was only mono-SUMOylated[161].

3.2.2 SUMOylation of ATRIP

Ataxia telangiectasia and Rad3 related (ATR) - interacting protein (ATRIP) is a protein involved in checkpoint signalling after DNA damage. ATRIP is SUMOylated at lysine 234 and lysine 289[162]. In UBC9 depleted cells, ATRIP SUMOylation was drastically reduced and failed to be recruited to the DNA damage site. Furthermore, SUMOylation of ATRIP serves as a glue for the interaction of multiple functional partners such as ATR, TopBP1, RPA and MRN in the ATR pathway and it is indispensable for activation of ATR signalling. Moreover, it is reported that the maintenance of ATR basal kinase activity upon DNA damage requires intact activity of the SUMO E3 ligase PIAS3[163].

3.2.3 SUMOylation of BLM

BLM is an ATP-dependent helicase that acts on DNA replication stress to ensure homologous recombination[164,165]. It belongs to the RecQ family. It is reported that two SUMO sites exist in BLM which are lysine 317 and lysine 331. SUMOylated BLM can bind RAD51 and regulates the recruitment of RAD51 at stalled replication forks. BLM can also regulate the accumulation of RPA at stalled replication forks and limits the generation of ssDNA[166].

3.2.4 SUMOylation of SLX4

The SLX4 Fanconi anemia protein is a tumor suppressor and acts as a scaffold for nucleases, it plays an important role in the maintenance of genomic stability[167]. The increased SUMOylation of SLX4 can be visualized during S and G2 phases and is decreased when the cell cycle is completed. It contains three SIMs that are needed for both SLX4 SUMOylation and its binding to SUMO-2. The SIM domains were also required for the localization of SLX4 to PML nuclear bodies and for proper interstrand DNA crosslink repair. Furthermore, SLX4 has been proposed as an E3 ligase that can SUMOylate itself and the XPF-ERCC1 endonuclease[168,169].

3.2.5 SUMOylation of BRCA1 and 53BP1

BRCA1 is a well-known ubiquitin E3 ligase and breast cancer tumor suppressor. It plays a vital role in the maintenances of genomic stability. It is poorly accumulated at the DNA damage foci in the absence of the SUMO E3 ligases PIAS1 and PIAS4, which means that BRCA1 is a SUMO regulated ubiquitin ligase. Moreover, BRCA1 is SUMOylated by PIAS1 and PIAS4 by both SUMO-1 and SUMO-2/3 in a DNA damage dependent way[170].

53BP1 is a DNA damage checkpoint protein that is SUMOylated upon DNA damage. 53BP1 co-localized with SUMO-1 at nuclear foci after four hours of ionizing radiation treatment. 53BP1 depletion only impaired SUMO-1 accumulation in DNA damaged laser tracks. Both PIAS4 and UBC9 depletion impaired 53BP1 accumulation at DNA damage sites[98].

3.2.6 SUMOylation of MDC1

Mediator of DNA damage checkpoint 1 (MDC1) is a central protein involved in the regulation of checkpoint activation and subsequent DNA repair following DNA damage. It mediates cell cycle arrest in response to DNA damage during S phase and G2/M phases of the cell cycle. Once MDC1 is recruited to DNA damage sites, it is SUMOylated by SUMO-1 or SUMO-2/3 at lysine 1840. PIAS4 is the major E3 ligase for MDC1 SUMOylation. To facilitate the DNA damage response, SUMOylated MDC1 can be further ubiquitinated and directed to 26S proteasome by the STUbL RNF4[171].

4 Cross talk among PTMs with focus on SUMO

In addition to modification by SUMO, other post translational modifications such as phosphorylation and ubiquitination can also collaborate and influence each other to fully control protein activity (Figure 5).

Figure 5. Crosstalk between post-translational modification (PTM). PTMs are able to regulated the function of targets not only by individual PTM types and sites but also by cooperatively action.

4.1 Cross talk between SUMOylation and Phosphorylation

More than ten years ago, a highly conserved PDSM (phosphorylation dependent SUMOylation motif) was discovered. The PDSM contains a SUMO consensus site ψKxE and a downstream proline directed phosphorylation site (ψKxExxSP), which regulates the SUMOylation of a substrate. Within this consensus motif, phosphorylation close to SUMOylation sites could positively regulate SUMOylation of several substrates[172]. Of the 46 human proteins that were found containing a PDSM, 71% are transcriptional regulators, such as heat-shock factors (HSFs) and the estrogen-related receptor nuclear receptors.

