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Conjugation and Deconjugation of Ubiquitin Family Modifiers

Edited by
Marcus Groettrup

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Edited by

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DEDICATION

For Claudia, Hanna and Tim

ABOUT THE EDITOR...

MARCUS GROETTRUP is a Professor for Immunology in the Department of Biology of the Konstanz University in Germany. His main interests are in the role of the immunoproteasome in antigen fragmentation and autoimmunity. Moreover, he has studied the function and conjugation of the ubiquitin-like protein FAT10. He studied biochemistry in Tübingen and ETH Zürich and did his diploma thesis in the laboratory of H. Hengartner and R. Zinkernagel. During his PhD at the Basel Institute for Immunology with H. von Boehmer he discovered the pre T cell receptor. After habilitation at Humboldt University Berlin in the group of P-M. Kloetzel on the topic of antigen processing he founded his own group at the Cantonal Hospital St. Gallen, Switzerland. Since 2002 he holds the Chair of Immunology at the University of Konstanz, Germany. Several prizes were awarded to Dr. Groettrup like the Award of the Sandoz Foundation for Therapeutic Research, the Karl Lohmann Prize of the German Society for Biological Chemistry, the Langener Science Prize of the Paul Ehrlich Institute, and the Research Award by the CaP CURE foundation.

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PREFACE

New Paradigms in the Conjugation of Ubiquitin Family Modifiers

The first conference on the ubiquitin-proteasome system (UPS) I attended took place in 1994 in Copenhagen. At this meeting, organized by Prof. Klavs Hendil, the UPS community was still quite small and almost all of the key questions in the field were awaiting answers. It was not yet clear what type of protease the proteasome would be (cysteine protease, serine protease or maybe something else…[1]), the crystal structure of the proteasome was not yet solved[2,3] and only the tip of the ice berg of the ubiquitin conjugating enzymes had been discovered including the ubiquitin activating enzyme UBE1,[4,5] a dozen of ubiquitin conjugating enzymes (E2)[6], and the first ubiquitin ligases.[7-10] In particular it was not clear how substrate specificity was achieved in ubiquitin-dependent degradation, which was not even a pressing question at that time with only a hand full of ubiquitylation substrates having been discovered. I remember a young PhD student asking Prof. Avram Hershko, at that time not yet a Nobel Laureate, what aspect of the UPS he would recommend to study in the future. I heard his answer and I thought for myself that entering such a field sounded a bit too risky. He recommended searching for ubiquitin ligases which would bring together a particular substrate and an E2 enzyme charged with activated ubiquitin. In the meantime hundreds of E3 ligases have been discovered and hundreds of ubiquitylation substrates are known.[11] Today it is hard to find a cell biologist who does not study or at least thinks about studying the ubiquitylation of his or her favourite protein.

Already in 1986/87 the groups of Ernest Knight and Arthur L. Haas were studying an interferon-induced 'ubiquitin cross reactive protein', today known as ISG15, and A. Haas speculated in his abstract that 'These observations suggest that the 15-kDa protein may represent one example of a functionally distinct family of ubiquitin-like proteins'.[12,13] Ten years later, when SUMO1,[14] NEDD8,[15-18] and FAT10[19] (at that time still named 'diubiquitin') were described, it turned out that this was a wise prediction. Each of the newly found ubiquitin-like modifiers turned out to be conjugated by dedicated cascades of E1, E2, and E3 enzymes further contributing to the expansion and complexity of the field of ubiquitin conjugation and the search for new E1, E2, and E3 enzymes is still ongoing. To cope with the steadily growing body of knowledge and for organizational reasons the articles in this book are focussed on the modifiers ubiquitin, SUMO1/2/3,

NEDD8, ISG15 and FAT10 but it should be at least mentioned here that enzymes involved in conjugation and deconjugation of URM1,[20] UFM1,[21] ATG8[22] and ATG12,[23] have been reported and continue to be investigated.

Several new paradigms have been discovered in the ubiquitin conjugation field throughout the past few years which are reviewed and discussed in this book by scientists who have made major contributions to these developments. The previous conception that one type of modifier is activated by one type of activating enzyme as well as modifier-selective conjugating enzymes and ligases has recently been challenged. The discovery of UBA6 as a second ubiquitin activating enzyme opens the possibility that separate conjugation cascades originate from the two ubiquitin activating enzymes UBA6 and UBE1.[24-26] On the other hand evidence accumulates that a single E1 type enzyme can activate several modifiers. Prominent examples are the activation of ubiquitin and FAT10 by UBA6, the activation of ATG8 and ATG12 by ATG7 or the activation of SUMO-1, -2, and -3 by the heterodimeric E1 enzyme UBA2/AOS1. For several years one could read in the introductions to manuscripts on ubiquitin that there are two functionally important kinds of ubiquitin linkages: the one via lysine 48 of ubiquitin signals proteasomal degradation whereas K63 linked ubiquitin trees serve as scaffolds for the cooperation of enzymes, e.g., in NF-κB activation or endocytosis. Advances in mass spectrometric analysis of ubiquitin linkages have now allowed to correct this simplified view by showing that ubiquitin linkages via K6, K11, K27, K29, or K33 all signal proteasomal degradation not as rare events but comparable in their abundance to K48 linkages.[27] How conjugation enzymes determine specific linkages and how they are interpreted by ubiquitin binding domains is a new theme discussed in this book. To add to the complexity of the system, chains with mixed linkages have been identified[28] and it will be a major challenge to understand how the formation of mixed linkages is accomplished and for what purpose they are formed.

Another emerging paradigm which is covered in several chapters of this book is the cross-talk between the ubiquitin family modification systems. The archetype of such a cross-talk is the modification of SCF ubiquitin ligases with NEDD8[29] but other facets of this principle have been discovered as for example the sumoylation of the ubiquitin conjugating enzyme E2-25K[30] or the SUMO-specific E2 enzyme UBC9.[31] In all these cases, a ubiquitin family modifier controls the activity of a conjugating enzyme or a ligase. Cooperation between modification systems has also been shown in proteasomal targeting where sumoylation of a substrate protein enables recognition by a ubiquitin ligase which then leads to polyubiquitylation of the substrate and its degradation by the proteasome.[32]

Following a ubiquitin conference today is quite demanding as striking insights are reported from virtually all fields of cell biology. On the other hand, keeping an eye on the latest developments and new concepts in UPS research is important for the broad audience of life scientists. I hope that this book will invite the reader to join the current debate on ubiquitin family modifiers. Last but not least, I would like to thank all authors and contributors who made this project possible, and I would like to acknowledge Brigitte Schanze for her excellent secretarial assistance, and Erin O'Brien and Celeste Carlton from Landes Bioscience for their fast and reliable cooperation. While reading the chapters of this book I learned a lot myself and I hope this will be true for other readers as well.

Marcus Groettrup, PhD

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CHAPTER 1

ACTIVATION OF UBIQUITIN AND UBIQUITIN-LIKE PROTEINS

Frederick C. Streich, Jr. and Arthur L. Haas*

Abstract: Attachment of ubiquitin and ubiquitin-like proteins to cellular targets represents a fundamental regulatory strategy within eukaryotes and exhibits remarkably pleiotropic effects on cell function. These posttranslational modifications share a common mechanism comprised of three steps: an activating enzyme to couple ATP hydrolysis to formation of a high-energy intermediate at the carboxyl terminus of ubiquitin or the ubiquitin-like protein, a ligase to couple aminolysis of the activated polypeptide to formation of the new peptide bond and a carrier protein to link the two half reactions. The activating enzymes play pivotal roles in defining pathway specificity for ubiquitin or the ubiquitin-like protein and for target protein specificity in charging the cognate carrier protein supporting downstream ligation steps. Therefore, the family of activating enzymes are critical components of cell regulation that have only recently been recognized as important pharmacological targets.

INTRODUCTION

The post translational modification of cellular proteins by conjugation of polyubiquitin degradation signals serves to target these proteins for 26S proteasome-dependent degradation, altering their steady state levels to regulate cellular function or regulate genetic programs.[1] Conjugation of ubiquitin to protein targets can alter cell localization[2] and activity[3] in addition to influencing epigenetic effects of gene expression, heterochromatin structure[4] and mitochondrial inheritance.[5] These remarkably pleiotropic consequences derive from the inherently large information content represented by the solvent accessible surface of ubiquitin that is transduced by binding of a diverse subset of ubiquitin interacting proteins.[6] The success of ubiquitin modification as a regulatory strategy has

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driven subsequent divergence of the polypeptide into the superfamily of ubiquitin-like proteins: Neural precursor cell expressed, developmentally down-regulated gene 8 kDa (Nedd8), Small ubiquitin-like modifier (Sumo1/2/3/4) and Interferon stimulated gene 15 kDa (ISG15) among others.[7] Recent work demonstrates that the conjugation of these ubiquitin-like proteins utilizes mechanistically similar pathways comprised of distinct components in order to minimize crosstalk.

GENERAL MECHANISM OF CONJUGATION

Conjugation of ubiquitin or ubiquitin-like proteins results in formation of a new peptide bond between the C-terminal glycine of these polypeptides and ε- or α-amino groups on the target protein.[8] More recent work has identified unconventional pathways of ubiquitination involving modification of serine/threonine and cysteine residues, although these are typically exploited as mechanisms for viral evasion of cellular innate immune responses.[9,10] Both conventional and unconventional conjugation mechanisms formally belong to the class of ligase reactions, which typically occur in two half reactions: an activation step forming a high energy intermediate at the expense of ATP hydrolysis and a ligation step that couples cleavage of the high energy intermediate to formation of the new bond. For most ligases such as the aminoacyl tRNA-synthetases, the two half reactions are catalyzed by the same enzyme;[11] however, in the conjugation of ubiquitin and ubiquitin-like proteins, these half reactions are catalyzed by different enzymes, resulting in the three step mechanism characteristic of these pathways, Figure 1A.[12]

The activating enzyme (E1) binds ATP as its Mg^{2+} chelate[13] and ubiquitin (or cognate ubiquitin-like protein) then catalyzes formation of a tightly-bound ($K_d \leq 8 \times 10^{-12}$ M)[14] ubiquitin adenylate intermediate, a high energy mixed acid anhydride linked between the carboxylate of the ubiquitin C-terminal glycine and the α-phosphate of ATP, resulting in release of PP_i.[15,16] The E1 then catalyzes formation of a covalent enzyme-bound ubiquitin thiolester by nucleophilic attack of an active site cysteine on the ubiquitin C-terminal glycine-AMP acid anhydride, resulting in release of AMP.[15,16] The E1 subsequently undergoes a second round of ubiquitin adenylate formation to generate the E1 ternary complex.[15,16] The presence of the E1 ternary complex requires two spatially distinct active sites on the enzyme, one for formation of the ubiquitin adenylate and one for formation of the covalent enzyme-ubiquitin thiolester and a conformational transition to physically transfer the activated polypeptide between the sites in the rate limiting step of the activation reaction.[15,16] The E1 ternary complex in turn binds one of several members of a large family of ubiquitin conjugating enzymes (Ubc/E2) and catalyzes transthiolation of the activated ubiquitin thiolester from E1 ternary complex to a conserved cysteine on the E2.[17,18] Aminolytic cleavage of the E2-ubiquitin thiolester bond ultimately drives ubiquitin isopeptide bond formation catalyzed by the ubiquitin-protein isopeptide ligases (E3).[15,16]

The ubiquitin conjugation mechanism explains the unusual separation of the half reactions onto distinct enzymes by providing a node for divergent evolution of distinct ligation pathways. Thus, pathway specificity for ubiquitin or any of the ubiquitin-like proteins becomes functionally defined by their cognate activating enzyme. In addition, specificity for the cognate E2 allows the activating enzyme to serve as a licensing factor for correct downstream modification(s) of the target protein and to minimize cross talk among different ubiquitin-like pathways. Finally, the hierarchical mechanism

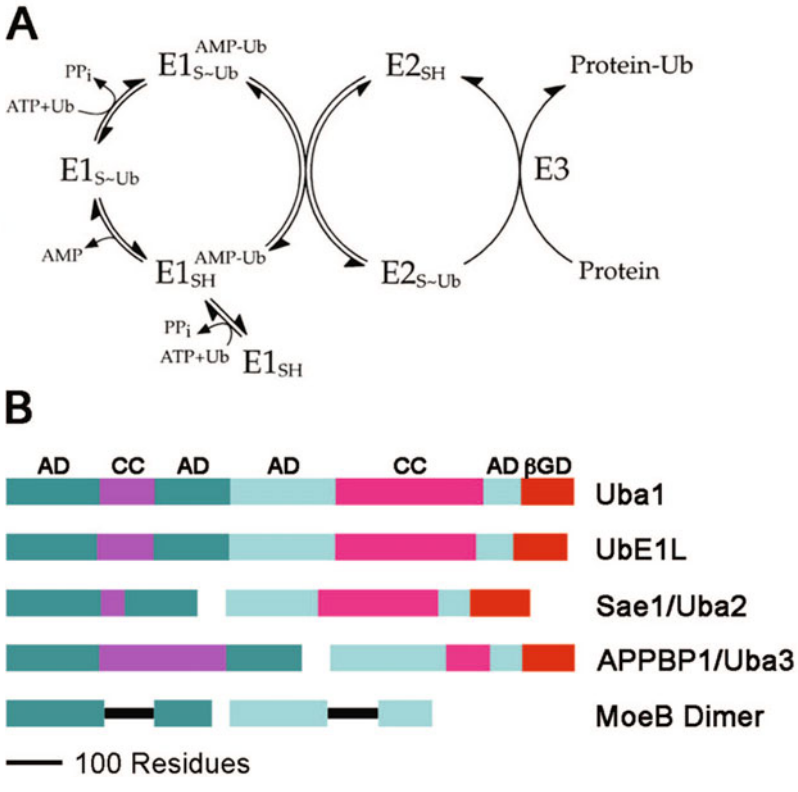

Figure 1. The mechanism for ubiquitin conjugation and the role of ubiquitin activation. A) The mechanism for the conjugation of ubiquitin requires the activities of three proteins: activating enzyme (E1), carrier protein/conjugating enzyme (E2) and ligase (E3), as described in the text. B) Domain alignment of the activating enzymes for ubiquitin (Uba1), ISG15 (UbE1L), Sumo (Sae1/Uba2) and Nedd8 (APPBP1/Uba3) compared to the related prokaryotic MoeB homodimer. AD-adenylate domain, βGD-β grasp domain, CC-catalytic cysteine domain.

allows a single activating enzyme to support multiple conjugation pathways defined by mutually-specific E2-E3 pairs.

CO-EVOLUTION OF E1 AND UBIQUITIN

The ubiquitin system has long represented an enigma since no evolutionary precursors were immediately evident among prokaryotes. However, more recent sequence analysis suggests a provenance for ubiquitin and its activating enzyme in earlier bacterial enzymes involved in sulfur metabolism.[19-21] It is now clear that the ubiquitin family diverged from a common ancestor of the bacterial β-grasp proteins MoaD and ThiS, polypeptides involved in sulphur incorporation during molybdenum cofactor and thiamine biosynthesis, respectively.[19,20] The MoaD and ThiS proteins undergo similar chemistries of carboxyl terminal adenylate formation that is reminiscent of ubiquitin adenylate formation and that are catalyzed by the larger bacterial enzymes MoeB and ThiF, respectively, of which

they are subunits.[20,22] The MoeB and ThiF enzymes are preceded by a still earlier MccB enzyme that synthesizes the bacterial antibiotic microcin C7.[23] The MccB, MoeB and ThiF enzymes share a common adenylate active site that is highly conserved in both sequence and fold with the E1 family of activating enzymes, reflecting their common mechanisms and ancestry.[23] Subsequent divergence of the ubiquitin activating enzyme accounts for the corresponding E1 activities specific for the larger family of ubiquitin-like proteins, all of which share a well-conserved sequence and structure,[24] Figure 1B.

The evolutionary relationship among members of the E1 superfamily is reflected in their shared mechanisms and remarkably similar kinetics. Human and rabbit ubiquitin activating enzymes (Uba1) share similar affinities (K_d) for ubiquitin of 800 and 580 nM, respectively,[16,25] that are nearly identical to the K_d values of the heterodimeric Nedd8 activating enzyme (APPBP1/Uba3) of 950 nM for Nedd8[26] and of the ISG15 activating enzyme (UbE1L) of 500 nM for ISG15.[27] These K_d values are well below the normal intracellular concentrations of their respective polypeptides, indicating that the activating enzymes are likely to be saturating with respect to their cognate polypeptides under normal conditions.[26-28] Although the Uba1, APPBP1/Uba3 and UbE1L paralogs exhibit somewhat disparate affinities for ATP•Mg^{2+} of 7, 103 and 17 μM, respectively,[25-27] all remain saturating with respect to normal intracellular concentration of the nucleotide. These properties anticipate well-conserved binding sites for the initial nucleotide and polypeptide cosubstrates among the E1 paralogs, which has been borne out by the subsequent crystal structures. Within the nucleotide binding pocket, 10 of 13 residues in the MoeB-MoaD-ATP crystal structure are absolutely conserved among the activating enzyme family,[29] suggesting that the disparate APPBP1 residues (Ile54, Ile27 and Ser147) likely account for the large difference in K_d for ATP exhibited by the Nedd8-specific paralog.[26]

The bacterial precursors of the E1 paralogs do not catalyze a step analogous to that of E2 transthiolation found among the ligation pathways for ubiquitin and the ubiquitin-like proteins. However, careful measurement of E2 transthiolation kinetics demonstrates that this step is highly conserved among E1 paralogs. Human and rabbit Uba1 catalyze Ubc2/Rad6 transthiolation with k_{cat} values of 4.5 and 4.8 s^{-1}, respectively, which compare well with a value of 3.5 s^{-1} for human APPBP1/Uba3-catalyzed transthiolation of Ubc12.[25,26] Because k_{cat} reflects the activation energy for the E2-bound E1 ternary complex at the point of ubiquitin transfer, the very similar k_{cat} values indicate nearly identical geometries for the respective transition states. In addition, since the k_{cat} for E2 transthiolation is less than the lower limit for the net forward rate for E1 ternary complex formation (9.6 s^{-1})[16] determined by ATP:AMP exchange studies of Uba1, the rate limiting step of E2 transthiolation must reside in the transfer step and not an internal step of E1 ternary complex formation.[25]

OVERALL STRUCTURAL TOPOLOGY OF E1

The available structural data demonstrate that Uba1, APPBP1/Uba3 and Sae1/Uba2share a very similar domain topology and tertiary structure even though the latter E1 paralogs exists as heterodimers, Figures 1B-3.[30-33] The activating enzymes all adopt a shape resembling a cupped hand (as shown when the structure is rotated 90 degrees along the vertical axis) that defines the adenylate active site and has a dimension of 50 Å × 30 Å × 20 Å in the case of APPBP1/Uba3, Figure 2.[29] When looking down into the groove from above, a length of 8-11 residues forms a crossover loop that transverses the

groove and divides it into two spatially distinct sites that are connected by free space above and below the crossover loop, Figure 2. Using compass points for orientation, the nucleotide binding site (Site 1) sits to the west of the crossover loop and harbors a pocket into which the nucleotide binds. Site 2, sitting to the east of the crossover loop, is the site into which the ubiquitin-like protein docks with the C-terminal tail of the ubiquitin-like protein laying in a shallow channel running under the crossover loop and extending into Site 1, poised for nucleophilic attack of the carboxyl terminus on the α-phosphate of ATP, Figure 2.[30-33] The nucleotide binding site is comprised of two structurally homologous domains [root mean square deviation (rmsd) of 1.6 Å over 412 residues] that each adopt a Rossman-fold variation, composed of 8 α-helices and 8 mixed β-sheets, Figures 1B-3.[29] For comparison, the structures of Nedd8 and ubiquitin share 60% sequence identity and have a rmsd of 0.8 Å for the α-carbons over 72 residues.[34,35] These two homologous domains and their interface share structural homology with the MoeB and ThiF homodimers in complex with MoaD and ThiS, respectively, even in regions of low sequence homology.[19,29,32,36]

The north wall of the groove is defined by the catalytic cysteine domain, provided by disjointed sections of the amino acids from different regions of the same polypeptide (Uba1 and UbE1L) or different polypeptides (APPBP1/Uba3 and Sae1/Uba2). The first (inactive) catalytic cysteine domain forms the northeast wall of the groove and is relatively large in APPBP1/Uba3, for which it provides considerable contact surface for bound Nedd8 poised for adenylation.[30] The paralogous region in Uba1 is considerably smaller and provides a diminished number of contacts for ubiquitin while the corresponding region is nearly absent in Sae1/Uba2, retaining approximately 25 residues in a disordered unresolved loop with no observed contacts to Sumo1.[32,33] The second (active) catalytic cysteine domain forms the northwest wall of the groove and harbors the conserved active site cysteine that is poised above the groove on the upper lip of the wall. The catalytic cysteine domain segments, present only in the ubiquitin and ubiquitin-like protein activating enzymes, represent insertions into the original nucleotide binding domains of MoeB and ThiF. The congruent regions in MoeB and ThiF consist of short mobile loops that contain a cysteine that is not utilized in MoeB-MoaD catalytic cycles but is used in ThiF-ThiS reaction via a different mechanism involving a disulfide.[19,29,36,37] The southern wall of the groove is defined by the β-grasp domain (βGD), which shares structural homology to ubiquitin (rmsd 1.9 Å over 50 residues).[29] Structural evidence suggests the βGD is involved in part in binding the cognate E2 and, in coordination with additional E1 surfaces, guides E1-catalyzed E2 transthiolation, Figures 2 and 3.[32]

APPBP1/Uba3 and Sae1/Uba2 both contain zinc binding domains that sit at the base of the βGD on the side of the crossover loop adjacent to Site 1.[30-32] The bipartite zinc binding motif consists of two C-X-X-C motifs on Uba3 and Uba2, separated by 141 and 280 amino acids, respectively. The zinc motif is absent in Uba1, replaced by a short β-hairpin structure intercalated between the loop linker to the βGD and the β-strands forming the base of Site 2.[33] The function of these zinc motifs is unclear since genetic disruption of zinc coordination is without effect in vivo while deletion of the βGD is lethal by comparison.[32]

Uba1 and Sae1/Uba2 both contain extensions harboring nuclear localization signals, although on different regions of the protein. Two forms of Uba1 arise from alternative translational start sites with the shorter cytoplasmic form missing the N-terminal nuclear localization signal.[38] Further evidence suggests the longer form of Uba1 undergoes phosphorylation at a serine within the extension in order to promote nuclear localization.[38]

Sequence alignment demonstrates that yeast do not contain the N-terminal extension peptide; in addition, that both full length and truncated forms of human Uba1 can rescue mutant yeast phenotypes, suggesting the 40-residue peptide represents a recent evolutionary addition.[38] In contrast, Sae1/Uba2 has a C-terminal extension that contains a

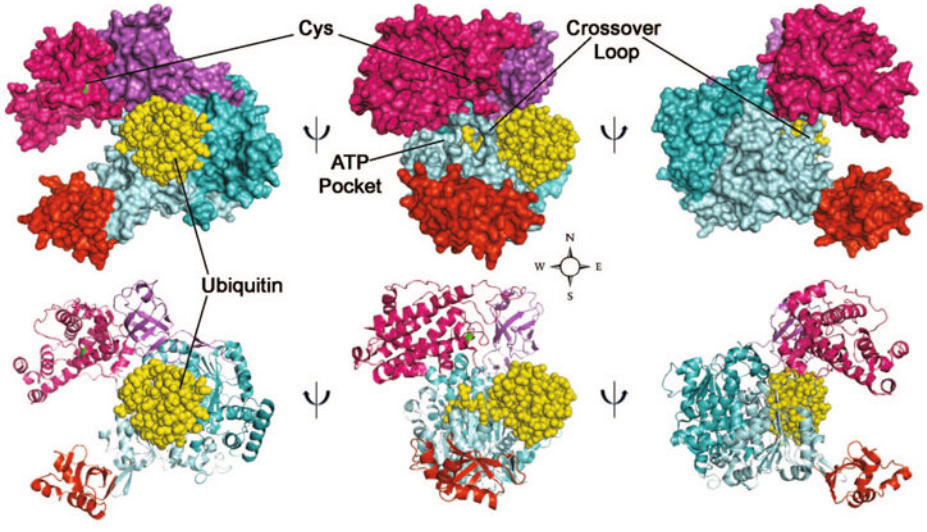

Figure 2. Topology of the yeast Ubal activating enzyme. Three views of the structural model for *Saccharomyces cerevisiae* ubiquitin activating enzyme (Uba1) with bound ubiquitin (yellow). Structural domains are color coded to match those shown in Figure 1B. Ribbon diagrams corresponding to the surface modeled structure are shown below each structure. Structures to the left and right of the central pair are rotated 90° to the left or right, respectively. A compass symbol is presented for orientation, as discussed in the text. Structural images generated with PyMOL (DeLano Scientific) from PDB file 3CMM.