Later on, the negatively charged amino acid - dependent SUMOylation motif (NDSM) was also proposed to promote substrate SUMOylation. The acidic residues adjacent to the core SUMO motif and the negative charge character of the downstream amino acid residues are required to maintain SUMOylation levels of several target proteins[173].

4.2 Cross talk between SUMOylation and Ubiquitination

Published studies indicated that SUMO and ubiquitin can collaborate or counteract each other. Mixed SUMO and ubiquitin chains have been identified[84,174-176]. Recently, a combined immune-affinity enrichment approach was used to determine the crosstalk between SUMOylation and ubiquitination[177,178].

4.2.1 STUBLs

Research on SUMO-targeted ubiquitin ligases (STUbLs) highlights connections between SUMOylation and ubiquitination. STUbLs are a subset of ubiquitin E3 ligases that contain SUMO interaction motifs (SIMs) to interact with SUMO, and a RING finger domain to catalyse direct transfer of ubiquitin from the E2 conjugating enzyme to the substrates (Figure 6). STUbLs recognize and specifically ubiquitinate SUMO conjugates[179-181].

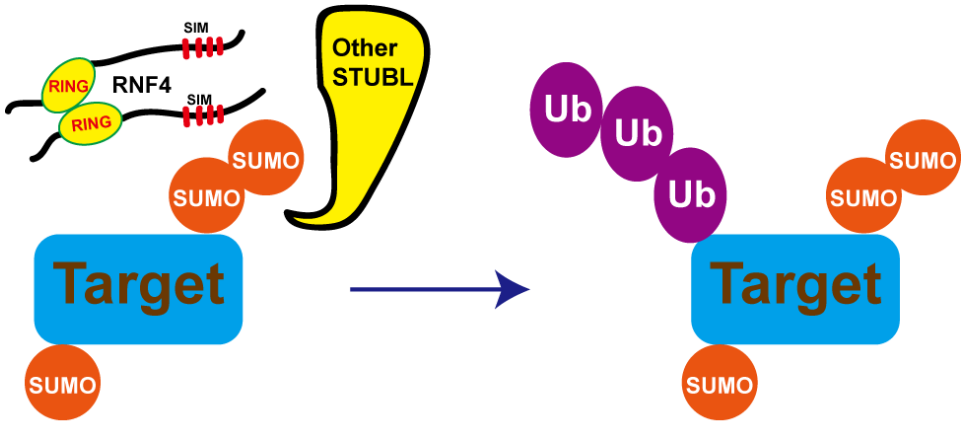

Figure 6. SUMO-targeted ubiquitin ligases (STUbLs). STUbLs recognize and ubiquitinate SUMOylated proteins to regulate their functions and therefore link SUMO modification to the ubiquitin/proteasome system**.**

4.2.2 SUMO-interacting motifs (SIMs)

SUMO-interacting motifs (SIMs) were first identified in many SUMO-1 interacting proteins in yeast through yeast two-hybrid screening[182,183]. Later on, this motif has been found in a wide range of proteins, including SUMO-binding proteins, SUMO substrates, SUMO enzymes and STUbLs. SIMs are generally characterized as a motif composed of hydrophobic amino acids ((V/I)X(V/I)(V/I)) and flanking acidic residues. This motif could bind SUMOs and mediate non-covalent interactions between SUMOs and SIM-containing proteins[184].

4.2.3 STUbLs in yeast

The first STUbL identified in yeast via two hybrid interaction screening is Uls1. Uls1 contains four predicted SIMs in its N-terminus and a RING finger domain in its C-terminus. Mutant strains lacking Uls1 show accumulation of SUMO conjugates but efficient ubiquitination of SUMOylated conjugates by Uls1 has not been reported yet[185,186]. Uls2 was identified as a second STUbL in yeast via two hybrid interaction with SUMO. It is a heterodimer consisting of two RING finger proteins Slx5 and Slx8. Mutations in either of these proteins were lethal and affected the levels of SUMO conjugates. Genetic data showed the connection of Uls1 and Uls2 with DNA damage repair and genome stability[187].

In budding yeast, Rad18 was also identified as a STUbl that can ubiquitinate SUMOylated proliferating cell nuclear antigen (PCNA)[188]. PCNA works as a sliding clamp on DNA and attracts and tethers replicative polymerases during DNA replication. It also plays its role in the DNA damage response. Rad18 contains only one SIM with a specificity for mono-SUMOylated conjugates, which is absent from human Rad18.

4.2.4 STUbLs in human

4.2.4.1 RNF4 and RNF4 substrates

The human RNF4 protein was the first identified STUbl in mammalian cells. It resides predominantly in the nucleus and contains only 190 amino acids with four potential SIMs, among which the C-terminal three are functional SIMs that can recognize poly SUMO chains (Figure 6)[189]. In the presence of SUMO chains, RNF4 is activated through dimerization. Activated RNF4 binds the UBCH4/5 family of E2 enzymes and directs the ubiquitination machinery to SUMOylated proteins and thereby promotes the ubiquitination of SUMO conjugates. *In vitro* experiments showed that RNF4 can also cooperate with the ubiquitin E2 UBC13-UEV1 and synthesize K63 linked chains on its substrate. Furthermore, RNF4 has the ability to rescue yeast cells deficient for Slx5/8, demonstrating the functional conservation in STUbls among eukaryotes[190].

The promyelocytic leukemia protein (PML) together with its oncogenic fusion product PML-RARα were identified as the first substrates of RNF4[191]. In the presence of arsenic trioxide, PML is degraded in a SUMO-dependent manner by the 26S proteasome. RNF4 was shown to promote K48 linked ubiquitin chain formation on PML, leading to the recognition and degradation of PML by the 26S proteasome. In cell culture RNF4 was also reported to disrupt PML nuclear bodies (PML-NBs) in cells treated with arsenic trioxide[192].

RNF4 was furthermore proposed to target SUMOylated RPA for proteasomal degradation based on the fact that in RNF4 depleted cells, RPA persists at the DNA damage lesion[193]. It was proposed that RNF4 mediates RPA degradation to promote the exchange of RPA for RAD51 on ssDNA. However, direct ubiquitination of SUMOylated RPA1 by RNF4 needs to be demonstrated[194].

Kinetochore protein CENP-I has also been reported as a target for RNF4. In SENP6 depleted cells, the SUMOylated levels of CENP-I were increased when RNF4 was co-depleted. In RNF4 depleted cells as well as in cells where 26S proteasome activity was blocked, SUMOylated CENP-I also accumulated[195].

The tumor suppressor proteins FANCI and FANCD2 (ID complex) are the central components of the Fanconi Anemia (FA) pathway. They are mono-ubiquitinated to enable nuclease recruitment and this mono-ubiquitination will promote their loading onto the chromatin. PIAS1 and PIAS4 can SUMOylate the chromatin-loaded ID complex which is then recognized and polyubiquitinated by RNF4[196].

MDC1 and BRCA1 have also been identified as SUMOylated RNF4 targets relevant for genome stability[194]. SUMOylation of MDC1 and BRCA1 were increased upon exposure of cells to ionizing radiation and knocking down RNF4 increased the amount of SUMOylated MDC1 and BRCA1[197]. Additionally, RNF4 regulates the degradation of the histone demethylase JARID1B/KDM5B in response to MMS to mediate transcriptional repression[198].

Although there is an increase in RNF4 targets, we are still restricted in our understanding of the role of RNF4 because of limited insight into the RNF4-regulated SUMO target proteins. The task to uncover a comprehensive network of RNF4 regulated SUMO targets is therefore evident.

4.2.4.2 RNF111 and RNF111 substrates

A second mammalian STUbL that has been identified is RNF111/Arkadia[199]. RNF111 contains three adjacent SIMs and shows a similar activity of RNF4 to ubiquitinate PML upon arsenic trioxide treatment. RNF111 and RNF4 cannot form heterodimers and so far they were only known to work independently on PML.

RNF111/Arkadia uses UBC13-MMS2 as its E2 conjugating enzyme and promotes non-proteolytic, K63-linked poly-ubiquitin chains on SUMOylated xeroderma pigmentosum group C (XPC), which is the generic initiator of global genomic nucleotide excision repair (GG-NER)[200].