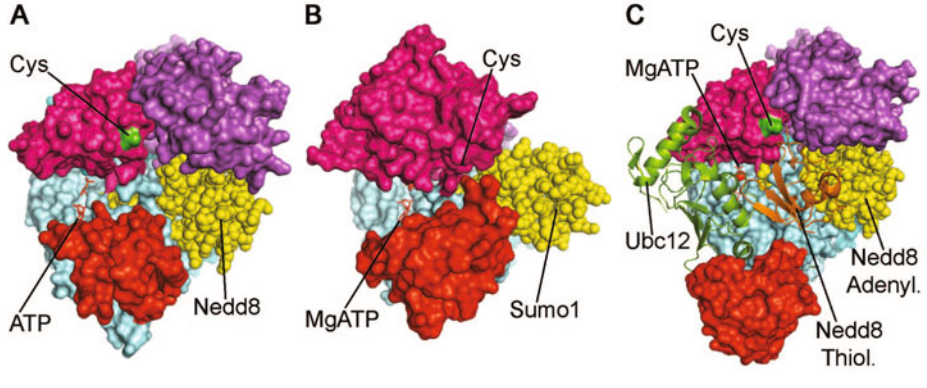

Figure 3. Structural models of APPBP1/Uba3 and Sae1/Uba2. A) APPBP1/Uba3 with bound Nedd8. B) Sae1/Uba2 with bound Sumo1 and ATP•Mg^{2+}. C) APPBP1/Uba3 ternary complex with bound Ubc12. Domains are color coded to match those in Figure 1B. Structural images created with PyMOL (DeLano Scientific) from PDB files 1R4M, 1Y8R and 2NVU, respectively.

nuclear localization signal.[39] This extension is not resolved in the crystal structure of the Sae1/Uba2 and is not lethal when deleted; however, the profile of ubiquitinated proteins is affected by deletion of the nuclear localization peptide, suggesting the presence of nuclear-specific Sumo conjugation pathways.[32]

TERNARY COMPLEX FORMATION

Early equilibrium and kinetic studies of Uba1 indicated ordered substrate binding, with ATP•Mg^{2+} as the leading substrate and ubiquitin as the trailing substrate and product release, with PP_i dissociating before AMP.[16] Ordered substrate binding and product release typically indicate obligatory structural transitions during the catalytic cycle. That mutation of Arg72 on ubiquitin obviates ordered substrate addition, based on ATP:PP_i exchange kinetics, argues that ordered addition reflects the relative affinities of ATP•Mg^{2+} binding versus that of ubiquitin as the leading substrate.[14] Subsequent mechanistic work in which mutation of Asp576 within human Uba1, predicted to interact with the coordinated Mg^{2+} of the nucleotide, also results in a formally random addition mechanism, supporting an affinity rather than structural model of ordered substrate binding.[40] That wild type APPBP1/Uba3 exhibits pseudo ordered addition, with ATP•Mg^{2+} as the preferred leading substrate, rules out ordered addition as a prerequisite for a catalytically competent enzyme.[26]

The mode of ATP binding among the E1 paralogs is highly conserved and consists of a subset of interactions with the adenine ring and phosphate groups.[29-33] Several lines of evidence suggest that there are coordinated domain movements throughout the catalytic cycle of ubiquitin-like protein activation beginning with ATP binding. Binding of ATP to Sae1/Uba2 results in Arg21, Asp347 and Lys346 (the latter incorrectly labeled as Lys348 in the paper) forming a network of polar interactions with each other and the γ-phosphate of the nucleotide.[32] Subsequent binding of Sumo1 breaks the interactions of Asp347 and Lys346 (which are present on a loop hanging over the γ-phosphate) with the phosphate, leaving Arg21 alone to interact with the γ-phosphate.[32] Because these residues are conserved among the E1 paralogs, similar binding-dependent conformational changes are anticipated for the other paralogs. Additionally, there are global differences observed between the APPBP1/Uba3 and the APPBP1/Uba3-Nedd8-ATP complex. In the presence of ligands, the catalytic cysteine domain (northeast wall region, residues 178-280 of APPBP1) rotates 10 Å out of Site 2 in order to accommodate Nedd8 binding. Additionally, the crossover loop shifts approximately 2.5 Å closer to the base of the active site groove, clamping the C-terminus of Nedd8.[30]

The domain movements required during the catalytic cycle of ubiquitin adenylate formation appear to be linked to coordination with the chelated metal of ATP•Mg^{2+}. Tokgöz et al has demonstrated that Asp576 of human Uba1 (paralogous to Asp130 of MoeB, Asp146 of Uba3 and Asp117 of Uba2)[19,29,32] is responsible for triggering these catalytic transitions during ubiquitin adenylate formation.[40] Earlier studies had suggested that the paralogous positions on MoeB, Uba3 and Uba2 were important for ATP•Mg^{2+} binding since mutation to alanine abolished biological activity.[19,29,32] Tokgöz et al demonstrated that the analogous mutation of Asp576 within human Uba1 decreased the affinity for ATP by 38-fold (K_d = 208 μM), consistent with a role in nucleotide binding. More important, the mutation resulted in a >10^5-fold decrease in the catalytic efficiency for ubiquitin adenylate formation, leading to an altered stoichiometry for the ternary complex and a change in rate limiting step for transthiolation to that of ubiquitin adenylate formation.[40]

Other mechanistic studies suggest that Asp576 functions to stabilize the predicted pentacoordinate transition state of ubiquitin adenylate formation and that abrogation of this role is responsible for the loss of biological activity previously ascribed to effects on ATP•Mg^{2+} binding.[40] At least some of the catalytic contribution of Asp576 is a function of the enhanced affinity of the Uba1 active site for the pentacoordinate transition state, as originally predicted by Wolfenden and subsequently shown to be an intrinsic feature of enzyme catalysis.[41] As such, one anticipates that other residues that bind the ATP•Mg^{2+} substrate likely also contribute to binding of the incipient transition state and correspondingly to the observed k_{cat} for ubiquitin adenylate formation. As such an example, Lys528 of human Uba1 is absolutely conserved among E1 paralogs and functions in hydrogen bonding to the β-phosphoryl oxygen of ATP. As predicted by the model, mutation of Lys528 to alanine results in a kinetic phenotype for ubiquitin adenylate formation that is indistinguishable from that of the Asp576 mutation, consistent with a role in enhanced binding of the transition state.[40] Presumably other residues required in ATP binding will yield the same effect on E1 function.

One unanticipated consequence of mutating Asp576 is that the resulting enzyme exhibits random substrate addition.[40] This observation indicates that coordination to the metal of bound ATP•Mg^{2+} is responsible in part for conformational transitions associated with the catalytic cycle of the enzyme. These conformation transitions in part define the binding site for ubiquitin since the Asp576 point mutant also possesses a reduced affinity for binding the polypeptide.[40] It has been known for some time that occupancy of the nucleotide binding site, either with ATP•Mg^{2+} or ubiquitin adenylate, enhances the observed rate of E2 transthiolation, indicating that the ternary complex is the catalytically competent conformation of the enzyme and supporting the idea of large conformational transitions during the catalytic cycle.[42] Similarly, binding of active E2 also stimulates the otherwise depressed rate of ubiquitin adenylate formation resulting from Asp576 mutation.[40] While seemingly unexpected, this observation is a direct consequence of microscopic reversibility in the catalytic cycle and the corresponding conformational transitions.

RECOGNITION OF THE UBIQUITIN-LIKE PROTEIN

Ubiquitin and Nedd8 share 60% sequence identity[43] and, not surprisingly, share many features of recognition by their cognate activating enzymes. The three main recognition motifs center around the conserved C-terminal tail, the conserved hydrophobic patch (Leu8, Ile44, Val70, ubiquitin numbering)[35,44,45] and polar contacts between the ubiquitin-like protein and the catalytic cysteine domain (northeast wall) of Site 2. These areas of recognition are less conserved with Sumo1, as expected for a protein with less sequence homology. However, in all cases, the respective E1 paralogs exhibit near absolute specificity for their cognate polypeptides.

Early experiments suggested the C-terminal tail of ubiquitin was necessary for efficient activation. Pickart et al observed a 10-fold decrease in affinity for ubiquitin when the C-terminal Gly76 was mutated to alanine.[42] Similarly, truncation of the C-terminal di-glycine inactivated ubiquitin,[46] while a hexapeptide consisting of the C-terminal residues of ubiquitin was a low affinity substrate for Uba1-catalyzed adenylate formation but was not transferred to the active site cysteine.[47] Similarly, mutation of the corresponding C-terminal ubiquitin residues also blocked Uba1 activation, as measured by ATP:PP_i

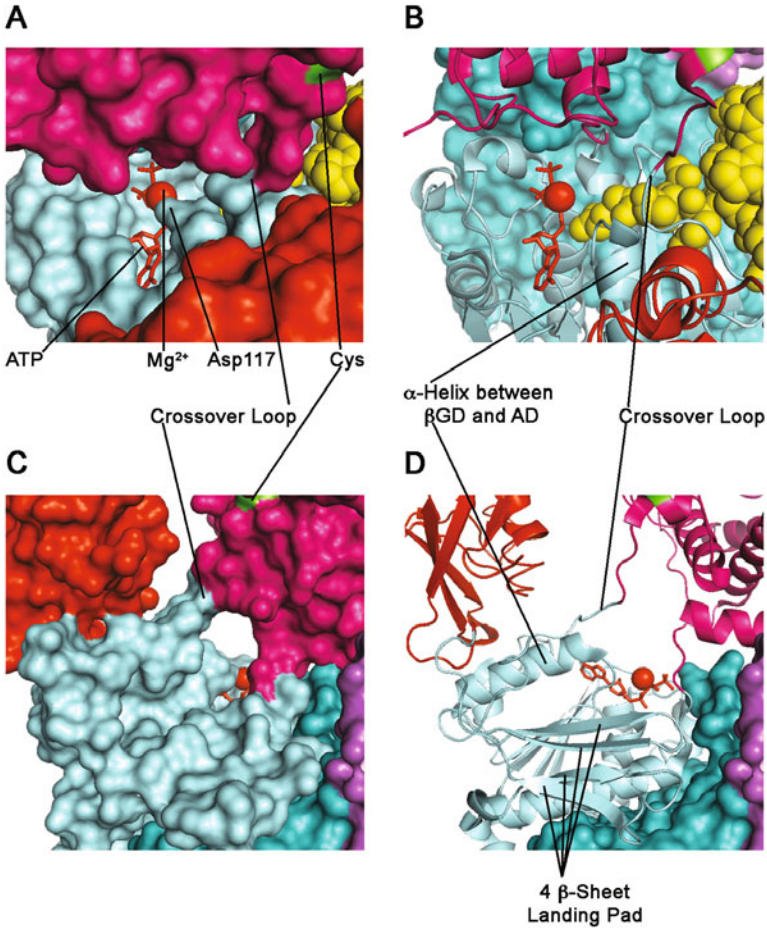

Figure 4. Close up views of ATP binding site (Site 1) and ubiquitin-like protein binding site (Site 2). A) Surface representation of the ATP binding pocket (Site 1) with bound ATP•Mg^{2+} from the structural model of Sae1/Uba2.[32] B) Structural model of panel A with the surface representation removed. C) Surface representation of the ubiquitin-like protein binding site (Site 2) with Sumo1 removed for clarity; from the structural model of Sae1/Uba2.[32] D) Structural model of panel C with the surface representation removed. Structure images created with PyMOL (DeLano Scientific) from PDB file 1Y8R.

exchange.[48] Point mutation demonstrated that Arg72 of ubiquitin was critical for binding to and the catalytic efficiency of Uba1.[14] The contribution to catalysis results in part from the role of Arg72 in binding the ubiquitin adenylate intermediate.[14] Mutation of the other four arginine residues had less dramatic effects that suggested an order of relative importance for recognition by Uba1 of Arg72 > Arg54 > Arg42 > Arg74.[14]

Solution of the structures for ubiquitin and other ubiquitin-like proteins bound to their cognate activating enzymes provides a clear understanding of how polypeptide recognition is achieved, Figures 4C and 4D. In the case of ubiquitin binding to yeast Uba1, the structure reveals the C-terminal tail winding through a shallow channel under the crossover loop and terminating next to the ATP binding pocket adjacent to the

α-phosphate of the bound ATP•Mg^{2+}.[33] The Arg72 residue sits in a pocket of negative charge stabilized by a network of hydrogen bonds to residues in the crossover loop, including Glu576, Asp591, Tyr586 and the backbone of Ser589.[33] Specificity for the cognate polypeptide is defined by the nature of the paralogous residues at position 72 of ubiquitin. The paralogous positions in Nedd8 and Sumo are occupied by alanine and either glutamate or glutamine, depending on the Sumo isoform. Witby et al demonstrated that a Nedd8 Ala72Arg point mutant can bind with low efficiency to Uba1 and form an E1 ~ Nedd8 thiolester, suggesting that the reside at that position in the tail is critical for defining specificity.[35] The structure of APPBP1/Uba3 with bound Nedd8, demonstrates the C-terminal residues adopt a similar conformation to that of ubiquitin, lying in a shallow channel weaving under the crossover loop into the ATP binding pocket. Alanine72 and Leu71 are enveloped by a hydrophobic sequence comprised of Leu206, Tyr207 and Pro208 within the crossover loop; mutation of Leu206 and Tyr207 to aspartate ablates Nedd8 adenylation but is not sufficient to allow ubiquitin activation.[29,30] This structure predicts Arg72 in ubiquitin would repel Arg190 of Uba3 (which is Gln576 in Uba1).[30] Modeling suggests a network of bonds lock Arg190 into a position that accommodates only a relatively small residue such as alanine at position 72 that is not accommodated by the arginine in ubiquitin or the glutamine/glutamate of Sumo paralogs.[49] In the Sae1/Uba2 structure, the glutamine/glutamate residue interacts with Tyr159 in the crossover loop and Arg119 on the α-helix in Site 1 adjacent to the crossover. This α-helix and the preceding loop form a portion of the ATP binding pocket that cradles the adenine ring as well as harbors the conserved aspartate residue that coordinates to Mg^{2+}. Like the other structures, the C-terminus of Sumo extends under the crossover loop in a shallow channel and into the ATP binding pocket.[32]

The crystal structure of ubiquitin reveals a hydrophobic patch consisting of Leu8, Ile44 and Val70[45] that packs into repeating units within chains greater than four ubiquitins, as observed in the crystal structure of tetraubiquitin.[44,50] Beal et al demonstrated that this hydrophobic patch is a central component of polyubiquitin binding to the S5a subunit of the regulatory complex in the 26S proteasome.[44] Numerous studies have implicated involvement of this hydrophobic patch in the binding interaction of several proteins and motifs, including CUE domains,[51] ubiquitin associated domains (UAD),[52] ubiquitin interaction motifs (UIM),[53] NEMO[54] and Rabex.[55] These three ubiquitin residues interact with a conserved hydrophobic patch on Uba1 present on a 4 β-sheet surface formed from one of the Rossman-folds from the nucleotide binding domain that forms the base of the active site groove and Site 2. This surface resembles a "landing pad" for ubiquitin, Figures 4C and 4D.[33] A paralogous hydrophobic patch on Nedd8 interacts with a complementary surface on Uba3 in addition to observed ring stacking between His68 of Nedd8 and Tyr333 of Uba3.[30] Mutation of residues within the complementary hydrophobic interacting surface on Uba3 ablates Nedd8 adenylate formation.[29] In contrast, Sumo1 lacks a well-defined hydrophobic patch;[56] consequently, the interaction of Sumo1 with Sae1/Uba2 involves fewer hydrophobic interactions but a more defined network of hydrogen bonds that specify binding fidelity.[32]

The remaining determinant of polypeptide binding specificity resides with interactions between the surface of the ubiquitin-like protein and northeast wall of Site 2, Figure 2. Nedd8 makes extensive polar interactions with the catalytic cysteine domain in APPBP1/Uba3, while less extensive interactions are present between ubiquitin and Uba1, Figure 2 and 3A.[30,33] These interactions are absent in Sae1/Uba2 due to differences in the relative size of that portion of the catalytic cysteine domain insertion.[32]

E1 THIOLESTER FORMATION

The most interesting structural questions at present relate to how the activated ubiquitin is transferred from the corresponding bound adenylate to the thiolester sites since no crystal structure has yet captured this intermediate. Because of the requirement for topologically distinct adenylate and thiolester active sites, the catalytic cycle for E1 requires large conformation transitions to transfer activated ubiquitin from the adenylate active site to the thiolester site. Interestingly, the conformations for all structures without a ubiquitin-like protein thiolester are remarkably similar to the single structure with a ubiquitin-like protein thiolester, the APPBP1/Uba3 ternary complex with bound Ubc12, Figures 2 and 3A and 3B compared with Figure 3C.[29-33] The structure with a Nedd8 thiolester features Nedd8 tethered to the catalytic cysteine perched atop the middle of the north wall of the active site groove. The global positions of the APPBP1/Uba3 domains are in much the same conformation as the APPBP1/Uba3 structures without thiolester present, except for modest movement of the βGD to allow room for Ubc12 binding.[31] In these structures the distance between the C-terminal glycine of the ubiquitin-like protein and the catalytic cysteine is approximately 28-35 Å.[29,30,32]

Because the C-terminal tails of the ubiquitin-like proteins are disordered, it is proposed that a shift of the Nedd8 C-terminal tail to the conformation similar to that seen in the crystal structure could reduce the distance between the Nedd8 adenylate and the catalytic cysteine to approximately 10 Å.[29,35] Modest shifts in the catalytic cysteine domain could potentially closed the remaining gap; however, the model does not resolve the steric problems associated with having the C-terminus of Nedd8 threading beneath the crossover loop, an issue that is more acute with Uba1. While it is possible that conformational changes could open the channel under the crossover loop, allowing the ubiquitin-like protein globular domain to pass under the crossover loop, it seems more likely that the crossover loop moves out of the way prior to transthiolation. This latter model is supported by differences between the Sae1/Uba2 structures in the presence and absence of Sumo1 binding which suggest that the βGD and the catalytic cysteine domains shift and form a kink in the crossover loop that could allow the tail to free itself from under the loop and rotate up toward the active site cysteine.[32] Further work is required to trap such a transitional intermediate of the catalytic cycle in order to resolve the mechanism of ubiquitin transfer between the distinct active sites.

E2 TRANSTHIOLATION

The crystal structure of the APPBP1/Uba3 ternary complex bound to Ubc12 has provided the most compelling model for the conformation of a bound E2 prior to transthiolation, Figure 3C.[31] The E2 conjugating enzymes adopt a conserved fold that results in an overall elongated shape with N-terminal α-helix and β1 and β2 loops on one end and a C-terminal helix-loop-helix defining the opposite end, with the active cysteine in the middle.[57] In the structure, Ubc12 is bound to the βGD through the N-terminal α-helix and β1 and β2 loops.[31] This interaction is recapitulated in a structure of the Ubc12 core domain (missing the N-terminal extension) bound to the βGD. The βGD interaction surface is comprised of mixed β-sheets and kinked α-helix that adopt a fold with two grooves, one of which binds the N-terminal α-helix while the other binds the β1 and β2 loops on Ubc12.[58] Complementary interactions between these interacting surfaces define binding

specificity and point mutation results in diminished Ubc12 transthiolation relative to the wild type proteins.[58] Many of these interactions are conserved on other E2 proteins and where there are differences, the alterations are compensated by changes to the appropriate interacting residues on the βGD. For example, Leu32 of Ubc12 is substituted by Arg or Lys in most ubiquitin specific E2 conjugating enzymes and the Uba1 orthologs have either Glu or Asp instead of the Ala424 or Ala426 present in Uba3.[58] Observations that deletion of the βGD abrogates E1 activity in vivo by preventing Sae1/Uba2-catalyzed E2 transthiolation is consistent with conservation of βGD-E2 interactions.[32] Lee and Schindelin modeled the N-terminal α-helix of Ubc1 onto the βGD of yeast Uba1 and predicted Lys5 and Lys9 on Ubc1 to likely interact with Glu1004, Asp1014 and Glu1016 of the βGD. Mutation of Glu1004 to lysine decreased E2 transthiolation, as did similar mutations of Asp1014 and Glu1016.[33] Finally, detailed transthiolation kinetic analysis shows that the affinities of E1 paralogs for their cognate E2 proteins are remarkably well conserved. Human Uba1exhibits a K_d of 123 nM for Ubc2b[25] and an affinity of 100-185 nM for UbcH7.[27,59] Similarly, APPBP1/Uba3 shows an affinity of 43 nM for Ubc12[26] and UbE1L has an affinity of 66-100 nM for its cognate UbcH8 conjugating enzyme.[27,59] Agreement among these K_d values indicate a well conserved E2 binding site among the various E1 paralogs.