Ubiquitination by Arkadia regulates the recruitment of XPC and locates XPC to UV induced DNA damage sites, highlighting a fundamental non-proteolytic function of a STUbL, coupling ubiquitination and SUMOylation in response to DNA damage[201].

5 Technical developments in SUMO proteomics

5.1 SUMO proteomic challenges

The process of SUMOylation is essential in nearly all eukaryotic cells, it has been involved in regulating many cellular functions such as DNA damage repair and cell cycle. In order to understand SUMOylation, it is important to decipher networks of SUMO targets in the cell and identify their SUMO acceptor sites. That is why over the last few years, mass spectrometry analyses of SUMO proteomes has attracted much attention and has been advanced greatly[81-83,202-207]. However, due to PTMs including SUMOylation, proteomes become highly complex, which means that sensitive mass spectrometers are required for their analysis.

Moreover, SUMO proteomics analysis is challenging for many reasons. First of all, because of the low stoichiometry of SUMOylation and low abundance of SUMOylated proteins. Although, several SUMOylated proteins such as PML and RanGAP1 are quite stable in their SUMOylated form and the steady state SUMOylation level is large in relation to the total level of protein[105,191,192]. However for the majority of other proteins, only a small proportion of them is SUMOylated and thus their SUMOylated forms are hard to detected[77].

A second challenge is the high activity of SUMO proteases that can cleave SUMO from its target proteins[208,209]. In the cell, SUMO proteases are well controlled. These proteases are quite active in many standard buffers used to lyse cells. Furthermore, there are no specific and effective inhibitors for those SUMO proteases available yet. As a result, when processing cells or tissues under these conditions, SUMOylated proteins are largely de-SUMOylated by active SUMO proteases.

Thirdly, a long C-terminal remnant of SUMO after trypsin digestion remains. In contrast to ubiquitin, which after trypsin digestion leaves only a di-glycine remnant, the SUMO-2/3 remnant is 32 amino acids long and cannot be efficiently analysed by current mass spectrometry[73,210,211].

To address these issues, several approaches have been established and optimized within the last decade to inactivate SUMO proteases and to enrich SUMOylated proteins from cells or tissue extracts (Figure 7). These cells were grown under normal conditions as well as treated with DNA damaging agents or treated otherwise to study SUMOylation dynamics. At the same time, mass spectrometers have improved significantly during recent years. By combining these two factors, this has led to the identification of thousands of SUMO sites within thousands of SUMO conjugates[73,80,82,198,205,211,212].

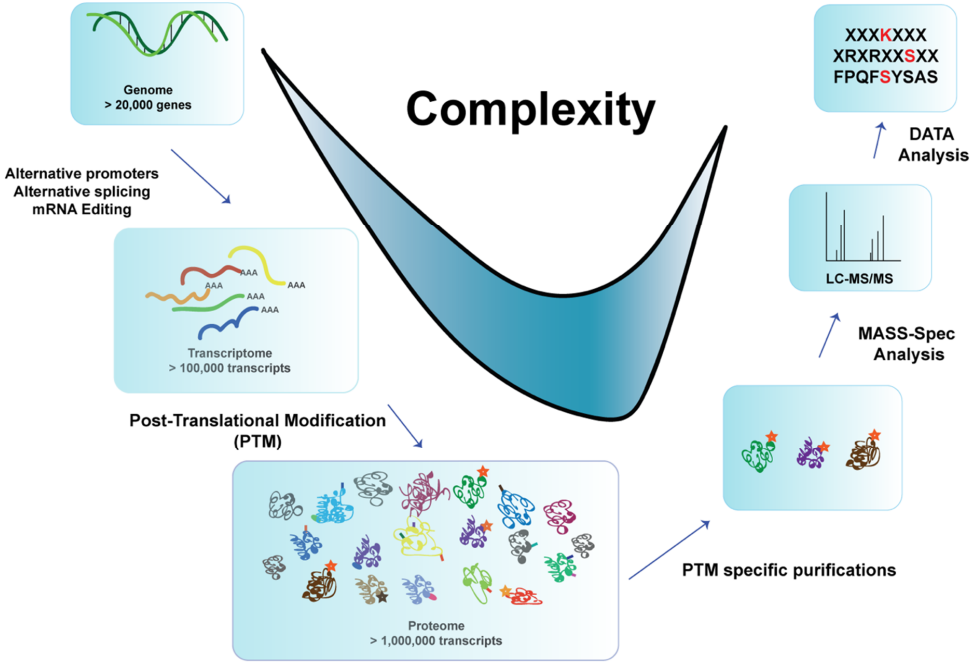

Figure 7. PTM specific purification and identification. Proteomes are much more complex than genomes and transcriptomes due to PTMs. PTM specific approaches have been established and optimized within the last decade to enable their analysis.

5.2 Purifying endogenous SUMO target proteins

The first affinity purification method for the isolation of endogenous poly SUMO conjugates was developed using an RNF4 fragment containing four SIMs. 339 putative endogenous poly SUMO conjugates were identified from HeLa cells by this method after heat shock The method is not efficient for purification of mono-SUMOylated proteins and has a relatively high background due to the non-specific binding of the SIMs[213].

To identify a number of endogenous SUMOylated proteins from more complex organs and tissues, monoclonal antibodies to SUMO-1 and SUMO-2 have been used to highly enrich endogenous SUMO conjugates from HeLa cells and from mouse liver. 584 endogenous SUMO target protein candidates were identified in this manner. However, this method requires very large amounts of starting material and very large amounts of antibodies, which serves as major disadvantages of this method[210].

5.3 Purifying SUMO target proteins using tagged SUMO

To counteract these disadvantages and to get more efficient purification of SUMO targets, SUMO affinity purification was developed by exogenously expressing N-terminally tagged SUMO family

members to purify epitope-tagged SUMO conjugates. These tags including his6, HA, Myc, His6-FLAG and so on[205]. Some tags such as histidine or biotin work well under denaturing conditions where proteases are largely blocked. Other epitope tags such as HA and FLAG can be recognized by specific antibodies with relatively higher affinity compared with SUMO antibodies and work well in more stringent buffers. However, exogenously overexpressing N-terminally tagged SUMO usually increases the number of false positives and can only be performed in cultured cells. Thus, SUMOylated candidates characterized by this methodology should be strictly verified through testing SUMOylation of endogenous proteins identified[79,80,205,211 214].

5.4 Purifying and identifying SUMO acceptor lysines

Even though plentiful SUMO acceptor lysines in target proteins reside within the consensus motif, bioinformatics analysis of protein sequence is far from sufficient to identify SUMO sites[215]. This consensus motif also occurs without evidence for SUMOylation and SUMOylation occurs on non-consensus sequences. Thus, there is a demand for direct identification and characterization of SUMO acceptor lysines by proteomics approaches in SUMO targets purified from cells. However, identification of SUMO acceptor lysines is even more challenging because of the relative low stoichiometry of SUMOylation and the inefficient identification of the large proteolytic C-terminal remnants of SUMOs by mass spectrometry. To overcome these difficulties, epitope tagged SUMOs have been used with an additional proteolytic cleavage site introduced. These SUMO mutants were exogenously expressed in cells. Using this strategy, thousands of SUMO sites have been identified so far. Endogenous identification of SUMO sites is still very challenging[82,203,204,219].

5.5 Proteomic techniques that were used in this thesis

5.5.1 Protein level and Site specific level

In order to study SUMOylation at the protein level, a method has been developed by Ivo Hendriks in our group, employing His10 tagged SUMO-2 (Figure 8)[73]. A major advantage of the His10 tag over the His6 tag is that a higher concentration of competing imidazole can be introduced during the purification procedure as well as rigorous washing procedures to reduce the binding of contaminating proteins.

To be able to identify SUMO conjugates as well as SUMO acceptor lysines, U2OS cell lines that stably expresses His10-tagged SUMO-2 or lysine-deficient His10-SUMO-2 with a conserved mutation at its C-terminal part, Q87R were generated. SUMO-2 conjugates were enriched by Ni-NTA beads. A label free quantitative proteomics approach was used to study SUMOylated proteins in a system-wide manner as well as at the site-specific level.