A second interaction interface has been observed by NMR for Ubc9 binding to Sae1/Uba2 that is probably conserved among the other E1 enzymes.[60] This surface involves residues comprising the C-terminal half of Ubc9, near the active site cysteine and residues contributed by the catalytic cysteine domain forming the northwest active site groove of Uba2, Figure 3C.[60] Most of the later residues face inward toward the opening of the groove in the Sae1/Uba2 structure; however, some are on the backside of this domain. Residues 218-240 form an unresolved disordered loop that is particularly important for the interaction with Ubc9, specifically residues 129-134.[32] With Ubc9 modeled onto the βGD using the N-terminal residues as an interacting face, the residues contiguous with the catalytic cysteine face the majority of the interacting residues on the catalytic cysteine domain identified by NMR.[32,60] Modeling the interface of this binding suggests the interaction would bring the two catalytic cysteines within 14-17 Å of each other; additional refinement or movement of the catalytic cysteine domain could further reduce this distance. Mutation of residues in the disordered loop on the catalytic cysteine domain reduces binding affinity of Ubc9 as well as the rate of transthiolation compared to wild type protein. Mutation of the complementary Ile235 and Ile238 residues on Uba2 abrogate transthiolation without affecting the stoichiometry of ternary complex formation.[60] A similar interaction face in yeast Uba1 has been noted and deletion of residues 776-793 (part of which is an unresolved loop in the yeast Uba1 structure) decreased the ability of the activating enzyme to catalyze single turnover E2 transthiolation.[33]

In the structure of yeast Uba1, two slightly different conformations were observed between the two proteins in the asymmetric unit, suggesting an approximately 10 Å rotation of the βGD as a unit in relation to the rest of the protein, Figure 2.[33] Likewise, a 120 Å rotation of the βGD is observed in the structure for APPBP1/Uba3 ternary complex with bound Ubc12 versus that in the absence of the E2, Figure 3A versus 3C.[31] Analogous domain rotations of the βGD and catalytic cysteine domains are observed in the Sae1/Uba2 structures with and without Sumo1 bound, Figure 3B.[32] In the absence of Sumo1, Lys472 on the βGD hydrogen bonds to Arg119 on the adenylate domain α-helix between the βGD and adenylate domain, in aggregate constituting part of the ATP binding pocket. Additionally, Tyr442 on the βGD interacts with Glu160 on the

crossover loop in the absence of Sumo1. When Sumo1 is bound, the interaction between Tyr442 and Glu160 is disrupted and Arg119 participates in an interaction with Glu93 on Sumo1. The shifting of these domains produces changes in the residues within the ATP binding pocket.[32] Similarly, rotation of the βGD that allows Ubc12 binding also reveals an additional binding surface on Uba3 consisting of residues Ile149, Arg152 and Trp153 present on the same α-helix between the βGD and adenylate domain and Glu201 on the crossover loop, providing a potential mechanical link between E2 transthiolation and adenylate formation.[31]

These structural observations begin to suggest how E1 accomplishes the allosteric communication between the rate of E2 transthiolation and adenylate formation.[40,42] In Uba1, replacement of the zinc binding motif found in APPBP1/Uba3 and Sae1-Uba2 with the β-hairpin appears to result in a more open conformation of the βGD similar to that seen in APPBP1/Uba3 with bound Ubc12, Figures 2 versus 3C. Modeling of Ubc1 binding to the βGD of yeast Uba1 suggests that a 40 Å rotation of the βGD could diminish the gap between the two catalytic cysteine residues from 38 Å to 8 Å since relatively small rotations of the βGD translate into larger movements at the far end of the oblong E2 structure, where the catalytic E2 cysteine resides, Figure 3C.[31,33] Since the βGD moves within different structures as a single unit and the linker to the βGD involves residues that are not buried and that only minimally interact with the adenylate domain, such rotations are quite plausible. Furthermore, experiments that stiffened the linkers on Uba1 and Sae1/Uba2 both result in enzymes defective in catalyzing E2 transthiolation.[33,58] Consistent with this model, the APPBP1/Uba3 ternary complex with bound Ubc12 structure, suggests there is room for the E1 ~ Nedd8 thiolester to move out of the way of the incoming E2. It has been suggested that once transthiolated, reverse rotation of the βGD with bound Ubc12 ~ Nedd8 thiolester would cause a steric hindrance between Ubc12 ~ Nedd8 and E1 domains, resulting in dissociation of the charged E2 product.[31] An additional attractive aspect of the model for βGD rotation is that the second interaction surface, between the C-terminal end of the E2 and the catalytic cysteine domain could serve as an attractive force that guides proper orientation of the E2 as the βGD swings it across the canyon.[60]

CONCLUSION

Activation of ubiquitin and the family of ubiquitin-like proteins is catalyzed by the family of E1 paralogs. The ubiquitin-like proteins and their corresponding activating enzymes share sequence and structural homology, as expected for proteins that arose from divergent co-evolution from common ancestors. Not surprisingly, the activating enzymes share a common mechanism of cognate ubiquitin-like protein activation and similar affinities for cosubstrates. The superfamily of E2 conjugating proteins similarly arose through divergent evolution and thus share considerable sequence and structural homology.[12] Considerable progress had been made in understanding the structure-function relationships among the E1 paralogs in their common mechanism of action. The current structural information provides a framework for understanding substrate specificity with respect to ubiquitin and its paralogs. Much less is known regarding the substrate specificity of the E1 enzymes for their cognate E2 carrier proteins. Major advances are required in capturing intermediates between the catalytic extremes of uncharged E1 and ternary complex in order to understand the internal transthiolation reaction involving ubiquitin transfer between the two active sites. Because E1 is a potential

therapeutic target, understanding the mechanism of these enzymes is critical. At present, a mechanism-based high affinity inhibitor based on adenosyl sulphamate shows significant promise as a lead compound.[61]

Because of the marked conservation among the paralogs for E1, the ubiquitin-like proteins and their corresponding E2 conjugating enzymes, it becomes possible under extremely high concentrations of E1, ubiquitin-like protein, or E2 as well as assay conditions using extremely long incubation times that spurious interactions can be forced to occur between noncognate components. This is especially the case in assigning pathway specificity for the E2 conjugating enzymes. In contrast to many enzymatic reactions, the affinity of the E2 substrate is nearly the same as the E2 ~ ubiquitin-like protein thiolester product.[25] This phenomena results in potential problems when in vitro assays utilize abnormally high concentrations, like those seen in over expression assays, to probe functionality of pathways, since it can result in global inhibition of E1 activity.[25] Likewise, over expression can force interactions between noncognate E1-E2 (and E2-E3) pairs, resulting in functional assignment of conjugating enzymes to incorrect pathways or to erroneous dual functions, as in the case of UbcH8.[27,59] This caution also holds for the identification of novel ubiquitin-like proteins and novel activating enzymes. Therefore, reliable functional assignment requires biochemically-defined kinetic assays using concentrations of enzymes determined by stoichiometric functional assays in order to unambiguously determine specificity.

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CHAPTER 2

CONTROL OF UBIQUITIN CONJUGATION BY Cdc48 AND ITS COFACTORS

Alexander Buchberger*

Abstract: Cdc48 (alias p97, VCP) is an important motor and regulator for the turnover of ubiquitylated proteins, both in proteasomal degradation and in nonproteolytic pathways. The diverse cellular tasks of Cdc48 are controlled by a large number of cofactors. Substrate-recruiting cofactors mediate the specific recognition of ubiquitylated target proteins, whereas substrate-processing cofactors often exhibit ubiquitin ligase or deubiquitylating activities that enable them to modulate the ubiquitylation state of substrates. This chapter introduces the major groups of Cdc48 cofactors and discusses the versatile options of substrate-processing cofactors to control the fate of Cdc48 substrates.

INTRODUCTION

Cdc48 (also known as p97 or—a misnomer—valosin-containing protein/VCP in vertebrates)[†] is a highly conserved, chaperone-related protein involved in a variety of cellular processes, including ubiquitin-dependent protein degradation in protein quality control, cell cycle regulation, signal transduction and development, and membrane fusion in organelle biogenesis.[1] Cdc48 is a member of the AAA (ATPases associated with various cellular activities) family of ATPases.[2,3] It forms a ring-shaped complex of six identical subunits comprising an amino-terminal N domain and two ATPase domains, D1 and D2 (Fig. 1A). The common molecular basis underlying the multifaceted cellular functions of Cdc48 is believed to be a "segregase" activity,[4] where ATP-driven domain

[†] For the sake of clarity, the term "Cdc48" is used throughout this chapter collectively for all eukaryotic Cdc48 homologues.

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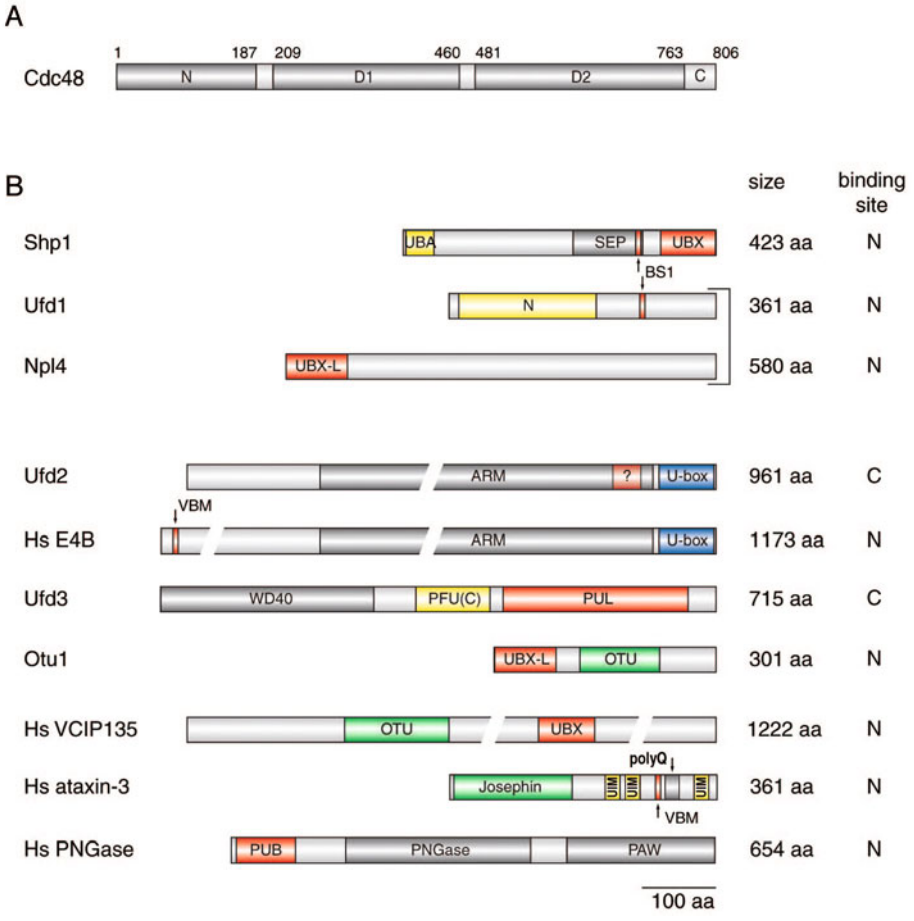

Figure 1. Domain architecture of Cdc48 and important cofactors. A) Cdc48. Structural domains (N, D1, D2) and the unstructured carboxy-terminal region (C) are indicated. Residue numbers refer to the mammalian Cdc48 homologue p97. B) Selected yeast and human (Hs) cofactors discussed in the text. Shp1, Ufd1 and Npl4 are substrate-recruiting, the others substrate-processing cofactors. Ufd1 and Npl4 form a stable heterodimer, as indicated by a bracket. The binding site on Cdc48 is indicated at the right (N, N domain; C, carboxy terminus). Cdc48 binding modules including the UBX and UBX-like (UBX-L) domains, the PUL domain, the PUB domain and the short linear binding site 1 (BS1) and VCP binding motif (VBM) are shown in red. The exact position and nature of the Cdc48 binding motif in Ufd2 are unknown (red box labeled with question mark). Ubiquitin binding modules including the UBA, N and PFU(C) domains and the UIM motif are shown in yellow. The U-box ubiquitin ligase domain of Ufd2 and human E4B is in blue, whereas the OTU and Josephin deubiquitylating domains are in green. Further domains of interest are shown in dark grey. polyQ, polyglutamine stretch.

motions generate mechanical force for the extraction of substrate proteins from protein complexes, membranes and chromatin.[2,3,5]

Cdc48 recognizes primarily ubiquitylated substrates, both in the context of proteasomal protein degradation and in proteolysis-independent pathways of the ubiquitin system. The best-studied nonproteolytic process requiring Cdc48 is the fusion of homotypic Golgi and ER membranes, where Cdc48 appears to act on SNARE complexes and/or their

regulators.[6-8] In the budding yeast OLE (oleic acid desaturase Ole1) signal transduction pathway, the segregase activity of Cdc48 is required to release the active, processed p90 form of the transcription factor Spt23 from complexes with the unprocessed p120 precursor, which is anchored in the ER membrane.[4,9] Furthermore, Cdc48 extracts Aurora B kinase from chromatin as a prerequisite for postmitotic nuclear envelope reassembly in metazoans.[10]

Proteasomal degradation pathways requiring Cdc48 include, among others, the endoplasmic reticulum-associated protein degradation (ERAD) pathway, where Cdc48 dislocates substrates from the ER membrane to the cytosol;[11,12] the ubiquitin fusion degradation (UFD) pathway for the degradation of engineered linear fusion proteins with ubiquitin;[13,14] degradation of the budding yeast transcription factor Spt23 p90[15] and of the metazoan myosin chaperone UNC-45.[16] The central importance of Cdc48 for cellular protein homeostasis is underscored by the fact that mutations in the *VCP* gene give rise to the multisystemic protein aggregation disease "Inclusion body myopathy with Paget's disease of the bone and frontotemporal dementia" (IBMPFD).[17]

In order to provide specificity to its various cellular functions, Cdc48 is tightly controlled by numerous cofactors. This chapter first gives an overview about Cdc48 cofactor families and functions and then focuses on the regulation of substrate ubiquitylation by substrate-processing cofactors. Additional aspects of Cdc48 biology and regulation are covered in more detail in a number of recent reviews.[1,18-21]

Cdc48 COFACTORS

There are several hundred Cdc48 cofactors known in total and any given eukaryotic species possesses typically more than ten. Cofactors interact with Cdc48 through a small number of conserved, modular binding motifs. These Cdc48 binding modules are found in combination with ubiquitin binding domains and other domains known or assumed to interact with substrates, or with catalytic domains possessing ubiquitin ligase, deubiquitylating, or deglycosylating activities. Cdc48 possesses two major binding regions for cofactors: the amino-terminal N domain and the flexible carboxy-terminal tail (Fig. 1A).

N Domain Binding Cofactors

The majority of cofactors interacts with the N domain of Cdc48, either by virtue of a UBX(-like) domain or one of several linear binding motifs, or both (Fig. 1B).

The UBX domain was the first Cdc48 binding domain identified.[22,23] It has an ubiquitin-like fold and high overall structural homology to ubiquitin. A distinctive feature of the UBX domain is a highly conserved surface patch with the consensus sequence R ... FPR (dots indicate separation in primary sequence) that is required for the binding to the Cdc48 N domain and is absent in ubiquitin.[22,24] There is a wealth of data establishing the UBX domain as general Cdc48 binding module,[25-28] and UBX domain containing proteins constitute by far the largest family of Cdc48 cofactors (reviewed in ref. 18). The cofactors Npl4 and Otu1 interact with Cdc48 via a different ubiquitin-related domain that lacks significant sequence homology to UBX domains.[29,30] Based on the similar three-dimensional structure and binding site on the Cdc48 N domain, this domain was proposed to be termed "UBX-like".[18]

The second group of N domain binding cofactors binds to Cdc48 using linear binding motifs, including the "Binding site 1" (BS1) or "SHP box" motif,[29,31,32] the "VCP binding motif" (VBM)[33] and the "VCP interacting motif" (VIM).[34] The BS1 (consensus sequence FxGxGQRn; x, any amino acid; n, nonpolar) is found in Shp1, Ufd1, members of the Derlin family involved in ERAD, and some poorly characterized proteins. The VBM (consensus sequence E(I/L)RRRR) was first identified in the deubiquitylating enzyme ataxin-3 and is also present in vertebrate homologues of the ubiquitin ligases Ufd2 and Hrd1.[19,33,35] The VIM (consensus sequence (K/R)RxxLAxAAERRxQ) has been identified in the vertebrate Hrd1 homologue gp78 and in small VCP-interacting protein (SVIP), a negative regulator of ERAD in mammals.[34] Whereas a peptide spanning the BS1 motif of Ufd1 was shown to bind to a region of the N domain that partially overlaps with the binding site of the UBX domain,[36] the binding sites of the VBM and VIM motifs on the N domain are not precisely known.

Carboxy Terminus Binding Cofactors

The highly conserved carboxy-terminal region of Cdc48 is structurally disordered[37,38] and probably well exposed. It is the docking site for a relatively small number of cofactors, including PUB domain containing proteins, Ufd2 and Ufd3 (Fig. 1B).

The PUB (peptide:N-glycanase/UBA- or UBX-containing proteins) domain was first identified at the amino-terminus of mammalian PNGase (peptide:N-glycanase)[39,40] as a domain of unknown function and subsequently found to be a p97 interaction domain.[41,42] Structure determination of the PUB domain did not reveal significant similarity to other known structures,[41] but provided atomic detail of the interaction with the five carboxy-terminal residues of Cdc48.[42] Intriguingly, this interaction is completely abolished upon phosphorylation of the penultimate tyrosine residue of Cdc48,[42] suggesting a tight control of PUB domain cofactor binding by cellular tyrosine kinases and phosphatases.[43] The PUB domain is found in the conserved metazoan proteins PNGase, UBXD1 and RNF31, as well as in quite diverse plant and protozoan proteins linked to the ubiquitin proteasome system.[41,44]

Ufd3 (also known as Doa1) is a highly conserved Cdc48 cofactor of insufficiently understood function that interacts with Cdc48 through a so-called PUL (PLAP, Ufd3, Lub1) domain.[30,45,46] While the three-dimensional structure of the PUL domain is unknown, the human Ufd3 homologue PLAP was shown to interact with the carboxy-terminal region of Cdc48 in a tyrosine phosphorylation-sensitive manner resembling the PUB domain.[42]

Ufd2 belongs to a family of ubiquitin ligases that bind E2 ubiquitin conjugating enzymes through a so-called U-box,[47] a variant RING finger domain lacking Zn binding.[48,49] Binding of Ufd2 to Cdc48 shows an interesting evolutionary divergence (Fig. 1B). Ufd2 homologues from budding yeast and *C. elegans* interact, through a yet unknown motif next to the U-box, with a carboxy-terminal region of Cdc48 overlapping the Ufd3 and PUB domain binding sites (our unpublished data).[16] In contrast, homologues from higher eukaryotes interact with the N domain of Cdc48 via a VBM.[33,35] The evolutionary driving forces for the different binding modes as well as the consequences for Cdc48 function at the molecular and cellular level are still unknown.

Substrate-Recruiting and Substrate-Processing Cofactors

Most Cdc48 cofactors can be categorized as either substrate-recruiting or substrate-processing cofactors on the basis of known function or suggestive domain composition (Fig. 2).[18,30]

Substrate-recruiting cofactors are typically adaptor proteins combining modules for the binding of ubiquitin (and thus ubiquitylated substrates) and Cdc48. They recognize

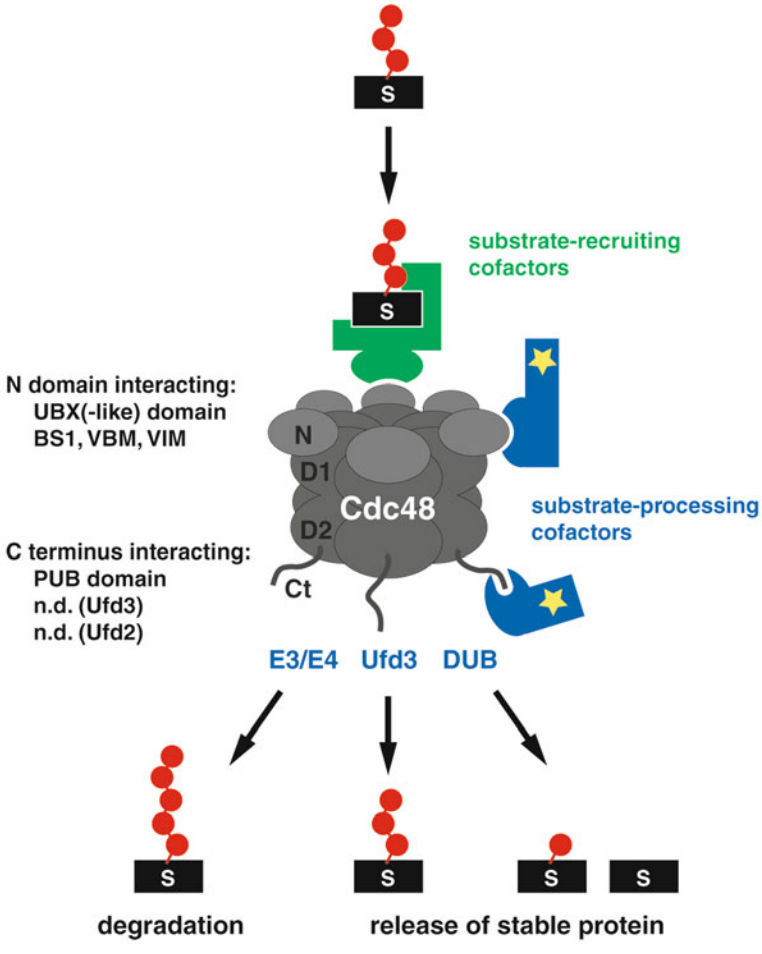

Figure 2. Cdc48 cofactor binding. Substrates (S) marked by short ubiquitin chains (red circles) are recognized by substrate-recruiting cofactors (green) and segregated from protein complexes, lipid membranes, or chromatin (not shown). Substrate-processing cofactors (blue) catalyze substrate polyubiquitylation targeting it for degradation by the 26S proteasome (E3/E4; left); prevent polyubiquitylation (Ufd3; middle); or catalyze deubiquitylation (DUBs; right). The latter two options result in the release of metabolically stable substrates. Enzymatic activity of substrate-processing cofactors is indicated by yellow asterisks. The domain organisation of Cdc48 (N, D1, D2; Ct: carboxy-terminus) and interaction modules of cofactors are indicated. n.d., the Cdc48 binding motifs of Ufd2 and Ufd3 are not known in detail. Modified from reference 30.

substrates apparently carrying relatively short ubiquitin chains[4,15,30] and deliver them to Cdc48 (Fig. 2). Prototypical examples are the UBA-UBX domain protein Shp1 and the heterodimeric Ufd1-Npl4 cofactor, which combines a ubiquitin binding N domain and two different Cdc48 binding motifs in its two subunits (Fig. 1B).

Shp1 and its mammalian homologue p47 function in the Cdc48-dependent fusion of Golgi fragments,[50] ER membranes,[51] nuclear envelope vesicles[52] and the yeast vacuolar membrane.[53] The critical function of Cdc48 in Golgi and ER membrane fusion was proposed to be the disassembly of SNARE complexes containing the SNARE syntaxin 5, thereby priming syntaxin 5 for another round of membrane fusion.[6,54] However, there is to date no proof that Cdc48 and p47 are directly involved in the disassembly of syntaxin 5-containing SNARE complexes in vivo (see refs. 8, 55 for discussion), and physiological substrates of Shp1/p47 are still elusive. Nevertheless, biochemical analyses of postmitotic Golgi vesicle fusion provided convincing evidence for the existence of a critical p47 target that is ubiquitylated, but not degraded by the 26S proteasome.[7,56]

In contrast to Shp1/p47, a number of targets of the Ufd1-Npl4 adaptor are known. These include substrates of the ERAD[57-61] and UFD[13] pathways, but also the processed p90 form of the transcription factor Spt23[4] and the chromatin-associated kinase Aurora B at mitotic exit,[10] which are not destined for immediate degradation. In addition, Ufd1-Npl4 has additional functions during the cell cycle, even though specific targets have not been identified in all cases.[62-66]

Shp1 and Ufd1-Npl4 bind to Cdc48 in a mutual exclusive manner, suggesting that they specify distinctly different cellular functions and/or molecular activities of Cdc48.[18,67] Besides these major substrate-recruiting cofactors, a larger number of additional substrate-recruiting cofactors appears to exist. They bind Cdc48 in concert with Ufd1-Npl4 or Shp1 and thus provide a further layer of specificity control.[18] This is best exemplified by the budding yeast UBA-UBX protein Ubx2. Ubx2 is an integral ER membrane protein that recruits the $Cdc48^{Ufd1\text{-}Npl4}$ complex to the ER, coordinates its interactions with ERAD substrates and ubiquitin ligases and thereby ensures efficient degradation of ERAD substrates.[68,69] Several other Cdc48 cofactors have been proposed to serve similar functions in yet to be identified cellular processes (discussed in ref. 18).

Substrate-processing cofactors assist the turnover of substrates subsequent to their recruitment to Cdc48 (Fig. 2). In general, these cofactors are enzymes, even though Ufd3 has been classified as substrate-processing based on its ability to negatively regulate binding of Ufd2.[30] Enzymatic activities include the glycanase activity of PNGase that catalyzes removal of sugars from glycosylated ERAD substrates,[70,71] but more frequently ubiquitin ligase or deubiquitylating activities (Fig. 1B). This suggests that the ubiquitylation state of Cdc48 substrates is actively controlled by substrate-processing cofactors, which will be the focus of the following section.

CONTROL OF UBIQUITYLATION STATE BY SUBSTRATE-PROCESSING COFACTORS

Cofactors with E3/E4 Ubiquitin Ligase Activity

Budding yeast Ufd2 was the first substrate-processing cofactor to be characterized.[47] It had initially been identified in a genetic screen for yeast mutants that stabilize the UFD

substrate ubiquitin-proline-β-galactosidase.[13] Intriguingly, Ufd2 was found to be unable to catalyze the de novo ubiquitylation of UFD substrates on its own.[47] Rather, the E3 ubiquitin ligase Ufd4 has to catalyze the conjugation of one or few ubiquitin moieties to UFD substrates first. UFD substrates carrying such short ubiquitin tags are subsequently recognized by the $Cdc48^{Ufd1\text{-}Npl4}$ complex and polyubiquitylated by Ufd2 (Fig. 3A).[47] This ubiquitin chain-elongating activity of Ufd2 has been termed "E4 activity" to indicate its dependence on prior E1, E2 and E3 action.[47] Mass spectrometric analyses demonstrated that Ufd2 catalyzes the formation of K48-linked polyubiquitin chains,[72] in line with its role in proteasomal degradation. In addition to its enzymatic activity, Ufd2 binds the proteasomal receptor proteins Rad23 and Dsk2, thereby ensuring that polyubiquitylated substrates destined for degradation are escorted from the $Cdc48^{Ufd1\text{-}Npl4\text{-}Ufd2}$ complex to the 26S proteasome.[15]

Besides its essential role in the UFD pathway, Ufd2 is also important for the degradation of ERAD substrates[15,73] and of Spt23 p90.[15] In both cases, the initial ubiquitylation is catalyzed by E3 ubiquitin ligases at the ER membrane. Importantly, this two-step ubiquitylation of substrates by consecutive E3 and E4 activities provides additional opportunities for the regulated degradation of Cdc48 substrates, as will be discussed below.

Analysis of Ufd2 homologues in metazoans revealed additional interesting roles of Ufd2 and Cdc48. In *C. elegans*, UFD-2 and CDC-48 are required for the developmentally regulated degradation of the myosin chaperone UNC-45.[16,74] UNC-45 has to be degraded in the young adult stage of worms in order for normal myofiber differentiation to proceed. Besides UFD-2, this process requires an additional U-box family ubiquitin ligase, the CHIP homologue CHN-1, which forms a complex with CDC-48 and UFD-2 but does not directly bind to CDC-48.[16,74] Intriguingly, the simultaneous presence of UFD-2 or CHN-1 results in the efficient polyubiquitylation of UNC-45 in vitro, whereas either ubiquitin ligase alone can only catalyze the formation of short ubiquitin chains on UNC-45.[74] Thus, both proteins can be considered as hybrid E3/E4 ubiquitin ligases whose precise catalytic activity depends on each other and, presumably, on the positioning of target lysine residues on their substrates. Importantly, the role of Cdc48 and Ufd2 in myosin chaperone degradation appears to be conserved in mammals and has been linked to the myopathy manifestation of IBMPFD.[16]

Recently, the human PUB domain containing RING finger protein RNF31 emerged as a further *bona fide* substrate-processing cofactor of Cdc48.[41,44] RNF31 forms a complex with the RING finger E3 ubiquitin ligase HOIL-1.[75] Surprisingly, this complex was found to catalyze the formation of linear, head-to-tail-linked ubiquitin chains on substrates and has therefore been termed LUBAC (linear chain ubiquitin assembly complex).[75] Importantly, the NEMO (also known as IKKγ) regulator of the canonical NF-κB pathway has been shown to be modified with linear Ub chains by LUBAC.[76] This modification does not target NEMO for degradation, but serves as a positive signal in the activation of the canonical NF-κB pathway.[76] It will be interesting to see if Cdc48 influences the ubiquitin ligase activity of LUBAC and/or the turnover of LUBAC substrates.

In addition to Ufd2 and RNF31, the mammalian ERAD E3 ubiquitin ligases Hrd1 and gp78 possess Cdc48 binding motifs and thus may be considered substrate-processing cofactors.[35,77] However, budding yeast Hrd1 lacks these motifs and interacts with Cdc48 through Ubx2,[68,69] suggesting that direct Cdc48 binding by the mammalian homologues may merely stabilize the interaction with Cdc48 rather than influencing the ubiquitylation state of ERAD substrates subsequent to their recruitment to Cdc48.

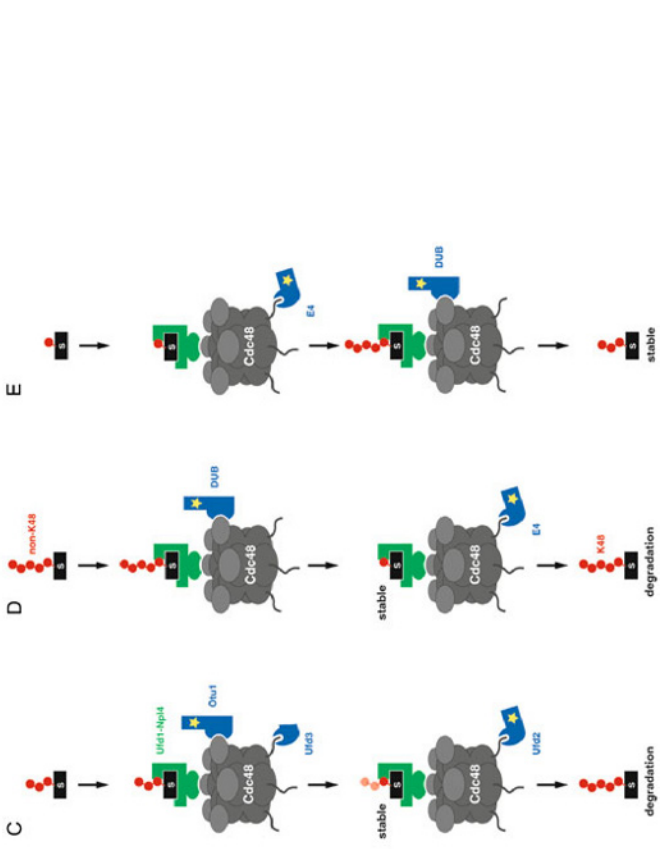

Figure 3. Regulation of ubiquitylation state by substrate-processing cofactors. Different fates of Cdc48 substrates regulated by substrate-processing cofactors are illustrated according to the schematic from Figure 2. A) Ubiquitin chain elongation. In the ERAD and UFD pathways, substrates are recruited by Ufd1-Npl4, polyubiquitylated by Ufd2 and degraded. B) Ubiquitin chain trimming. In postmitotic reassembly of Golgi fragments, an unknown substrate is recruited by p47, deubiquitylated by VCIP135 and released as stable protein. C) Delayed ubiquitin chain elongation. In the OLE pathway, the substrate Spt23 p90 is recruited by Ufd1-Npl4, mobilized from the ER membrane and maintained in a stable state by Otu1 and Ufd3. Subsequent to transport into the nucleus and activating transcription (not shown), p90 is polyubiquitylated by Ufd2 and degraded. It is unknown if p90 remains bound to the same Cdc48 complex along the entire pathway, or whether it is handed over from a $Cdc48^{Ufd3\text{-}Otu1}$ complex to a $Cdc48^{Ufd2}$ complex. The actual signal for cofactor switching is also unknown. D) Ubiquitin chain editing. Substrate modified by a nonK48-linked ubiquitin chain is recruited to Cdc48, deubiquitylated by a DUB cofactor, polyubiquitylated via K48 linkages by an E4 and degraded. Note that polyubiquitylation via nonK48 linkages by a different E4 may result in the release of a stable protein carrying a nondegradative ubiquitin signal. E) Ubiquitin chain proofreading. Substrate excessively polyubiquitylated by an E4 is trimmed by a DUB and released as a stable protein carrying a nondegradative ubiquitin signal.

Cofactors with Deubiquitylation Activity

VCIP135 and Otu1 are members of the otubain family of deubiquitylating enzymes (DUBs)[78,79] and bind Cdc48 by virtue of a UBX(-like) domain. VCIP135 was identified as a p47 interacting protein, but forms a relatively unstable ternary complex with Cdc48 and p47.[54] It is required for the fusion of Golgi and ER membranes and has been implicated in the turnover of syntaxin 5 by Cdc48 and p47 (see above).[54] In a biochemical assay for the Cdc48- and p47-dependent reassembly of postmitotic Golgi fragments, the DUB activity of VCIP135 was shown to reverse a poorly defined ubiquitylation event occuring during mitotic Golgi disassembly.[7] Because the ubiquitin-binding UBA domain of p47 is required in the same assay,[56] the most straightforward interpretation is that VCIP135 deubiquitylates a critical substrate after its recruitment by $Cdc48^{p47}$ (Fig. 3B).[7] Of note, the DUB activity of VCIP135 does not merely prevent proteasomal degradation of this substrate, but rather appears to remove an inhibitory effect of its ubiquitylation.[7] Consistent with this interpretation, Golgi and ER assembly during interphase, which does not appear to involve substrate ubiquitylation, requires VCIP135, but not its DUB activity.[80]

Budding yeast Otu1 was identified in a candidate search for DUBs that genetically interact with Ufd3.[30] Otu1 cleaves K48-linked ubiquitin chains in vitro and can negatively regulate the degradation of Cdc48 substrates in vivo, presumably by reducing the length of their ubiquitin chain.[30] Otu1 and Ufd3 appear to cooperate in antagonizing the Ufd2-catalyzed polyubiquitylation of some Cdc48 substrates, but it is not known if Otu1 activity depends on Ufd3 in general. While the opposing activities of Ufd2 and Otu1-Ufd3 serve as a paradigm for the regulation of Cdc48 substrate turnover by substrate-processing cofactors (see below), the physiological role of Otu1 is still unclear as cellular pathways critically dependent on Otu1 have not been identified yet.

The DUB ataxin-3 consists of an amino-terminal, catalytic Josephin domain[78,79] and a carboxy-terminal part containing a VBM and three ubiquitin interacting motifs (UIMs) (Fig. 1). In addition, the carboxy-terminal region harbours a polyglutamine stretch whose expansion gives rise to the neurodegenerative disease spinocerebellar ataxia Type 3 (SCA3; also known as Machado-Joseph disease; OMIM 607047).[81] Ataxin-3 binds K48- and K63-linked chains of at least six ubiquitin moieties with comparable affinity, but cleaves only K63 linkages efficiently.[82] Remarkably, mixed K48-/K63-linked chains turned out to be the preferred substrate for cleavage of K63 linkages, leading to the proposal that the cellular function of ataxin-3 involves trimming of non-K48 linkages from ubiquitylated substrates to ensure efficient proteasomal degradation.[82]

Ataxin-3 has been implicated in the degradation of ERAD substrates,[83,84] but its precise role in ERAD is unclear. Overexpression of wildtype ataxin-3 interfered with the degradation of the ERAD substrates TCRα and CD3δ, while siRNA-induced depletion slightly accelerated their degradation and decreased the sensitivity against the ER stress-inducing drug tunicamycin.[84,85] At face value, these results do not support the hypothesis that ataxin-3 ensures optimal proteasomal delivery of ERAD substrates, but rather suggest that ataxin-3 is a negative regulator of ERAD. Further studies are needed to clarify if ataxin-3 may act on certain (ERAD) substrates decorated with mixed-linkage ubiquitin chains to prepare them for proteasomal degradation.

COMBINED ACTION OF E3/E4 AND DUB SUBSTRATE-PROCESSING COFACTORS

The different effects of the budding yeast cofactors Ufd2, Ufd3 and Otu1 on the ubiquitylation state of substrates led to the formulation of a "gearbox" model for substrate turnover by Cdc48.[30,86] According to this model, Ufd2 activity represents the "forward" position for polyubiquitylation and, consequently, degradation of substrates. Ufd3 binding is "neutral" in that the initial ubiquitylation state of substrates remains unchanged, whereas Otu1-catalyzed deubiquitylation is "reverse" because it prevents degradation. The gearbox model is a powerful illustration of the fundamental possibilities of substrate-processing cofactors to regulate the fate of Cdc48 substrates. However, whereas binding of single substrate-processing cofactors is sufficient to switch between different fates according to the gearbox model, combinatorial binding of substrate-processing cofactors provides additional regulatory potential through antagonistic or synergistic effects.

Antagonistic interactions of substrate-processing cofactors can either rely on competitive binding to Cdc48 or on opposing enzymatic activities of the cofactors. Both mechanisms have been demonstrated to be in operation in the regulation of Cdc48 by Ufd2, Ufd3 and Otu1.[30] Ufd2 and Ufd3 compete for the same binding site on Cdc48 and overexpression of *UFD3* inhibits the Ufd2-dependent degradation of ubiquitin-proline-β-galactosidase, most likely by blocking access of Ufd2 to Cdc48. *OTU1* overexpression, on the other hand, inhibits the Ufd2-mediated degradation of Spt23 p90 in a manner dependent on its DUB activity, suggesting that it catalyzes the removal of ubiquitin chains from Spt23 p90. It should, however, be noted that these experiments relied on overexpression of one of the cofactors. Given the abundance of Cdc48 in the cell, it is not clear whether Ufd2 and Ufd3-Otu1 are competitors at their physiological expression levels as well. For instance, it has not been shown that loss of Ufd3 and/or Otu1 leads to accelerated degradation of Cdc48 substrates in Ufd2-dependent pathways. In protein quality control pathways like ERAD, where Cdc48 turns over misfolded proteins definitely destined for proteasomal degradation, a competition between Ufd2 and Ufd3-Otu1 would be counterproductive, as it would delay rapid delivery to the 26S proteasome and prolong the half-life of aggregation-prone substrates.

From a physiological point of view, it could be argued that Ufd2 and Ufd3-Otu1, rather than being antagonists, in fact act synergistically in the OLE pathway because they ensure the correct integration of ubiquitylation events into cellular signal transduction. Mono- or oligo-ubiquitylated p90 is initially dislocated from the ER membrane by $Cdc48^{Ufd1\text{-}Npl4}$,[4] and Ufd3 and Otu1 presumably preserve its ubiquitylation state (Fig. 3C, upper half).[30] Consequently, p90 is transported as a (meta-)stable transcription factor to the nucleus, where it activates transcription of *OLE1*.[4] The transcription factor activity of p90 is terminated by Ufd2, which polyubiquitylates p90 and thus triggers its proteasomal degradation (Fig. 3C, lower half).[15] The overall relationship of the substrate-processing cofactors in this pathway can be viewed as synergistic in that they mediate a correctly timed and localized "delayed elongation" of the ubiquitin chain. While the OLE pathway is so far the only example for the cooperation of cofactors possessing opposing activities, similar regulatory mechanisms are likely to exist in other pathways involving substrates whose function is controlled by differential ubiquitylation.

Opposing enzymatic activities of Cdc48 substrate-processing cofactors are a specific manifestation of the more generally observed coexistence of ubiquitin ligases and DUBs in the same protein complex.[78,79] Consequently, several other models for the function of

combined ubiquitin ligase and DUB activities are also plausible for Cdc48 cofactors, though purely speculative at present. In a "chain editing" model, a DUB cofactor catalyzes the removal of one specific ubiquitin chain/linkage, e.g., K63-linked, from Cdc48 substrates in order to allow an E3/E4 cofactor to assemble a different type of ubiquitin chain, e.g., K48-linked (Fig. 3D). In principle, ataxin-3 with its specificity for K63 linkages in mixed chains and mammalian Ufd2a/E4B could be such a pair of cofactors and have indeed been found to form a complex with Cdc48.[87] While there is currently no convincing evidence for a positive function of either cofactor in mammalian ERAD, it is tempting to speculate that ataxin-3 may edit ubiquitin chains on other E4B-dependent substrates like the myosin chaperone UNC-45.

According to a "proofreading" model, a DUB cofactor corrects excessive E4 activity by trimming polyubiquitin chains, resulting in the release of a metabolically stable substrate with a short, nonproteolytic ubiquitin tag (Fig. 3E). Alternatively, the DUB cofactor could proofread branched or very long polyubiquitin chain products of E4 activity to release them with polyubiquitin chains taylored for optimal proteasomal degradation (not shown; see ref. 15 for discussion of chain length restriction).

Last, but not least, DUB cofactors may function to counteract E3/E4 side reactions like auto-ubiquitylation or ubiquitylation of Cdc48 and cofactors in order to prevent their accidental degradation, similar to a number of other E3-DUB pairs.[78,79]

CONCLUSION AND FUTURE PERSPECTIVES

Cdc48 interacts with a fascinating multitude of different cofactors whose precise cellular functions are still largely unknown. While there is clear evidence for the control of Cdc48 substrate ubiquitylation state by a small number of well-studied substrate-processing cofactors, the molecular basis for combinatorial cofactor binding and its consequences for substrate ubiquitylation remain a challenging and rewarding topic for future investigation. Intriguingly, several E3 ubiquitin ligases and DUBs lacking recognizeable Cdc48 binding motifs were recently identified as Cdc48 interactors[28,85] and are likely to provide even more diverse scenarios for the Cdc48-mediated recognition and regulation of ubiquitin chain plasticity.

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CHAPTER 3

REGULATION OF UBIQUITIN RECEPTORS BY COUPLED MONOUBIQUITINATION

Daniela Hoeller and Ivan Dikic*

Abstract: The regulation of a variety of cellular processes, such as endocytosis, DNA-repair or signal transduction relies on the inducible modification of proteins with Ubiquitin (Ub). Ub-receptors, i.e., effector proteins carrying Ub-binding domains (UBDs), recognize ubiquitinated proteins and trigger specific cellular responses. The activity of Ub-receptors is controlled by "coupled monoubiquitination" which provides an efficient switch from an active to an inactive conformation. In this chapter we discuss the molecular basis of the underlying processes of coupled monoubiquitination and their physiological significance.

UBIQUITINATION

Ubiquitination labels proteins in a highly specific manner by the covalent attachment of ubiquitin (Ub) to a lysine residue of a targeted protein. This normally occurs in a three-step process involving the sequential action of ubiquitin activating (E1), conjugating (E2) and ligating (E3) enzymes (Fig. 1).[1] Substrate proteins can be modified by ubiquitin in several ways: by monoubiquitination (addition of a single Ub molecule), multiubiquitination (addition of multiple single Ub molecules onto different lysines of the substrate) or polyubiquitination (assembly of a chain of Ub molecules onto one lysine residue of the substrate). Since Ub contains seven lysines that can serve as acceptor sites for chain elongation, Ub chains of different topologies exist. Whatever type of ubiquitin modification is attached to a protein it can be reversed by the action of deubiquitinating enzymes (DUBs) allowing the protein to escape from its fate imposed by the Ub tag.[2,3]

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Conjugation and Deconjugation of Ubiquitin Family Modifiers, edited by Marcus Groettrup.

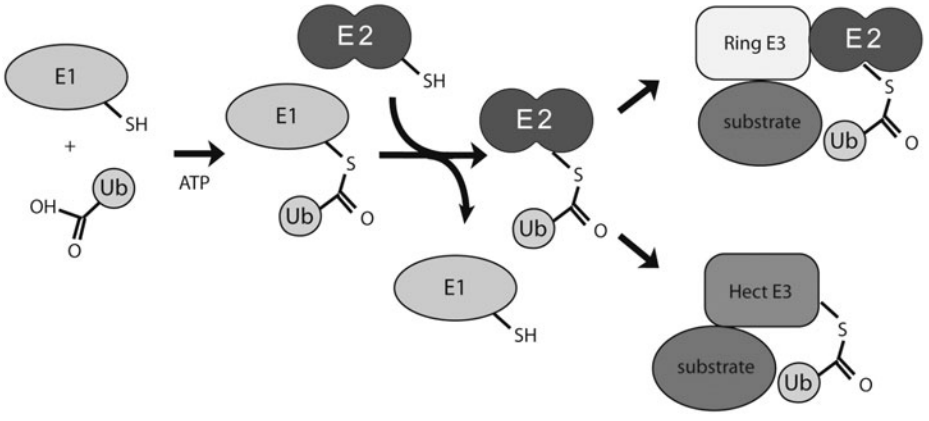

Figure 1. The covalent attachment of Ubiquitin (Ub) to a substrate requires the subsequent action of three enzymes. In the first step, the C-terminus of Ub is activated in an ATP-dependent manner by forming a thiolester with the E1 Ub-activating enzyme. It is then passed on to the E2 conjugating enzyme. Ubiquitination occurs when an E3 ligase enzyme binds to both substrate and E2 ~ Ub, bringing them in proximity so that the ubiquitin is transferred from the E2 to the ε-aminogroup of a lysine in the substrate. Whereas RING type ligases mediate the direct transfer of Ub from E2 to the substrate, HECT type ligase form themselves a thioester intermediate with Ub to achieve ubiquitination.