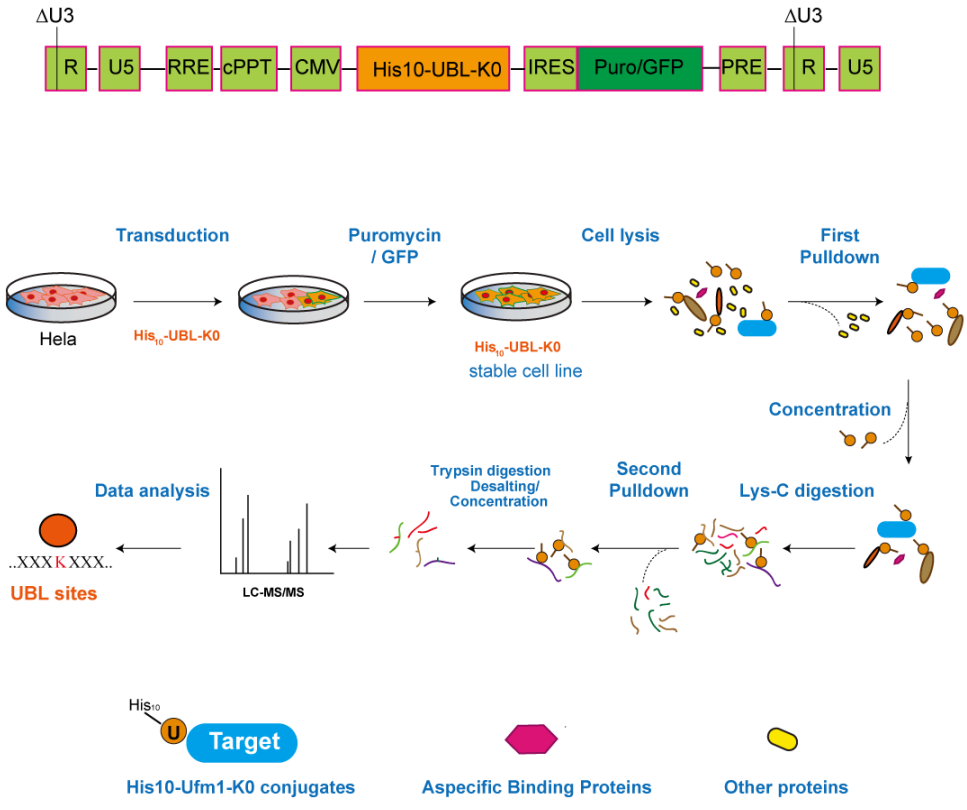

Figure 8. Strategy for identifying UBL modified proteins and acceptor lysines. Cartoon depicting the strategy to identify UBL targets as well as acceptor lysines developed by Alfred Vertegaal lab.

5.5.2 Label-Free Quantitation method

Label-free quantification is a robust strategy in quantitative proteomics that aims to determine the relative amount of proteins in more than one biological sample. Compared with stable isotope labelling, label-free quantification circumvents the use of a stable isotope labeling. Yet, this strategy also has its limitations such as it is relatively high sensitivity to variations in preparing samples and sensitivity to differences in performance of mass spectrometers[202,214].

6 Aim of the thesis

In this thesis, I aimed at decoding the role of SUMO in the DNA damage repair pathway. The SUMO system is believed to be involved in this process at several levels[159,162,168,171,193,196,198]. I focused on the most inevitable DNA obstacle causing DNA replication stress, and the cellular roles of SUMOylation in regulating the recruitment of DNA repair factors to sites of DNA replication damage to influence the choice of employed cellular repair mechanisms for efficient repair.

Post-translational modifications are essential regulators of proteins[216]. PTMs do not only play their roles solo but extensively interact with each other[217,218]. Our knowledge about proteins modified by a combination of SUMO and ubiquitin, SUMO and phosphate and crosstalk between them is quite limited. In this thesis we also aimed at deciphering the crosstalk between SUMOylation and ubiquitination during the DNA damage response and searching for indirect and direct targets for the human STUbL RNF4, which mediates the ubiquitination of SUMOylated target proteins.

Lastly, we adopted the strategy described for SUMO and introduced His10-tagged UFM-1-K0 to identify UFM-1 acceptor lysines. UFMylation has been found to promote the interactions between proteins[58]. However, limited targets have been found to be regulated by UFM-1[15,63,65]. We identified and confirmed RPL26 as a key UFM1 target and further confirmed that the UFMylated form of RPL26 can efficiently interact with the Signal Recognition Particle Receptor, implicating that UFMylation could regulate protein transfer to the Endoplasmic Reticulum.

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