It was observed that different Ub modifications have specific consequences for the tagged protein. Monoubiquitination has been implicated in endocytic protein transport and DNA-repair; K48-linked polyUb chains label the protein for proteasomal destruction; K63-linked chains function to promote the assembly of signalling complexes, e.g., during DNA-repair or NF-κB signalling (Fig. 2).[4-6] Recently, the newly described linear head-to-tail-linked Ub chains were shown to be involved in NF-κB signalling as well.[7] This variety of specific functions is supported by proteins that are able to recognize and distinguish different types of Ub-modifications and convert the Ub-encoded message into a cellular response. These proteins are called Ub-binding proteins or Ub-receptors and are key components in the cellular Ub-network. The deregulation of this elaborate network has been implicated in human pathogenesis, including the development of many types of tumours.[8,9]

UB-RECEPTORS ARE EQUIPPED WITH UB-BINDING DOMAINS (UBDs)

Ub-receptors are characterized by the presence of one or more Ub-binding domains (UBDs). The list of known UBDs is constantly growing. To date, around two dozen UBDs have been characterized structurally and functionally.[4,10,11] UBDs are rather small (20-150 amino acids) modular domains that fold predominantly into α-helical structures (UBA, UIM, MIU, DUIM, CUE, GAT, TOM) or Ub-binding zinc fingers (NZF, PAZ, UBZ). Other types of folds are represented by GLUE, Pru, Jab1/MPN and UEV domains. In most cases UBDs bind Ub with low or moderate affinity (K_d values range between 2-500 μM) allowing flexible and highly dynamic interactions. However, the presence of multiple copies of UBDs within an Ub receptor and/or the modification of substrates with multiple Ub moieties can significantly enhance the strength of interaction.

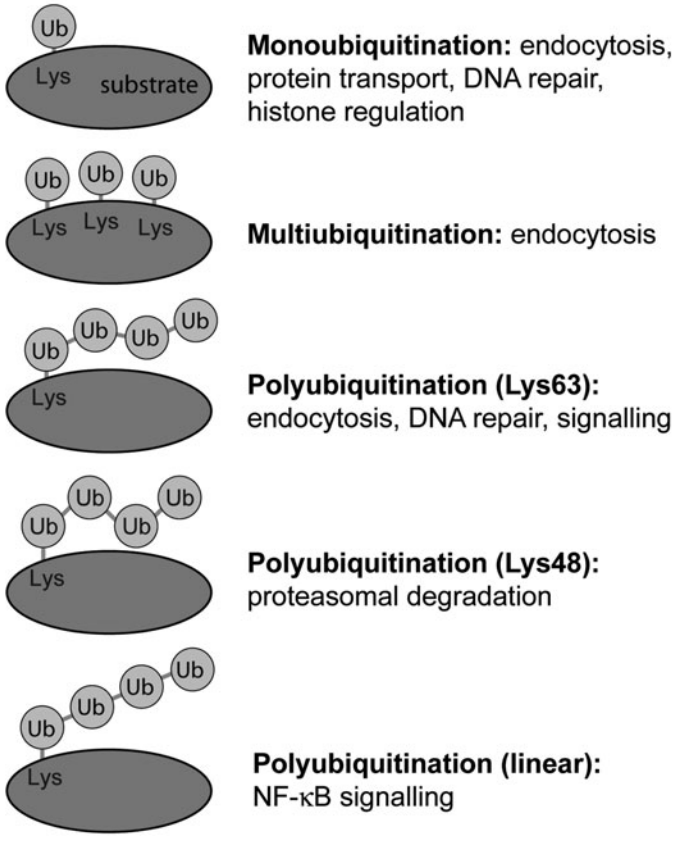

Figure 2. Various types of ubiquitination have been detected in cells and linked to the indicated cellular processes. The attachment of a single Ub-molecule is called "monoubiquitination". The monoubiquitination of multiple lysines within a substrate molecule results in "multiubiquitination". Polyubiquitination occurs when a lysine in ubiquitin serves as acceptor side in iterative rounds of ubiquitination. Dependent on the utilized lysine chains of different topologies are assembled. K48-linked and K63-linked chains are the most common types.

Although there is an obvious connection between Ub linkage type and cellular response there are only few examples of UBDs that show a clear-cut preference for a specific type of Ub-modification in vitro. It is therefore believed that the microenvironment (surrounding domains, local concentration of binding partners and/or presence of other proteins) in which Ub-UBD interaction takes place contributes to the observed specificities as well as affinities in physiological settings. Indeed, there is experimental evidence that a tandem-arrangement of UBDs in multi-valent Ub receptors (for example the two UIMs present in Rap80) allow high-affinity interactions only with a specific linkage type (in case of Rap80 with K63-linked polyUb chains) but not with others.[12] Similarly, it was observed that the GST-induced dimerization of UBDs leads to special positioning that greatly favours binding of a number of UBA domains to K63-linked Ub chains over K48-linked chains. This linkage-specificity was lost when the same UBA domains were analysed after GST-cleavage, i.e., in their monomeric state.[13]

Interestingly, most UBDs, including UIM, UBA, UBZ, UBM, VHS, CUE and GAT, can bind noncovalently to Ub that is attached to other proteins and can also mediate the covalent coupling of monoUb to their host protein.[14] This process has been termed "coupled monoubiquitination" because the self-ubiquitination of the Ub-receptor is intimately coupled to its Ub-binding ability. Intriguingly, the fusion of a functional UBD to a protein that is normally not ubiquitinated (such as GFP or GST) is sufficient to induce its monoubiquitination.[14-17]

FUNCTIONAL CONSEQUENCES OF COUPLED MONOUBIQUITINATION

The close link between Ub-binding ability and ubiquitination of UBD-proteins raises the question of the molecular and cellular meaning of coupled monoubiquitination. Crucial insights were gained by in vitro experiments showing that UBD-proteins lose their Ub-binding activity once they are monoubiquitinated.[15] Further analysis revealed that this is due to intramolecular interactions between the UBD and the attached Ub. This proved to be very efficient and robust even when Ub was artificially fused to the C-terminus of the protein. Apart from the functional inactivation of the UBD further protein-specific consequences of coupled monoubiquitination can be envisioned, including changes in enzymatic activity, binding properties or intracellular localization.

The cellular role of Ub-receptors as well as coupled monoubiquitination has been best studied in the Ub-dependent endo-lysosomal trafficking of receptor tyrosine kinases (RTKs). After growth-factor binding and signal initiation activated RTKs have to be downregulated by endocytosis and subsequently degraded in the lysosome to avoid hyperstimulation of the cell.[18] For lysosomal degradation RTKs need to be ubiquitinated and sorted into vesicles that bud into the lumen of endosomes during the biogenesis of multivesicular bodies (MVBs).[19] This process involves several Ub-receptors, such as Eps15 and epsins as well as Hrs, Stam2, Tsg101 and others that are part of large protein assemblies known as "endosomal sorting complexes required for transport" (ESCRT). These receptors are localized on endosomal membranes, recognize ubiquitinated RTKs via their UBDs and trap them in specialized membrane domains that will bud inwards to give rise to intraluminal vesicles. Unmodified proteins escape this process and recycle back to the plasma membrane instead of being degraded in the lysosome. The effects of coupled monoubiquitination on endosomal-lysosomal trafficking were assessed in some detail for Eps15 and Hrs. Both of them become monobubiquitinated in response to EGF stimulation. The functional analysis of Eps15-Ub in cells revealed that it failed to localize properly on EGF receptor-containing endocytic vesicles.[15] As a consequence, monoubiquitination interfered with the ability of the Eps15 UIMs to bind ubiquitinated EGF receptor, thereby delaying receptor internalization and degradation.[20]

A similar scenario has recently been proposed for Rabex-5, a guanine nucleotide exchange factor (GEF) for Rab5.[21] Rab5 is small GTPase that is enriched on clathrin-coated vesicles and endosomes. Together with Rabex-5 it is a key regulator of homotypic and heterotypic endosomal fusion.[22,23] Rabex-5 contains two UBDs: an A20-like ZnF and an inverted UIM domain (MIU, motif interacting with Ubiquitin), the latter required for coupled-monoubiquitination.[24,25] Although, Rabex-5 interacts with several endosomal proteins, including Rab-5 (Rabaptin-5), monoubiquitination of Rabex-5 was sufficient to preclude its recruitment to endosomes. It was thus unable to cooperate with Rab-5 and promote endosomal cargo trafficking.

In case of Hrs, which functions at later steps of endocytosis, monoubiquitination does not seem to affect its localization on endosomes nor the binding to its essential dimerization partner, Stam2.[15] However, using an artificially ubiquitinated Transferrin receptor (TfR-Ub) as a model substrate, it became evident that Hrs-Ub was unable to recognize and sort ubiquitinated cargo towards the lysosome.[15] Thus, in accordance with the in vitro model of monoubiquitinated Hrs, Rabex-5 as well as Eps15 seems to negatively modulate their UIM-dependent endocytic functions in cells. During endocytosis this might serve to fine-tune irreversible receptor down-regulation.

Another UBD-containing endocytic regulator undergoing coupled monoubiquitination is Sts-2.[26-28] Besides its N-terminal UBA, Sts-2 contains a SH3 domain that binds constitutively to the RING E3 ligase, Cbl. This interaction is required for recruiting Sts-2 to receptor tyrosine kinases (RTKs) upon ligand binding and receptor activation. Once in complex with the activated receptor the UBA domain seems to bind to the Ub moieties attached to the receptor by Cbl.[27] In this way, Sts-2 blocks the recognition of the ubiquitinated receptor by endocytic sorting proteins such as Eps15 and causes the accumulation of activated RTKs at the cell surface where they continue to emit signals instead of being down-regulated. In this way Sts-2 might contribute to oncogenic processes in vivo. In contrast to most monoubiquitinated proteins Sts-2 contains a single lysine that serves as the acceptor site for Ub (K202). It thus represents one of the rare examples of UBD-proteins where it was possible to analyse the functional consequences of a point mutation that renders the protein non-ubiquitinated while its UBD is fully functional. Importantly, this mutant corroborated the notion that monoubiquitination of Ub-receptors provides a switch-off mechanism as the ubiquitination-deficient Sts-2 K202R mutant was significantly more active than the wild type protein in stabilizing activated EGF receptor in cells.[15]

The concept of auto-inhibition by coupled monoubiquitination is not only valid for endocytic proteins but also for UBD-proteins involved in other cellular processes such as translesion polymerases that facilitate DNA-repair (Fig. 3). Translesion synthesis (TLS) past DNA lesions requires specialised DNA polymerases, belonging mostly to the Y-family of polymerases.[29,30] All the members of the human Y-family, polη, polι, polκ and Rev1 contain UBDs located in their C-terminal regions that are implicated in the onset of TLS.[31] In order to translocate to the site of DNA damage Y-polymerases need to sense the damage-induced monoubiquitination of PCNA (proliferating-cell nuclear antigen) that forms a ring around the DNA to facilitate and control DNA replication.[32] The same UBDs also mediate coupled monoubiqitination of a fraction of Y-polymerases rendering them unable to bind to monoubiquitinated PCNA. This has two reasons: (1) The UBD is blocked by the autoinhibited conformation[31] and (2) one of the identified Ub-sites in polη is located in a secondary PCNA binding surface.[33] The combination of both keeps polη away from replication forks and/or might facilitate the dissociation of the TLS polymerase from PCNA after TLS has been completed.

E3-DEPENDENT COUPLED MONOUBIQUITINATION

Given that the presence of an UBD is the prerequisite for coupled monoubiquitination it was speculated that it serves as binding site to recruit the ubiquitination machinery (or part of it) to the substrate. Indeed there was evidence that E3-ligases of the Nedd4 HECT-family are, at least in part, involved in the coupled monoubiquitination of

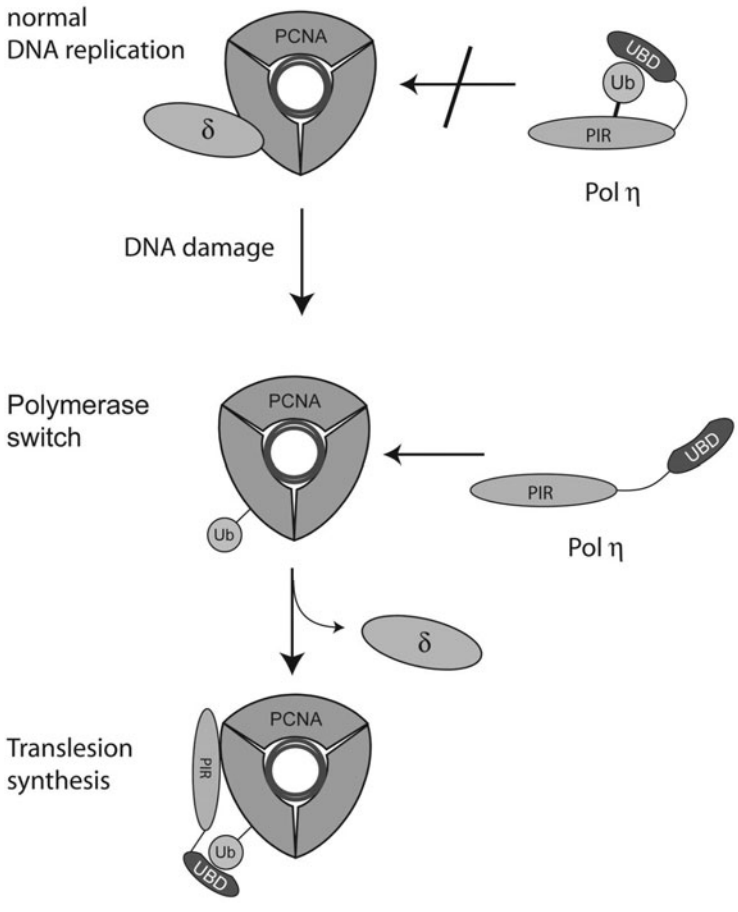

Figure 3. Regulation of translesion synthesis by monoubiquitination of PCNA and polη. UBD-mediated monoubiquitination of polη results in intramolecular interaction between Ub and the UBD keeping polη away from PCNA during normal replication by δ-polymerase. Following DNA-damage, monoubiquitination of PCNA and deubiquitination of polη facilitate the exchange of δ-polymerase by the Y-polymerase polη and the onset of translesion synthesis.

several endocytic adaptor proteins containing one or more UIM domains (Eps15, epsins, Hrs).[34] HECT type ligases do not only ubiquitinate their substrates but also monoubiquitinate themselves. It turned out that this event is crucial to engage the E3 ligase Nedd4 in coupled monoubiquitination reactions with Eps15 as the attached Ub-molecule is required for the binding to one of the UIM of the substrate (Fig. 4A). In contrast to RING E3 ligases (such as Cbl) that mediate the ubiquitination reaction by functioning as adaptors between E2 and substrate, HECT-type E3 ligases take over the activated Ub from the E2 before catalyzing its transfer to the substrate, also forming a thiolester intermediate with Ub. So, in principle there are two possible modes of how an Ub-dependent interaction of UBD-protein and HECT-type E3 might occur: (1) via the thiolester-bound Ub or (2) engaging the Ub that is covalently attached through and isopeptide bond. It was, however, experimentally excluded that

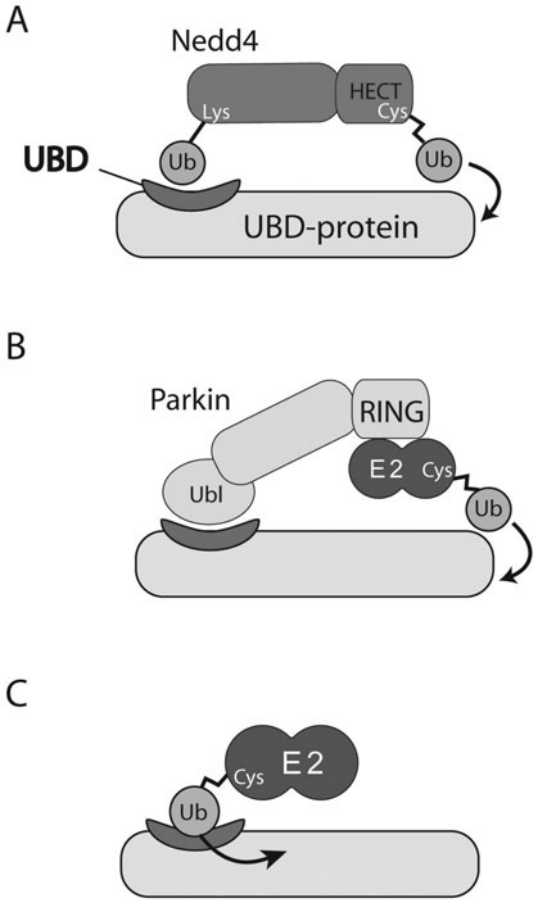

Figure 4. Mechanisms of coupled monoubiquitination. A) Monoubiquitinated HECT E3-ligases, such as Nedd4, are able to interact with the UIM of their substrate (for example Eps15) via the attached Ub molecule and transfer the thiolester-conjugated Ub on a substrate lysine. B) RING E3 ligases decorated with an Ubl domain, such as Parkin, bind to the UIM of the substrate via the Ubl. The RING domain recruits an Ub-charged E2 from which Ub is transferred to the substrate. C) Ub-charged E2 can interact directly with a variety of UBDs which allows the direct transfer of Ub from the E2 on a lysine in the substrate in the absence of an E3 ligase.

the 'thiolester Ub' plays a significant role in mediating the binding to the Ub-receptor. The 'isopeptide model' was further corroborated by the fact that only the subset of HECT-type ligases that are subjected to monoubiquitinated can mediate coupled monoubiquitination.[34]

The RING-type E3 ligase Parkin is a second ligase that has been implicated in the monoubiquitination of Eps15 (Fig. 4B).[20] The mechanism of how this occurs is very similar to Nedd4, although Parkin has no similarity with HECT-types ligase nor is it monoubiquitinated. Instead of being ubiquitinated posttranslationally, Parkin contains an aminoterminal Ub-like (Ubl) domain. In analogy to the attached Ub-molecule in Nedd4 the Ubl domain enables binding of Parkin to the UIM of Eps15.

E3-INDEPENDENT COUPLED MONOUBIQUITINATION

Although E3 ligases such as Nedd4 and Parkin are able to monoubiquitinate Eps15 in response to growth factor stimulation there is evidence that other mechanisms of coupled monoubiquitination exist. Firstly, the monoubiqitination of most UBD-containing proteins (including Eps15 and several other endocytic proteins) does not require cell stimulation and can happen independently of overexpression of known E3 ligases. Secondly, the extent of monoubiquitination of Eps15 is not changed in cells that have been depleted of Nedd4 and Parkin. Indeed, it was shown that UBDs confer cis-ligase activity on their host proteins by recruiting Ub-loaded E2 enzymes (Fig. 4C).[35] The ability to directly bind to and ubiquitinate substrates in the absence of E3 ligases was observed for several different E2 enzymes and was strictly dependent on a functional UBD/Ub-interface. Notably, the E2 enzymes seem to participate in the reaction with a different degree of efficiency and substrate specificity in vitro. The analysis of nonphysiological UBD-fusion proteins revealed that this specificity stems from the type of UBD as well as from residues located outside the Ub/UBD interface. This combination might ensure the optimal positioning of the acceptor lysine within a substrate towards the active site of the E2. Importantly, whereas UBD-proteins can act as self-ligases they do not show *trans*-ligase activity in vitro, i.e., they are no classical E3-ligases able to ubiquitinate other proteins or generate polyUb chains from monomeric Ub.

The E3-independent coupled monoubiquitinaton is both functionally and mechanistically distinct from the E3-dependent event described above. Since it is not inducible by cell stimulation it might represent a constitutively active homeostatic process that determines the amount of binding competent Ub-receptors in cells. Both mechanism are not mutually exclusive and can be responsible for coupled monoubiquitination of a certain Ub-receptor under specific cellular conditions, i.e., in a certain physiological state.

CONCLUSION AND FUTURE PERSPECTIVES

Regardless whether coupled monoubiquitination is brought via E3-dependent or independent mechanisms the outcome is the inactivation of the Ub-receptor. Changes in their ubiquitination status seem to induce a conformational switch from an ubiquitin-binding state of these proteins to an intramolecular monoubiquitin-inhibited state. This raises the question of whether and how the Ub-receptor can be re-activated. Given the key function of Ub-receptors in basic cellular process such as signal transduction, DNA-repair, inflammation and others, the regulation of their activity is of significant interest. However, strong experimental evidence to answer what counteracts coupled monoubiquitination is still missing. An obvious possibility would be the action of DUBs that are known to eliminate Ub-modifications of many proteins in a highly specific manner.[3] However, due to the robust intramolecular interaction between Ub and UBD an efficient de-ubiquitination might be sterically precluded. Thus, the re-activation of UBD-proteins might require binding of the UBD to Ub in trans prior to cleavage of the Ub moiety. According to biophysical calculations this can only happen when the UBD-protein is part of a larger protein complex offering alternative Ub-moieties to bind in close proximity.[15] The knowledge of the mechanisms underlying coupled monoubiquitination as well as de-ubiquitination might offer the basis for the highly specific manipulation of Ub-dependent cellular processes in the treatment of human disease.

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CHAPTER 4

CONTROL OF CULLIN-RING UBIQUITIN LIGASE ACTIVITY BY Nedd8

Raymond J. Deshaies,* Ethan D. Emberley and Anjanabha Saha

Abstract: The Cullin-RING ubiquitin ligase (CRL) family, which may number as many as 350 different enzymes, has an enormous impact on cellular regulation. CRL enzymes regulate cell biology by conjugating ubiquitin onto target proteins that are involved in a multitude of processes. In most cases this leads to degradation of the target, but in some cases CRL-dependent ubiquitination acts as a switch to activate or repress target function. The ubiquitin ligase activity of CRLs is controlled by cycles of attachment and removal of the ubiquitin-like protein Nedd8. Conjugation of Nedd8 onto the cullin subunit of CRLs promotes assembly of an intact CRL complex and switches on ubiquitin ligase activity. Conversely, removal of Nedd8 switches off ubiquitin ligase activity and initiates CRL disassembly. Continuous maintenance of CRL function in vivo requires the activities of both the Nedd8-conjugating and deconjugating enzymes, pointing to a critical role of complex dynamics in CRL function. Here, we review how the Nedd8 cycle controls CRL activity and how perturbations of this cycle can lead to disease.

THE UBIQUITIN-PROTEASOME SYSTEM

Conjugation of ubiquitin to cellular proteins plays a key role in regulating many cellular and organismal processes.[1] Ubiquitin is covalently attached to target proteins via an isopeptide bond that links the C-terminus of ubiquitin to a lysine residue of the acceptor substrate.[2] Additional ubiquitins can be conjugated to any of the seven lysine residues of ubiquitin to form a polyubiquitin chain on the substrate. Assembly of a chain of ≥4 ubiquitins linked via the lysine-48 residue of the substrate-proximal ubiquitin (referred to as a Lys48-linked chain) marks cellular proteins for degradation by the 26S proteasome.[3-4] Emerging evidence suggests that other linkages, including Lys11, can

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also target substrates for degradation by the proteasome.[5] In contrast, monoubiquitination serves as a non-proteolytic signal in intracellular trafficking, DNA repair and signal transduction pathways.[6]

Ubiquitination of proteins is achieved through an enzymatic cascade that comprises ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligating (E3) enzymes.[2,7] E1 activates ubiquitin for transfer by adenylating its C-terminus and then forming a thiolester linkage between the activated C-terminus of ubiquitin and an active site cysteine of E1. The activated ubiquitin is passed in a thioester linkage to the active site cysteine of an E2 enzyme. Substrate ubiquitination occurs when an E3 binds to both a molecule of substrate and an E2 thioesterified with ubiquitin (E2~Ub), bringing them into proximity with each other such that the ubiquitin can be transferred from the E2~Ub to the substrate. This transfer can either occur directly (i.e., with no intermediary) or via a covalent E3~ubiquitin thioester intermediate. The pairing of E2~Ub and substrates by E3s is the key determinant of substrate specificity in ubiquitination reactions. There are two major classes of E3s in eukaryotes, defined by the presence of either a HECT (Homologous to E6-AP C-terminus) domain or a RING (Really Interesting New Gene) fold.[8] HECT-domain E3s contain a conserved cysteine that accepts ubiquitin from E2~Ub and then passes it on to substrate, whereas RING ligases promote the direct transfer of ubiquitin from E2~Ub to the substrate.

CULLIN-RING UBIQUITIN LIGASES

General Principles of CRL Organization

RING ligases are conserved from yeast to human and human cells potentially express more than 650 different types of these enzymes.[9] The RING E3s that are perhaps the best understood of the members of the cullin-RING ligase (CRL) superfamily.[9-12] CRLs are modular multisubunit complexes that all contain a common core comprising a cullin subunit and a zinc-binding RING domain subunit. The cullin subunit folds into an extended structure that forms the backbone of CRLs. The C-terminal region of the cullin subunit forms a globular domain that wraps itself around the RING protein, which in turn recruits the E2~Ub to form the enzymatic core (Fig. 1). The N-terminal region of the cullin subunit, which resides at the opposite end of the elongated cullin structure, recruits substrate receptors via adapter proteins. SCF, the prototype of the CRLs, consists of the cullin Cul1, the RING domain protein Rbx1/Roc1/Hrt1, the adapter protein Skp1 and a substrate receptor protein such as Skp2 or β-TrCP. The substrate receptor proteins for SCF complexes share an F-box motif that links them to Skp1. The human genome encodes 69 F-box proteins[13] and thus human cells potentially assemble 69 distinct SCF complexes, each with different substrate specificity. Other CRLs are assembled using other cullin and adapter/substrate receptor subunits. All told, there are nine different proteins that contain a region homologous to the C-terminal domain of Cul1 that binds a RING subunit. The complexes they nucleate are specified in Figure 2. Interestingly, eight of these proteins (Cul1, Cul2, Cul3, Cul4a, Cul4b, Cul5, Cul7 and PARC), share one of only two different RING proteins (Rbx1/Roc1/Hrt1 and Rbx2/Roc2/Hrt2). Only Apc2, which is the most atypical member of the family, assembles with the dedicated RING protein Apc11.[14]

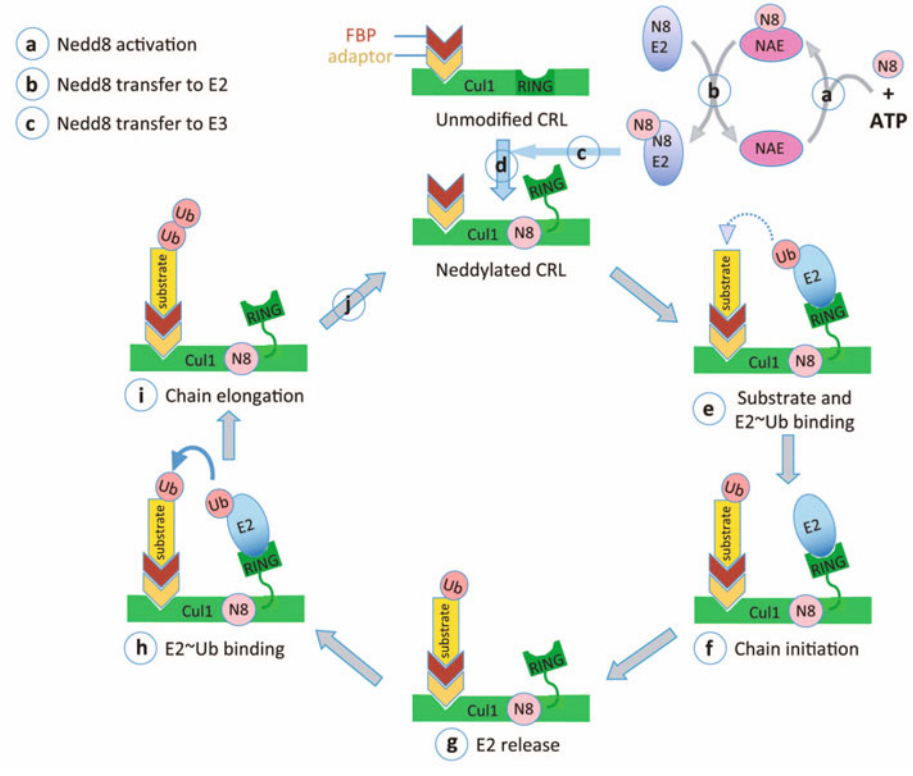

Figure 1. Reaction cycle for substrate ubiquitination by an activated cullin-RING ligase (CRL). a) Nedd8 (N8) is first activated when it reacts with Nedd8-activating enzyme (NAE) in the presence of ATP. b) Activated Nedd8 is then transferred from NAE to the Nedd8 conjugating enzyme (N8 E2). c) Nedd8 is subsequently transferred from Nedd8 E2 to CRL. Nedd8 is covalently attached to the cullin subunit via an isopeptide bond on a conserved lysine residue. d) Conjugation of Nedd8 causes the RING subunit to spring out from the cullin resulting in increased CRL activity. e) Members of the SCF subfamily of CRLs recruit E2~Ub via the RING domain and substrates via the F-box protein (FBP). The FBP is linked to the cullin subunit (Cul1) by the adaptor, Skp1. Ubiquitin (Ub) is transferred from E2~Ub to the lysine residue of the bound substrate to initiate chain formation (f). g) After ubiquitin is discharged to the substrate, the apoE2 dissociates from the CRL. h) Another E2~Ub is recruited to the RING for subsequent ubiquitin transfer. i) Ubiquitin is transferred from E2 to the lysine residue of ubiquitin conjugated to substrate resulting in chain elongation. Dissociation of discharged E2 clears the way for subsequent recruitment of E2~Ub to build a longer ubiquitin chain. j) At some point, the ubiquitinated product dissociates. The length of the ubiquitin chain acquired by the substrate is a function of the rates of E2 cycling and product dissociation. If the rate of E2~Ub binding, ubiquitin transfer and E2 dissociation are fast compared to product dissociation, long chains will be generated. Conversely, if the rate of E2 cycling is slow, product will dissociate before long chains can be acquired.

Recruitment of E2 Enzymes by CRLs

The E2~Ub that is recruited by SCF to ubiquitinate substrate has been the subject of some debate. Yeast SCF complexes specifically employ Cdc34 as the E2,[15-17] whereas human SCF utilizes either Cdc34 or UbcH5c in vitro.[18] The identity of the E2 used by human SCF in vivo remains uncertain and few studies address this issue.[19-20] UbcH5 and Cdc34 have markedly different biochemical properties. Cdc34 is relatively poor at

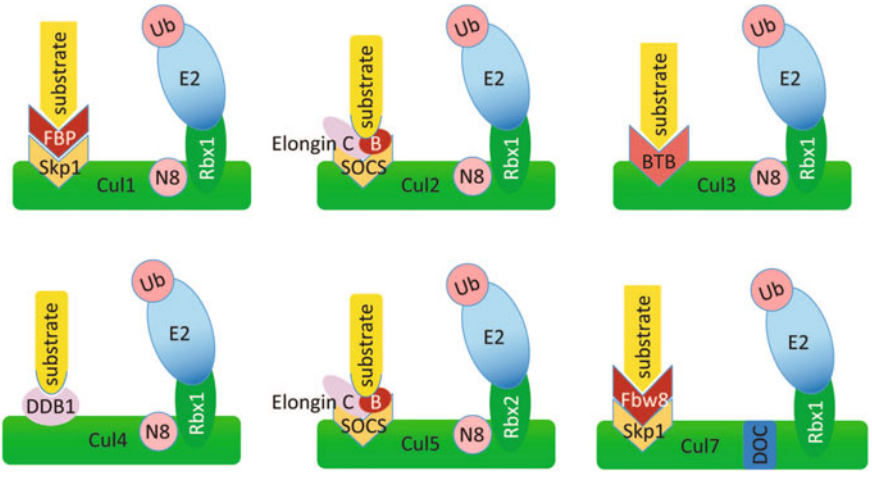

Figure 2. Modular assembly of cullin RING ligases (CRLs). The C-terminal domain of the cullin subunit associates with a RING domain protein (Rbx1 or Rbx2) to form the catalytic core that recruits E2~ubiquitin thioester. The N-terminal domain of cullin interacts with substrate receptor either directly or via an adaptor protein that recruits substrates. All cullins are known to be modulated by the conjugation of Nedd8.

transferring ubiquitin to unmodified substrate, but extends ubiquitin chains on modified substrate very rapidly—up to 100-times faster than it initiates them.[18,21] Another feature of Cdc34 is that it is very specific for forming Lys-48 linkages and this specificity is intrinsic to Cdc34. UbcH5, on the other hand, is adept at transferring ubiquitin to naïve substrate, but unlike Cdc34 shows no preference for elongating the attached ubiquitin into a chain.[18] Indeed, once a substrate bears a single ubiquitin modification, both the K_M and k_{cat} for subsequent ubiquitin transfers by UbcH5 decrease, suggesting that UbcH5 binds more tightly to the modified substrate, but in a configuration that has relatively low ubiquitin transfer activity.[18] A second difference between UbcH5 and Cdc34 is that the former exhibits no preference for Lys-48 linkages and forms polyubiquitin chains linked through multiple lysine residues.[22] It is unclear how this lack of chain linkage specificity would influence the metabolism of ubiquitin chains assembled by UbcH5.

It has been suggested for the yeast APC (Anaphase Promoting Complex) that one E2 (Ubc4) is used to initiate chains whereas a second E2 (Ubc1) elongates the chain.[23] A similar model could explain the conflicting observations that have been made with SCF. However, it remains unclear how chain-initiating and chain-elongating E2s would not interfere with each other's actions and SCF would know to recruit the proper E2 depending on the stage of substrate modification. Given this concern, other possibilities should be entertained. For example it is possible that UbcH5 is not a physiological E2 for SCF substrates. Two lines of argument support this proposal. First, high concentrations of UbcH5 are required to saturate SCF (K_M~1-2 μM) and the requisite effective concentrations may not be achievable in vivo due to titration of UbcH5 by numerous other RING domain proteins.[24] Meanwhile, Cdc34 has a far lower K_M for SCF (100-200 nM)[25] and could easily saturate SCF in vivo, based on estimates we have made of Cdc34 concentration in yeast cells.[26] Second, because UbcH5 is not fast at building chains, most substrates that are modified by UbcH5–SCF do not acquire a chain of ≥4 ubiquitins before they dissociate from SCF. By contrast, whereas Cdc34 is

inefficient at initiating ubiquitination, the majority of substrates that are modified by Cdc34 go on to acquire a chain of ≥4 ubiquitins prior to dissociating from SCF.[18] It should be borne in mind that not only are the ubiquitin chains generated by UbcH5 short, but most likely they comprise a mixture of different ubiquitin-ubiquitin linkages of uncertain potency in sustaining turnover by the proteasome in vivo.

Recent kinetic studies have helped to shed light on how Cdc34 can be so adept at building ubiquitin chains on substrate rapidly. First, it appears that Cdc34~Ub has a noncovalent binding site for the ubiquitin that accepts the thioester.[27] Although this binding site has low affinity (K_M ~ 500 μM for yeast Cdc34), it should be easily saturated in the context of an SCF complex bound simultaneously to ubiquitinated substrate and Cdc34~Ub, due to the proximity between these two molecules. A second feature of Cdc34 that enables rapid chain assembly is the exceptionally rapid dynamics of the Cdc34–SCF complex. Although this complex forms with extremely high affinity (K_D ~ 20-100 nM depending upon the modification state of Cdc34 and Cul1),[18] it is remarkably unstable. Discharged Cdc34 dissociates from SCF with an off-rate of ~30 sec^{-1}.[25] which leads to a predicted on-rate of ~4 × 10^8 M^{-1} sec^{-1}[25]. This exceeds by 2-3 orders of magnitude the predicted on-rate for protein–protein interaction based on random diffusion. Exceptionally fast binding of Cdc34~Ub and SCF is mediated by the acidic tail of Cdc34, which engages in an electrostatic interaction with a basic "canyon" on the underside of Cul1. The extremely rapid dynamics of Cdc34–SCF interaction enables chain assembly to occur at rates approaching 4-5 ubiquitin transfers per second.[21]

Substrate Recruitment to CRLs

Unlike the nature of the E2 used by SCF, the matter of how substrate is recruited is better understood. Substrate recruitment by ubiquitin ligases has been reviewed recently[28] and substrate recruitment by SCF ubiquitin ligases in particular has also been discussed in depth,[10,12] so we will not go into detail here. Substrates recruited to SCF for ubiquitination are usually covalently modified by phosphorylation, although other covalent modifications, including glycosylation and ribosylation, have been reported to serve as signals for recruitment. The structures of several phosphorylation-based degrons bound to their cognate substrate receptor subunit of SCF have been solved and in each case the covalent modification makes defined molecular contacts that enable its specific recognition.[29-32]

MECHANISM AND REGULATION OF CRLs

Whereas substrate recruitment to SCF is now understood in molecular detail for some complexes, the actual ubiquitination reaction has resisted detailed description. The ubiquitination reaction catalyzed by E2~Ub–SCF can be subdivided into two steps by both kinetic and mutational analysis: transfer of the first ubiquitin to substrate (chain initiation) and polymerization of ubiquitin chains by formation of ubiquitin-ubiquitin linkages (chain elongation).[18,21,27] Perplexingly, the original structural studies of SCF sub-complexes suggested that there should exist a ~50 Å gap between bound substrate and the active site cysteine of E2 within an E2~Ub–SCF–substrate ternary complex.[29-33] For SCF to facilitate chain initiation, the substrate lysine that is to be modified must come in close proximity with the thioester bond that joins ubiquitin to E2. Thus, publication of the structural studies served to highlight how little we know about how SCF works.

CRLs Are Activated by Nedd8 Conjugation

We now appreciate that (at least part of) the answer to the conundrum of how SCF and other CRLs can ubiquitinate substrate across a ~50 Å gap lies in the fact that these enzymes are activated by a reversible covalent modification of the cullin subunit with the ubiquitin-like protein, Nedd8.[34] Covalent modification of cullins with Nedd8, which is often referred to as neddylation, is essential in all eukaryotes tested to date,[35-36] with the exception of budding yeast.[37-38] The conjugation of Nedd8 requires a ubiquitin-like enzyme cascade involving the Nedd8-activating enzyme AppBp1-Uba3, one of two Nedd8-conjugating enzymes (Ubc12/UBE2M and UBE2F),[39-40] the RING protein Rbx1 or Rbx2 and the activator Dcn1,[41] resulting in neddylation of Cul1 at the highly conserved lysine 720[34] (Fig. 3). A lysine is found in the equivalent position in Cul2, Cul3, Cul4A, Cul4B, Cul5, Cul7 and PARC and

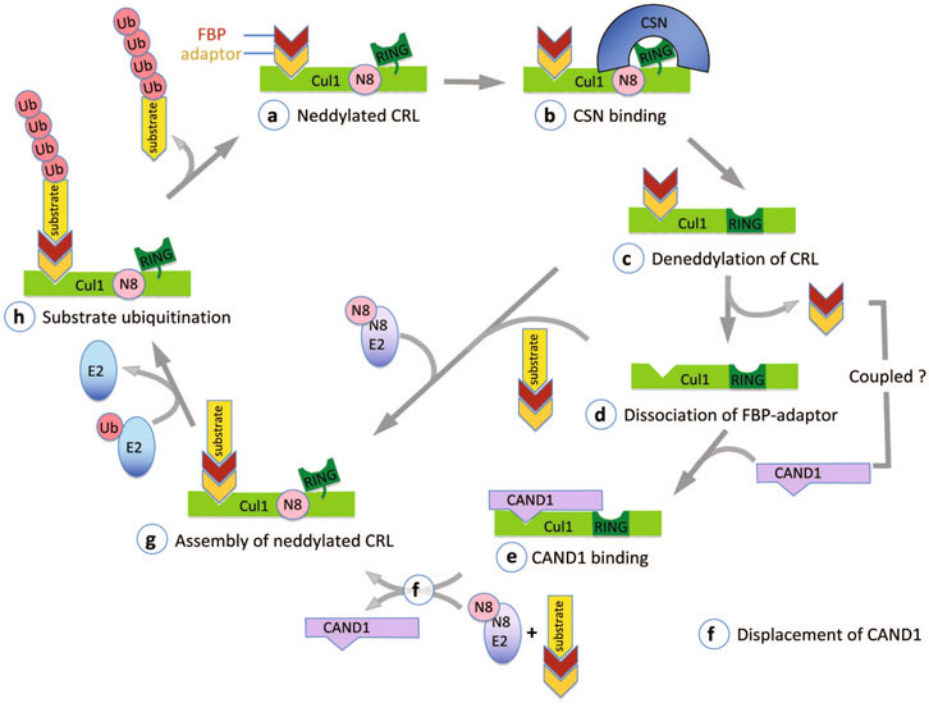

Figure 3. Neddylation and deneddylation cycle in substrate ubiquitination and CRL regulation. a) SCF in neddylated state possesses higher ligase activity for substrate ubiquitination. b) COP9-Signalosome (CSN) binds CRLs and recognizes neddylated cullin. The complementary binding surfaces on CRL and CSN are not known and thus the nature of the interaction depicted here is speculative. c) CSN deneddylates cullin to yield unmodified CRL with low ubiquitination activity. This deneddylated complex might be immediately reneddylated (shunt to (g)), or the F-box–Skp1 complex might dissociate from Cul1 (d) before reneddylation and binding of a new F-box–Skp1 occurs (shunt from step d to step g). Alternatively, Cul1–Rbx1 might become sequestered in a complex with CAND1 (e). Dissociation of F-box–Skp1 may or may not be coupled to binding of CAND1. The fluxes in vivo through the various sub-pathways linking steps c-g are not known. e) In the presence of neddylation machinery and substrate receptor–adaptor module, CAND1 is displaced from cullin. f) CAND1 displacement results in the assembly of active neddylated CRL complex. g) Neddylated CRL in the presence of E2~Ub builds ubiquitin chains on substrate, yielding ubiquitinated substrates.

all of these cullins are conjugated with Nedd8.[42-45] The cullin homology domain-containing Apc2 subunit of the Anaphase-Promoting Complex does not contain the equivalent lysine and does not appear to undergo modification with Nedd8.

Conjugation of cullins with Nedd8 has a range of effects on cullin function and assembly state. A substantial fraction of Cul1 in eukaryotic cells is sequestered into an inactive complex with CAND1.[46-47] A recent exception was noted in *S. pombe*[48] where only a minor fraction of Cul1 is sequestered. CAND1 binds Cul1 in an extended manner and thereby disrupts both the association of Cul1 with Skp1 and conjugation of Nedd8 to lysine-720.[49] Nedd8 conjugation coupled with F-box–Skp1 binding displaces CAND1 from Cul1, enabling the assembly of intact and functional SCF complexes.[49-50] However, the Nedd8 conjugation pathway remains essential even in the absence of CAND1 suggesting that Nedd8 regulates CRLs by other mechanisms that are critical for life.[51] Early studies reported that Nedd8 modification of Cul1 stimulates ubiquitination of the substrates $p27^{Kip1}$ and IκBα by SCF^{Skp2} and $SCF^{\beta\text{-}TrCP}$ respectively[52-56] and it was suggested that neddylation activates SCF by stabilizing its association with Ubc4, a close relative of UbcH5.[57] Subsequent NMR studies revealed a potential Nedd8 binding site on Ubc4,[58] suggesting a mechanism for how Nedd8 conjugation could stabilize E2 recruitment. However, the same site on UbcH5 binds ubiquitin and promotes ubiquitination by the E3 BRCA1, which is not modified by Nedd8.[59] Moreover, Cdc34 lacks the equivalent binding site and it was not shown by any direct measurement that Nedd8 conjugation improves the affinity for Ubc4 in a manner that depends on its putative noncovalent Nedd8 binding site. These observations raised the question of whether enhanced recruitment of E2~Ub is the primary mechanism by which neddylation activates SCF.

Nedd8 Conjugation Causes a Major Conformational Change in Cul5

The major mechanism by which Nedd8 conjugation stimulates ubiquitin transfer within the E2–E3–substrate complex was revealed by a confluence of X-ray crystallographic and biochemical studies. In a herculean effort, the Schulman laboratory solved the structure of the C-terminal domain (CTD) of Cul5 bound to Rbx1 in the unmodified and Nedd8-conjugated states.[60] Nedd8 conjugation induces a massive conformational change in the complex. The H29 helix of $Cul5^{CTD}$ rotates ~45° relative to the α/β portion of the $Cul5^{CTD}$. This results in a marked repositioning of the 4-helix bundle (4HB), winged-helix B (WHB) and α/β subdomains within $Cul5^{CTD}$. In the unmodified state, the WHB cradles the RING domain of Rbx1. Upon reorientation of the 4HB, WHB and α/β domains, the WHB and RING are levered apart, freeing the E2-binding RING domain of Rbx1 to spring forth from the surface of Cul5. Rbx1 remains tightly bound to Cul5 via a long β-strand (S1) that forms an extended β-sheet with the S1, S2 and S3 strands in $Cul5^{CTD}$. This β-sheet is connected to the RING domain via a flexible linker that is found in two different conformations in the crystal of Nedd8-conjugated $Cul5^{CTD}$–Rbx1. Taken together, these data suggest a model wherein Nedd8 conjugation releases the Rbx1 RING domain, which catapults from the surface of $Cul5^{CTD}$ like a jack-in-the-box. Although the RING domain remains tethered to $Cul5^{CTD}$, the linker is flexible, which allows the RING domain to sample three-dimensional space in the void that separates E2 from substrate in the unmodified SCF complex. Modeling suggests that the RING domain with its bound E2 could potentially come very close to substrate bound to the F-box subunit of SCF.

Analysis of a variety of mutants in the Rbx1 linker region as well as the Cul1–RING interface support the interpretation derived from the crystal structure.[60] Furthermore, small-angle X-ray scattering suggests that a similar conformational change occurs upon Nedd8 conjugation to Cul1. A particularly dramatic demonstration that this model is likely to be correct came from a cross-linking experiment.[18] The idea was to ask whether conjugation of Nedd8 influences the formation of a crosslink between a β-catenin substrate peptide and the active site of E2 enzyme within a UbcH5–$SCF^{\beta\text{-}TrCP}$–β-catenin complex. Whereas no cross-link was detected between β-catenin and UbcH5 when unmodified SCF was used, a strong cross-link was detected in the presence of Nedd8-conjugated SCF. The simplest explanation of this result is that Nedd8 conjugation induces a conformational change in SCF that brings the E2~Ub and the substrate into close approximation, as would need to occur during ubiquitin transfer. Detailed enzymological studies on Nedd8-conjugated SCF yielded additional data consistent with the idea that Nedd8 has a pervasive impact on the SCF complex. Nedd8 significantly enhances k_{cat} for ubiquitin transfer within the E2~Ub–SCF–substrate complex, particularly under single-turnover conditions. Binding analyses revealed that Nedd8 conjugation also stabilizes E2 recruitment (as measured by both K_M and K_D), but the effects on K_D are considerably smaller than the effects on k_{cat}.[18] Besides the effect on E2 affinity, Nedd8 even appears to have a modest effect on transfer of ubiquitin to the low molecular weight nucleophile hydroxylamine. However, it seems likely that both of these latter effects may be indirect consequences of modest changes in the dynamic properties of the RING domain depending upon whether or not it is ensconced within the cullin CTD via interactions with the WHB subdomain. In addition to its effects on CRL activity, Nedd8 conjugation disrupts the binding site for CAND1, thereby enforcing its dissociation and promoting the assembly of an intact CRL. The effects of Nedd8 conjugation indeed appear to permeate nearly every aspect of CRL function and regulation.

DECONJUGATION OF Nedd8 BY THE COP9-SIGNALOSOME (CSN)

Nedd8-mediated activation of CRLs is part of a cycle wherein Nedd8 is being continuously conjugated to and deconjugated from cullins (Fig. 3). The recent availability of a chemical inhibitor of the conjugation process reveals that this cycle operates at a high rate.[61] Thus, it is clear that to understand the impact of neddylation on CRLs, it is critical to understand the mechanism and regulation of Nedd8 deconjugation (deneddylation) as well as that of neddylation. Nedd8 conjugated to cullins is deneddylated by the COP9 signalosome complex (CSN).[62] The CSN is comprised of eight subunits (Csn1-Csn8) and is highly conserved throughout the eukaryotic kingdom.[63-64] Mutations in CSN components are manifest as defects in signal transduction,[65] transcription,[66] cell cycle progression[67] and development.[68-69] Overall, our best understanding of the physiological role of CSN derives from genetic studies of its role in photomorphogenesis in *Arabidopsis thaliana.*[70] Photomorphogenesis refers to the broad spectrum of physiological and developmental changes that occur when a seedling is exposed to light. Genetic data suggest that CSN regulates photomorphogenesis by stimulating turnover of the transcriptional regulator Hy5 via the presumptive E3, COP1. COP1 is the substrate-binding subunit of a CRL complex that contains Cul4A, which provides a pleasingly simple molecular model for photomorphogenesis that ties together the genetic and biochemical data on CSN.[71]

COP9-Signalosome Defines a Novel Class of Metalloproteases

Insight into the mechanism by which CSN promotes cleavage of Nedd8 from Cul1 came from bioinformatic analyses of CSN subunits. Csn5 and a subset of other proteins that contain the JAB1/MPN/Mov34 domain were found to contain a highly conserved sub-motif, $EX_nHS/THPX_7SX_2D$.[72] By analogy to zinc metalloproteases, we speculated that the His and Asp residues of this motif comprise a set of ligands that coordinate a catalytic zinc ion. Indeed, mutations in these conserved residues inactivate Csn5-dependent deneddylation of Cul1 in fission yeast and Nedd8 isopeptidase activity of purified pig CSN is sensitive to metal chelators. Based on these data we dubbed the conserved motif 'JAMM', for JAb1/Mpn domain Metalloenzyme. We and others went on to validate our predictions by solving the three-dimensional crystal structure of a JAMM domain protein from an archaebacterium.[73-74] Despite the insight into the active site of CSN, our overall understanding of the deneddylation reaction remains at a rudimentary level. Little is known about the molecular basis for substrate recognition or the dynamics of the process. The situation is exacerbated by the lack of structural data for the CSN complex or any of its individual subunits.

Consistent with its biochemical function as a Nedd8 isopeptidase, CSN behaves as an inhibitor of SCF in vitro. Deneddylation by CSN attenuates Cul1–RING-dependent ubiquitin chain synthesis[75] and also downregulates $p27^{Kip1}$ ubiquitination by SCF^{Skp2} in a cell-free extract.[76] Nevertheless, multiple genetic studies indicate that CSN is required for proper CRL function in vivo in *A. thaliana*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *S. cerevisiae*, *S. pombe*, *Neurospora* and human tissue culture cells.[48,77-83] It has been noted in these studies that cells lacking CSN function contain reduced levels of cullins and/or substrate receptor proteins due to increased turnover, possibly via an autoubiquitination mechanism. Likewise, although CAND1 can clearly inhibit active SCF ligases in vitro,[46-47] inactivation of CAND1 by mutation leads to a dramatic loss of the *Arabidopsis* F-box protein Ufo1 and consequent reduction in function of SCF^{Ufo1} ubiquitin ligase activity in vivo.[51,84] We and others have interpreted the contrasting negative roles of CSN and CAND1 in vitro versus their positive roles in vivo as evidence for an obligatory cycle of CRL assembly and disassembly, presumably involving reversible cycles of neddylation and deneddylation coupled with CAND1-dependent sequestration of cullin.[63,85] If any part of this cycle is interrupted, CRL activity is downregulated.

COP9-Signalosome as a Regulator of Human Disease

CSN is emerging as a potential player in human disease with several different connections having been made, particularly in cancer. Early studies noted a correlation in cancer cells between elevated expression of Csn5 and reduced levels of the SCF substrate and cyclin-dependent kinase inhibitor $p27^{Kip1}$.[86-88] Csn5 overexpression promotes $p27^{Kip1}$ turnover, but it remains unclear how this relates to CSN function. Subsequently, it was shown that overexpression of Csn5 and Myc in human breast cancer cells induces a regulon of 512 genes linked to the wound healing response in untransformed cells.[89] An activated wound healing response in cancer cells is a powerful predictor of metastasis and death in multiple primary human tumors.[90] Recent studies from the same lab reveal that proliferation of breast cancer cells in vitro requires Csn1, Csn5 and Csn6.[91] Moreover, defective proliferation of Csn5-depleted cells is not rescued by expression of a JAMM domain mutant. These data suggest that the isopeptidase activity of Csn5 promotes an

increase in Myc transcriptional activity, which in turn activates the wound response regulon.[91] It is tempting to speculate that this effect is mediated by deneddylation and downregulation of SCF^{FBW7}, which recognizes N-terminal Myc Box I and antagonizes Myc transcriptional function by enhancing its proteasomal turnover.[92-93] Another case in which CSN has been implicated to act in an oncogenic capacity is osteosarcoma. Analysis of an amplicon located at 17p11.2 suggests that overexpression of the COPS3 (Csn3) gene can be a causative factor in osteosarcoma.[94] More recently, colorectal cancer cells expressing oncogenic K-Ras but not isogenic cells deleted for the K-Ras oncogene were shown to depend on several CSN subunits for their survival.[95] Interestingly, genes in the neddylation pathway are also required for survival of cells expressing oncogenic K-Ras. Together, the studies on the wound response and K-Ras implicate CSN as an excellent candidate target for treatment of a subset of breast cancers as well as colorectal cancers powered by a mutant K-Ras.

Another link between CSN and cancer was suggested by investigations on the nucleotide excision repair proteins Csa and Ddb2.[96] Loss of Csa is a cause of Cockayne's syndrome whereas loss of Ddb2 is found in a subset of patients with the cancer-prone syndrome Xeroderma pigmentosum. These proteins serve as the putative substrate recognition subunits of CRL complexes comprising Ddb1, Cul4A and Rbx1. In the absence of UV irradiation, the assembled $CRL4^{DDB2}$ ubiquitin ligase (Ddb2–Ddb1–Cul4A–Rbx1) exists as a soluble complex in the nucleus and is bound to CSN. When the nucleotide excision repair pathway is activated by UV damage to DNA, $CRL4^{DDB2}$ dissociates from CSN and binds tightly to the damaged chromatin. Chromatin-bound $CRL4^{DDB2}$ becomes neddylated and is now competent to ubiquitinate its substrates. By contrast, in the absence of UV irradiation, the $CRL4^{CSA}$ (Csa–Ddb1–Cul4A–Rbx1) is not complexed with CSN. When UV irradiation damages chromatin, $CRL4^{CSA}$ binds to RNA polymerase IIo that is stalled at DNA lesions and recruits CSN, presumably resulting in inactivation of $CRL4^{CSA}$ ubiquitin ligase E3 activity.

CONCLUSION AND FUTURE PERSPECTIVES

Other Functions for the Nedd8 Regulatory System

A number of Nedd8-conjugated proteins other than the cullins have been described in the literature, including Mdm2,[97] p53,[97] VHL[98] and ribosomal proteins.[99] The physiological ramifications of these modifications (which are typically found only on a very small fraction of target molecules) remain to be fully explored. As has been pointed out by Rabut and Peter, none of the Nedd8-modified proteins (besides the cullins) discovered to date satisfy fully a set of criteria that they proposed for the validation of physiologic targets of the Nedd8 conjugation pathway.[100] A matter of particular concern is that the ubiquitin conjugation system has demonstrated the capacity to conjugate Nedd8 in vitro and so it is critical to establish by functional ablation of the Nedd8 conjugation pathway that the neddylation of a given protein is indeed specific. Currently, the noncullin Nedd8-modified proteins for which there exists the most convincing evidence are p53 and VHL. Clearly, more work on alternative targets of Nedd8 is urgently needed.

One potentially powerful approach to search for alternative Nedd8 conjugation targets is to evaluate neddylation in cells lacking deneddylation activity. Whereas blockade of CSN activity primarily induces the accumulation of Nedd8-modified cullins,[62,101]

blockade of the enzyme Den1/NEDP1 that processes the precursor form of Nedd8 to yield mature Nedd8 causes the accumulation of numerous unknown Nedd8-modified proteins in yeast[102] and *Drosophila* larvae.[101] The *Drosophila* data were particularly intriguing because the increase of neddylated proteins in mutants lacking Den1/NEDP1 was shown to depend on the Nedd8 conjugation pathway and the pattern of accumulation differs greatly from that of mutants deficient in Csn5. These observations suggest that CSN may be dedicated for cullin regulation, but Den1/NEDP1 may control the deneddylation of a substantial pool of alternative Nedd8 targets. Several different proteomic searches for Nedd8 conjugates have been reported[45,99,103-104] and it will now be of particular interest to repeat these analyses in cells deprived of Den1/NEDP1 activity.

The Nedd8 Pathway as a Target for Therapeutic Intervention

An important development in the past year was the report of a small molecule, MLN4924 (Millennium/The Takeda Oncology Company), which inhibits the activity of the Nedd8-activating enzyme (NAE) that primes Nedd8 for transfer to protein targets.[61] MLN4924 effectively wipes out Nedd8 conjugation activity in vivo and this leads to extremely rapid (≤5 minutes) loss of neddylated cullins. This striking result suggests that cullins are being constantly neddylated and deneddylated at a blistering pace. In response to treatment with MLN4924, substrates for multiple CRLs begin to accumulate. Interestingly, different substrates show different dose-responses to MLN4924, suggesting that different CRLs may be differentially sensitive to depletion of NAE activity. The basis for such a differential response is not known. Based on its potency and specificity, MLN4924 promises to be of enormous value for basic research studies on the Nedd8 conjugation and deconjugation system. Apart from its utility as a research tool, MLN4924 shows excellent promise as a candidate therapeutic for treatment of cancer. MLN4924 showed marked activity in downregulating NAE activity in HCT-116 tumor cells xenografted into mice, resulting in deneddylation of cullins and accumulation of CRL substrates.[61] Most impressively, MLN4924 elicited a strong reduction in the growth of the xenografted cancer cells.

Unresolved Questions

Although dramatic progress has been made in the past two years in understanding how Nedd8 conjugation regulates CRLs, much remains to be done. The most important unresolved question about the Nedd8 conjugation system is, how is the entire Nedd8 cycle controlled from the perspective of individual CRL complexes? What is perplexing is that cells express two different E2s and a handful of E3 enzymes to conjugate Nedd8 and two enzymes to deconjugate it (CSN and NEDP1/Den1), but meanwhile there are eight different cullin targets, each of which has the potential to assemble multiple distinct ubiquitin ligases. Based on the number of different putative cullin substrate receptors that are known, there may be as many as 350 different CRLs that are expressed in human cells and regulated by cycles of Nedd8 conjugation and deconjugation. It seems paradoxical that the regulation of such a large set of CRLs would be relinquished to such a small number of Nedd8-conjugating and deconjugating enzymes. Any signal that would serve to alter either Nedd8 conjugation or deconjugation activity could be expected to influence the activity of dozens to hundreds of CRLs, thereby influencing the turnover of hundreds to thousands of proteins, many of which are likely to function at cross-purposes.

It seems logical that there must exist some way to regulate the neddylation cycle in a more fine-tuned manner, such that the neddylation and deneddylation of individual CRL complexes can be controlled independently, enabling one flavor of CRL to be activated by neddylation at the same time that a distinct CRL complex is being decommissioned by CSN-mediated deneddylation. Indeed, this is precisely what happens to the $CRL4^{DDB2}$ and $CRL4^{CSA}$ complexes upon UV irradiation.[96] Such a mechanism could be based on substrate-mediated regulation of the Nedd8 cycle or could be controlled by a covalent modification (e.g., phosphorylation) that marks individual CRL complexes for neddylation or deneddylation.[63] The latter mechanism would echo CRLs themselves, which can ubiquitinate substrates on different schedules dictated by the protein kinases that mark the CRL substrates for ubiquitination.[105]

Besides this major question about global versus complex-specific regulation of neddylation cycles, a number of more specific problems have so far resisted solution but seem primed for resolution. How does the putative Nedd8 E3, Dcn1, promote Nedd8 conjugation in vivo, even though it appears to have only very modest effects on this reaction in vitro? Is the Nedd8 conjugation reaction influenced by other polypeptides that engage the CRL (e.g., substrate, E2 enzyme)? What is the mechanism by which Nedd8 is cleaved from cullins by CSN (this includes the question of what is the molecular basis of substrate recognition)? Is CSN-mediated deconjugation regulated by factors that bind or modify CSN or that associate with the CRL substrate (e.g., Nedd8-conjugating factors, E2 enzymes and substrates)? Finally, will NAE prove to be a suitable target for cancer chemotherapy and might CSN and Den1/NEDP1 also be good candidates for pursuit? Clearly, much remains to be done to understand how the cycle of Nedd8 conjugation and deconjugation controls the repertoire of active CRLs and how modulation of this cycle might lead to new medicines to treat intractable diseases.

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CHAPTER 5

CONTROL OF DENEDDYLATION BY THE COP9 SIGNALOSOME

Tilo Schmaler and Wolfgang Dubiel*

Abstract: The interplay between ubiquitin (Ub) family modifiers creates a regulatory network of Ub family proteins which is essential for cell growth and differentiation. One of the best studied crosstalks between Ub family modifiers is the stimulation of ubiquitination by Nedd8 (neural precursor cell expressed developmentally down regulated 8) modification. The neddylation-deneddylation pathway controls the selective ubiquitination of important cellular regulators targeted for proteolysis by the Ub proteasome system (UPS). In this process the cullin scaffolds of cullin-RING Ub ligases (CRLs) are neddylated, which allosterically activates the transfer of Ub to substrates of the CRLs. A major reaction of the regulatory network is the removal of Nedd8 by the COP9 signalosome (CSN), which converts CRLs into an inactive state. The CSN is a conserved protein complex that interacts with CRLs and possesses an intrinsic metalloprotease with a Jab1/Pad1/MPN+ (JAMM) motif responsible for deneddylation.

In the present chapter we focus on the CSN-mediated deneddylation and its biological significance. We summarize latest developments on the mechanism of the CSN and its association with supercomplexes. In addition, data on the regulation of CSN-mediated deneddylation are described. Moreover, dysfunctions of the CSN and their implication in the pathogenesis of diseases are discussed.

INTRODUCTION

Deneddylation is the process of removing the Ub family modifier Nedd8 from target proteins by hydrolysis. Nedd8 was cloned in 1993.[1] Among the Ub family proteins, it is most homologous to Ub, nevertheless its function is different from that of Ub.[2] The yeast homologue is called Rub1 and has been first described by Jentsch

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and coworkers.[3] In analogy to other Ub family modifiers Nedd8 has to be C-terminally processed prior to conjugation to generate a Gly-Gly C-terminus. This motif is required for the formation of an isopeptide bond between the ε-NH_2 of a target protein lysine residue and the Nedd8 C-terminal carboxyl group. The best characterized deneddylating enzyme is the CSN, a protein complex that occurs in all eukaryotic cells.[4] Originally the CSN was discovered in plant cells as a repressor of light-dependent growth.[5,6] Later it was identified in mammalian cells and characterized as a complex involved in signal transduction with similarities to 26S proteasome subunits.[7,8] Structurally and perhaps functionally the CSN is related to the 26S proteasome lid subcomplex and to the translation initiation factor 3 (eIF3).[9-11] Analog structures of the CSN and the lid have been recently confirmed by a novel mass spectrometry approach.[12] In mammals the CSN consists of 8 subunits (CSN1-CSN8, see Fig. 1). It has functions in cell cycle,[13,14] DNA repair,[15,16] transcription,[17] signal transduction,[18-20] development[21,22] and angiogenesis.[23-26] CSN functions are determined by associated and intrinsic activities. From yeast to men the CSN is associated with the Ub specific protease 15 (USP15 or UBP12 in yeast) (see Fig. 1), which protects proteins in proximity to the CSN from ubiquitination and subsequent degradation.[23,27,28] In addition, the particle is associated with a variety of protein kinases such as casein kinase 2 (CK2) and protein kinase D (PKD)[29] (see Fig. 1), Akt[30] as well as inositol 1,3,4-triphosphate 5/6 kinase.[31] The kinases modify specific substrates of the UPS and determine their stability.[24] The most prominent activity of the CSN is its intrinsic metalloprotease localized to CSN5 and responsible for deneddylation.[32] In this chapter we will focus on the CSN5-mediated deneddylating activity and its function in eukaryotes.

THE NEDDYLATION-DENEDDYLATION SYSTEM AND ITS PHYSIOLOGICAL SIGNIFICANCE

As shown in Figure 2, similar to most Ub-like modifiers, the neddylation cascade consists of E1, E2 and E3s. Neddylation or rubylation has been first described by Jentsch and coworkers.[3] In the first step of this reaction processed Nedd8 possessing a free Gly-Gly C-terminus is activated by a heterodimeric E1 composed of UBA3 and APPBP1.[33-35] Activated Nedd8 is specifically transferred to a thioester linkage of an Ub conjugating enzyme called Ubc12.[3,36] UBA3/APPBP1 and Ubc12 are essential to transfer Nedd8 to a target protein. Recently a second E2 has been identified, which seems to expand the substrate selection for neddylation.[37] Whether Nedd8 conjugation needs E3s is still a matter of debate. The major neddylation targets are the cullins of the CRLs. The RING component of the CRLs, Rbx1, interacts with Ubc12 and can act as an Nedd8 E3 ligase for cullins.[38] Rbx1 is sufficient for neddylation in vitro.[39] For an efficient neddylation of some cullins in vivo the Dcn1 protein (defective in cullin neddylation 1) is needed.[40] Other targets of Nedd8 require specific E3s. For example, the tumor suppressor proteins p53 and p73 are neddylated by Mdm2[41] and the epidermal growth factor receptor (EGFR) is modified by another RING domain protein called c-Cbl (casitas B-lineage lymphoma).[42]

It is still unclear whether poly-Nedd8 chains have a function in vivo. Recently the formation of poly-Nedd8 chains has been demonstrated.[43,44] In vitro poly-Nedd8 chains can be built on the catalytic lysine residue of Ubc12 and Rbx1 is necessary for their transfer to Cullin 1 (Cul1).[45]

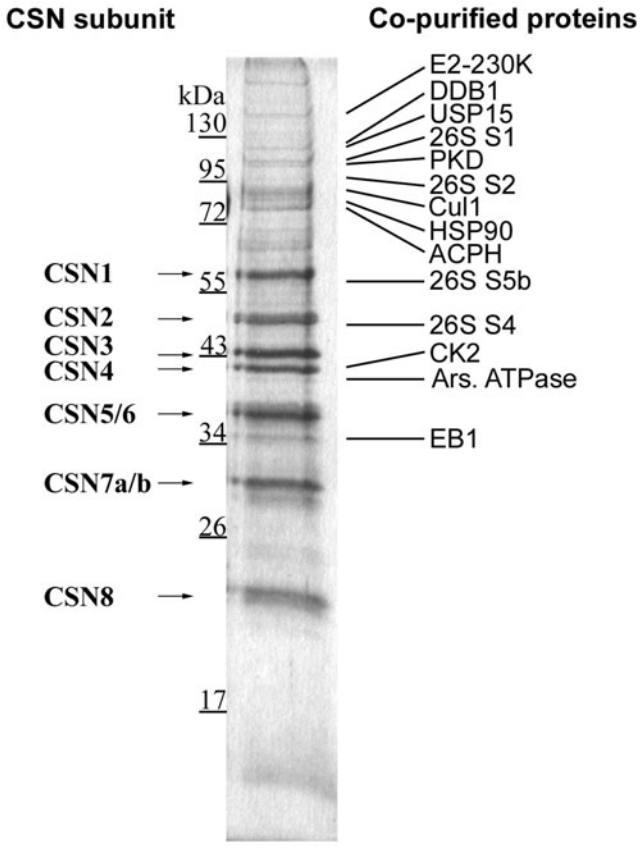

Figure 1. CSN subunits and copurified proteins. Silver-staining of the CSN purified from human erythrocytes according to Hetfeld et al[103] CSN subunits (CSN1-CSN8) were identified via mass spectrometry as published before.[8] Copurified proteins identified via mass spectrometry are the Ub-conjugating enzyme E2 O (E2-230K), the DNA damage-binding protein 1 (DDB1) and the arsenical pump-driving ATPase (Ars.ATPase) (Kähne and Naumann, unpublished data). The identification of the Ub-specific protease 15 (USP15) and of EB1 have been published before[27,28,104] and the 26S proteasome nonATPase regulatory subunit 1 (26S S1), the 26S S2, the heat shock protein 90 (HSP90), the 26S proteasome nonATPase regulatory subunit 5 (26S S5b) and the 26S regulatory subunit 4 (26S S4) were found by Kraft and Dubiel (unpublished data). The Acylamino-acid-releasing enzyme (ACPH) was identified by Overath (unpublished data). Copurified proteins identified via immunodetection: PKD and CK2[29] and Cul1 by Schmaler (unpublished data).

As shown in Figure 2 deconjugation of Nedd8 is catalyzed by the Nedd8 specific deconjugases CSN and Den1 (details see below) as well as by mixed isopeptidases cleaving peptide bonds between target proteins and Ub or Nedd8.

Despite its homology with Ub and the analogy of the ubiquitination-deubiquitination system conjugation with Nedd8 does not target proteins for degradation by the 26S proteasome. Major functional data came from studies on the neddylation-deneddylation of cullins in CRLs. In this respect conjugation-deconjugation of mono-Nedd8 acts as an allosteric regulatory mechanism (for details see below) comparable to phosphorylation-dephosphorylation.

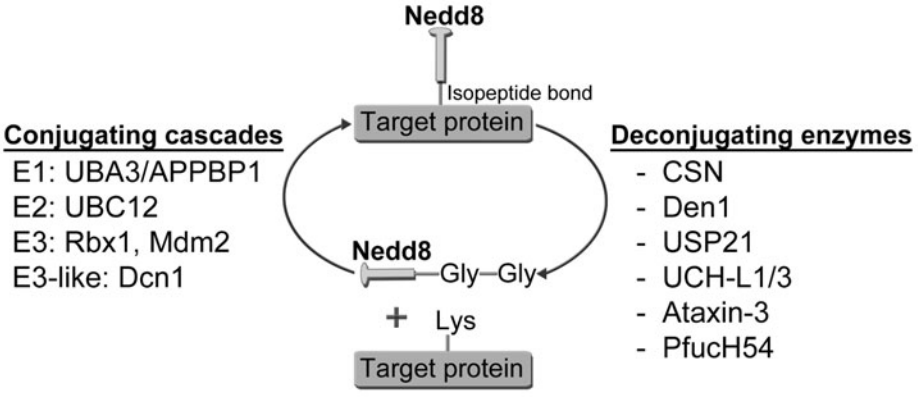

Figure 2. The neddylation-deneddylation pathway. The conjugation and deconjugation reactions of Nedd8 are very similar to those of other Ub family modifiers. The model shows currently known enzymes involved in Nedd8 conjugation as well as deneddylating enzymes, which are described in detail in the text. The best known target proteins for Nedd8 are cullins. However, other targets have been identified recently as outlined in the text.

TARGETS OF NEDDYLATION AND THEIR FUNCTIONS

Nedd8 as a Positive Regulator of Cullin-Ring Ub Ligases

Most prominent substrates of neddylation are members of the cullin protein family.[46] In recent years more targets of Nedd8 modification have been identified such as Mdm2 and p53[47] and others as reviewed before.[48] Nevertheless, the impact of neddylation in most cases remains poorly understood. All cullins shown so far can be modified by Nedd8.[49] The large class of E3s, the CRLs, consist of a scaffolding protein (one of the cullins 1-7), the RING domain proteins Rbx1 or Rbx2 and substrate recognition subunits[50] (see Fig. 3). The $CRL1^{Skp2}$ shown in Figure 3 belongs to the best studied E3 ligases examined to date. Neddylation of Cul1 is required for efficient ubiquitination of the cell cycle regulator $p27^{Kip}$. Nedd8 is covalently attached to Lys720 of Cul1 placing Nedd8 and the RING protein Rbx1 into close proximity. The attachment of Nedd8 increases the affinity between Rbx1 and the E2 necessary for ubiquitination.[51] The unneddylated Cul1/Rbx1 heterodimer is bound to CAND1 (cullin associated, Nedd8 dissociated). CAND1 competes with the substrate adaptor Skp1 for the binding to cullin[52] and sequesters the E3 ligase. Neddylation results in CAND1 dissociation and activation of the CRL. Recent studies point to a conformational control of CRL activity upon Nedd8 conjugation.[53] The neddylated form of the $CRL1^{Skp2}$ allows the initiator Ub to bridge a 50 Å gap between E2 and the substrate by a dramatic conformational change.[54] This process represents the rate limiting step in poly-ubiquitination.[55] Furthermore neddylation-deneddylation seems to influence the degradation of cullins via the UPS representing another layer of regulation.[56]

As reviewed before[48] further prominent neddylation targets are pVHL, BCA3, EGFR, APP and L11. Recent proteomic analysis identified additional potential targets.[44,57]

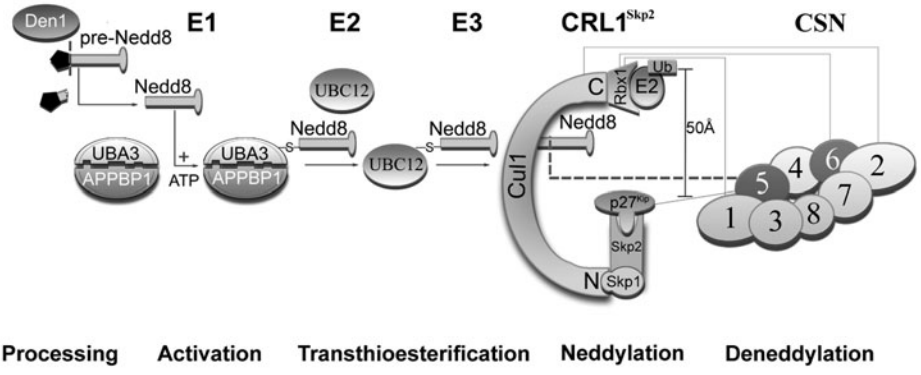

Figure 3. Neddylation and deneddylation cascade of Cul1^{Skp2}. Nedd8 is expressed as an inactive precursor that requires processing. The characteristic Gly-Gly motif is exposed by cleaving C-terminal five amino acids by Nedd8 processing proteases most likely including Den1. Mature Nedd8 is activated by a heterodimeric E1 composed of UBA3 and APPBP1 and transthioesterified to the specific E2. The involvement of an E3 such as Rbx1, Mdm2 or Dcn1 in the process of neddylation is in discussion. Nedd8 is conjugated to Cul1 by the formation of an isopeptide bond (Nedd8-Gly-Gly ↔ ε-NH_2-Lys). Nedd8 conjugation causes a conformational change of the CRL1^{Skp2} and allows the initiator Ub to bridge the 50 Å gap between E2 and the substrate (p27^{Kip}).[53,55] Deneddylation of Cul1 is performed by the isopeptidase activity of CSN5 integrated into the CSN. Interactions of the CSN with components of the CRL^{Skp2} are indicated by grey lines.

THE DIVERSITY OF DENEDDYLASES

Although enzymes of the neddylation cascade are unique and essential in most organisms,[48] deneddylases seem to be redundant. Deconjugation of Nedd8 from target proteins is performed by the isopeptidase activity of the CSN and by other deneddylases. Known deneddylases involved in the Nedd8 conjugation-deconjugation system are outlined in Figure 2. They all belong to the family of deubiquitinating enzymes (DUBs) that is further subdivided. Prominent family members are Ub-C-terminal hydrolases (UCH), Ub-specific processing proteases (USPs) and JAMM-domain-containing metalloenzymes.[58,59]

Den1, a Dual Functional Peptidase

Den1 is a highly conserved Nedd8 specific protease of the USP family type showing high substrate specificity for Nedd8 in comparison to Ub.[60-62] Den1 utilizes a Cys residue as the active site nucleophile (via a His/Asp/Cys catalytic triad) and catalyzes two important reactions. First, it is able to bind Nedd8 selectively and processes its C-terminus while transferring it into its active form (processing of a linear peptide bond). Secondly, Den1 is capable to deconjugate Nedd8 from cullins.[61,63] It is able to deneddylate Cul1 and Cul3 in a concentration dependent manner.[63] In addition, recently Den1 has been shown to deconjugate cellular proteins of unknown identity in vivo. Correspondingly, Den1 null mutants have been characterized by an increase of neddylated proteins.[64] This suggests that many cellular proteins are neddylated in vivo but may exist only transiently or at low steady state levels. Therefore, they are poorly described. Interestingly, the same studies revealed that knockout of Den1 does not enhance the portion of neddylated Cul1 or Cul3 suggesting that Den1 does not function as a cullin deneddylase in vivo.

Further Deneddylases

USP21 was the first ubiquitin-specific protease shown to have dual specificity for Nedd8 and Ub.[65] Further dual specific proteases include UCH-L1 and UCH-L3 which are Cys-proteases expressed in brain and other tissues.[66] Malfunction of UCH-L1 and its two isoforms have been implicated in Alzheimer's and Parkinson's disease.[67] Additional dual specific proteases for Nedd8 and Ub are Ataxin-3[68] and PfucH54.[69]

THE CSN-MEDIATED DENEDDYLATION AND ITS FUNCTIONS

Supercomplexes and Deneddylation

The mephistophelic principle of deneddylation by the CSN has been occupied researchers since its discovery in 2001.[70,71] In vitro CSN-mediated deneddylation of cullins negates CRL activity. In contrast, in vivo experiments clearly demonstrate that the CSN with its deneddylating activity positively influences CRL function.[50,72-74] This paradox is not yet solved. Perhaps CSN-mediated deneddylation allows the protection[75] and reassembly of CRLs[74] or additional deneddylating events are important for cell functions. Most of the genetic data obtained with knockouts of CSN subunits are associated with the disruption of the CSN complex and can not solely be due to a deficiency of deneddylation. Perhaps other features of the CSN such as associated kinases or USP15 are responsible for the observed effects. For example, the CSN can increase the efficiency of $CRL2^{pVHL}$ by facilitating the release of ubiquitinated substrates from pVHL (von Hippel-Lindau protein), which is independent of deneddylation.[76]

A prerequisite for cullin deneddylation is the interaction of the CSN and the CRLs. The formation of this type of supercomplexes has been demonstrated for most CRLs containing the known cullins 1-7 in all studied species.[15,70,71,77,78] Direct binding between cullins and CSN2 as well as Rbx1 and CSN6 and CSN1 seem to be the stabilizing connections in these supercomplexes. Interestingly, CSN-CRL supercomplexes can assemble into even larger particles together with the 26S proteasome.[79,80] Recently we have identified supercomplexes in mammalian cells consisting of the CSN, the $CRL1^{\beta\text{-}TrCP}$ and the β-catenin destruction complex.[23] The formation of these complexes is necessary for efficient β-catenin degradation and dependent on CSN-mediated deneddylation. Thus, deneddylation not only activates CRLs, it might also compartmentalize CRLs into supercomplexes that bring together all necessary parts of the proteolytic machinery. Notably, pulldowns of supercomplexes containing the CSN in most cases exhibit partially neddylated cullins indicating a tight regulation of deneddylation.

The MPN+/JAMM Motif

It has been shown by Cope et al that the metalloenzyme JAMM motif in the CSN5 subunit is responsible for the deneddylating activity of the CSN.[32] The MPN+ motif was predicted to have metalloprotease activity.[81] It possesses the His-X-His-X_{10}-Asp consensus sequence (where X indicates any residue) accompanied by an upstream conserved Glu.[32] The MPN+/JAMM motif is found in archaea, bacteria and eukaryots. The CSN5 paralog subunit of the 26S proteasome lid is S13/Rpn11, which is a deubiquitinating enzyme and essential for the functioning of the 26S proteasome.[82-85] Interestingly, S13/Rpn11 in

the context of the 19S regulator/PA700 of the 26S proteasome preferentially cleaves Ub chains linked via Lys63. Also the BRISC-associated Brcc36 protein disassembled Lys63 chains.[86] There are two additional MPN+/JAMM motif-containing proteins belonging to the family of deubiquitinating enzymes called AMSH and AMSH-LP. These proteins specifically cleave Lys63-linked poly-Ub chains from internalized receptors.[87] Surprisingly, AMSH-proteins are active as monomers, whereas S13/Rpn11[82,84] and CSN5[12] have to be complex-bound to display their proteolytic activities.

The MPN+/JAMM motif of CSN5 and of all the other MPN+/JAMM proteins is a typical Zn^{2+}-binding metalloprotease domain, which can be blocked by Zn^{2+} chelators such as o-phenanthroline. The specificity of CSN5 activity is most likely influenced by interacting subunits within the CSN. Our preliminary data reveal that the CSN exclusively removes Nedd8 from isopeptide bonds. It does not cleave Nedd8-AMC or Nedd8 linear extensions, indicating that the CSN is unable to process preNedd8 (Schmaler and Dubiel, unpublished data and as published before[63]). The deneddylating activity of the CSN can be knocked out by point mutations in the MPN+/JAMM motif of CSN5.[15,32] We recently found that CSN variants containing the CSN5D151N mutant are less efficient in forming supercomplexes necessary for β-catenin degradation.[23] Under these conditions cullins are mostly neddylated but supercomplexes fall apart and are not active anymore.

REGULATION OF CSN-MEDIATED DENEDDYLATION

Up to date little is known about regulation of deneddylation. During apoptosis deneddylation by the CSN is activated by caspase cleavage of CSN6.[88] This modification might be part of the general downregulation of the UPS during apoptosis[89] and accelerate the programmed cell death.

The CSN is phosphorylated[17,18] and it is conceivable to assume that phosphorylation-dephosphorylation might control CSN complex activities. Recently we found that presumably GSK3β-dependent phosphorylation of CSN1 is necessary for efficient assembly of the CSN into the β-catenin degrading supercomplex.[23] Interaction of the CSN with CRLs in larger supercomplexes might be driven by different kinases of signaling cascades. For example, UV irradiation can trigger the release of the CSN from $CRL4^{DDB2}$ complex, inducing its hyperneddylation. Simultaneously, UV light leads to association of the CSN with $CRL4^{CSA}$.[15]

The grade of cullin neddylation concomitantly with the multimodal activation of CRL-dependent ubiquitination[55] can be regulated by CRL substrates.[90] Hershko and coworkers demonstrated that the neddylation-deneddylation of Cul1 in $CRL1^{Skp2}$ is regulated by the availability of the F-box protein and/or of the substrate $p27^{Kip}$. In this model the substrate prevents CSN-mediated deneddylation by a yet unknown mechanism.[90] Accordingly the substrate controls its own ubiquitination and degradation. The regulation of cullin neddylation by adaptor proteins and substrates has been extended to CRL2, CRL3 and CRL4a complexes.[91] In these studies HIF-1α binding to VHL increased Cul2 neddylation presumably by a conformational change of the CRL2. The process was, however, independent of the CSN and of CAND1.[91]

We hypothesize that direct substrate interaction with the CSN might influence CSN-mediated deneddylation. There is a large number of CRL substrates interacting with CSN5 or with other subunits of the CSN.[24] We speculate that binding of these ligands might modulate CSN-mediated deneddylation. First preliminary data revealed that $p27^{Kip}$

inhibited cullin deneddylation by the CSN in vitro (Schmaler and Dubiel, unpublished data). Thus, direct substrate control of CSN-mediated deneddylation might be an important regulatory principle of CRL activity. Therefore, CSN-based supercomplexes can exhibit neddylated cullins.

INVOLVEMENT OF THE CSN-MEDIATED DENEDDYLATION IN THE PATHOGENESIS OF DISEASES

The CSN controls important cellular regulators including proto-oncogenes such as c-Jun and β-catenin or the tumor suppressors $p27^{Kip}$ and p53, known to be frequently malfunctioning in cancer.[23,26,30,92-94] Aggressive colorectal carcinomas are known to show decreased amounts of $p27^{Kip}$ due to accelerated Ub-dependent degradation.[95] Both p53 and $p27^{Kip}$ directly interact with CSN5, a necessary step for CSN-mediated phosphorylation, which targets them to degradation by the 26S proteasome.[30,93] Inhibitors of CSN associated kinases such as curcumin and curcumin-like substances stabilize the tumor suppressors and might drive tumor cells into apoptosis.[96] On the other hand, inhibition of CSN associated kinases destabilizes c-Jun, a major transcriptional regulator of tumor angiogenesis.[26] We and others found that curcumin and curcumin-like substances are potential anti-angiogenic agents and might be applied for novel tumor therapies.[97,98] A further promising approach would be to target the CSN-mediated deneddylation by specific metalloprotease inhibitors. Blocking deneddylation might lead to an unbalanced expression of tumor suppressors accompanied with apoptosis of tumor cells.

Dysregulation of CSN subunits has been often observed in tumor cells.[94,99-101] In particular high level of CSN5 has been detected in a variety of human cancers and is sometimes correlated with a poor prognosis. For example, CSN5 might play an important role in carcinogenesis of pancreatic cancer[101] and in breast cancer progression.[99] This is possibly based on its interaction with various tumor associated proteins suggesting that CSN5 contributes to cancer cell proliferation and could be a novel target of cancer therapy.[94,99] On the other hand, the CSN has been implicated in DNA repair either via the regulation of p53 or via other signaling pathways. It is involved in transcription-coupled repair as well as global genomic repair via the interaction with Cul4-DDB1-CSA and Cul4-DDB1-DDB2 complexes.[102] Recently, growth arrest in Arabidopsis CSN mutants were related to DNA damage, demonstrating a clear role of the CSN in DNA repair.[13] Moreover, overexpression of CSN2 caused genomic instability by influencing the CSN regulated degradation of CDC6 via anaphase promoting complex/cyclosome.[16] As a major regulator at the interface between signaling and proteolysis the CSN will be an important target of future therapies.

CONCLUSION AND FUTURE PERSPECTIVES

It is evident from the above survey that the mechanism and the function of the CSN-mediated deneddylation as well as its regulation are poorly understood. First of all the CSN paradox of deneddylation as a bad as well as a good process for CRL actions should be solved. Second, the mechanism of CSN-mediated deneddylation and the activation of CSN5 by its assembly into the complex are obscure. We need more information on the regulation of deneddylation. Which signaling cascades intervene with CSN-mediated

deneddylation by phosphorylation-dephosphorylation? Has the binding of diverse ligands to the CSN to do with the regulation of deneddylation? We need to answer these questions to understand how the CSN is involved in the pathogenesis of multiple diseases. This is a prerequisite to define the CSN as a target for novel therapies.

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CHAPTER 6

MECHANISM, SPECIFICITY AND STRUCTURE OF THE DEUBIQUITINASES

David Komander*

Abstract: Removal of ubiquitin from modified proteins is an important process to regulate the ubiquitin system. Roughly 100 dedicated enzymes for this purpose, the deubiquitinases, exist in human cells and are intricately involved in a wide variety of cellular processes, although many enzymes remain unstudied to date. The deubiquitinases consist of five enzyme families that contain USP, OTU, UCH, Josephin, or JAMM/MPN+ domains providing catalytic activity. We now understand the catalytic mechanisms of all deubiquitinase families from structural work and more importantly, have obtained insight into an unanticipated variety of ways to exercise specificity. It emerges that deubiquitinases exploit the entire complexity of the ubiquitin system by recognizing their substrates, particular ubiquitin chain linkages and even the position within a ubiquitin chain. This chapter describes the mechanisms of deubiquitination and the different layers of deubiquitinase specificity. The individual deubiquitinase families are discussed with a focus on structure, regulation and specificity features for selected enzymes.

INTRODUCTION

Protein ubiquitination is emerging as one of the most important regulatory posttranslational modifications. Most prominent and well-studied are its roles in protein degradation,[1] however, recent years have seen an explosion of data on nonproteolytic roles of ubiquitination in cell signalling processes, intracellular trafficking and the DNA damage response.[2] The versatility to modulate such diverse processes is achieved by the ability of ubiquitin to form at least eight different types of polymers (reviewed in refs. 3,4). In such ubiquitin chains, isopeptide bonds are formed between the ubiquitin C-terminus and one

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of seven lysine residues of a second ubiquitin (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63). Alternatively, also the N-terminal amino group can be used for ubiquitin linkages to generate linear ubiquitin chains.[5] The linkage type of the ubiquitin chain determines whether a ubiquitination event will trigger proteasomal degradation (mediated by Lys48- and Lys11-linked chains and possibly other chain types) or signalling processes such as protein kinase activation, or DNA repair pathways (mediated by Lys63-linked and linear chains).[4]

Like other posttranslational modifications, ubiquitination is reversible. The human genome encodes ~98 deubiquitinating enzymes, also known as deubiquitinases or DUBs, which provide different functionalities and specificities to carefully regulate ubiquitination events. These enzymes cluster in five structurally unrelated families:[6,7] the ubiquitin specific proteases (USP, 56 individual members in humans plus 11 additional genes from the USP17 multigene family),[8] the Ovarian Tumor (OTU) DUBs (15 members), the Ubiquitin C-terminal hydrolases (UCH, 4 members), the Josephin domain DUBs (4 members) and the JAB1/MPN/MOV34 (JAMM/MPN+) DUBs (8 members).[6,7]

An important role of DUBs is the maintenance of a free ubiquitin pool in cells. Ubiquitin genes produce polyubiquitin precursor proteins and specialized DUBs such as USP5/IsoT are required to process these precursors into monoubiquitin.[9] Ubiquitin has a half-life of several days in cells, which is achieved by recycling of ubiquitin from degraded substrates. The proteasome itself harbors three DUBs (USP14, UCHL5 and POH1) that hydrolyze the chains prior to degrading of the substrate, hence recycling ubiquitin for further use.[10] These roles of DUBs in maintaining a stable pool of monoubiquitin are performed by a handful of dedicated enzymes.

The majority of DUBs however directly regulate protein ubiquitination events. Most commonly, ubiquitination will lead to protein degradation and hence deubiquitination has a stabilizing effect, actively increasing protein levels in cells. Deubiquitination can also inhibit cellular signalling cascades that are activated by nondegradative chains types. As protein homeostasis as well as cell signalling often requires tight temporal and spatial regulation, the DUBs affecting these pathways are also regulated in many different ways. Furthermore, DUBs have maintained remarkable specificity, with regard to the selection of substrates, their preference for particular chain types and even their positioning on a ubiquitin chain.

De-regulation of deubiquitination can lead to imbalances of protein levels and hence to disease. For example, the degradation of the oncogene c-myc is mediated by USP28, which retains MYC in the nucleus and prevents it from entering the nucleolus, where it is degraded.[11] Proliferation of some cancer cell lines depends on high MYC levels and knock-down of USP28 inhibits growth of these cell lines, suggesting an oncogenic role of USP28.[11] However, USP28 also stabilizes several important mediators of the DNA damage response, including Chk2 and 53BP1, after DNA damage has occurred.[12] Hence, loss of USP28 attenuates the cellular response to DNA damage, rendering USP28 a likely tumor suppressor candidate. A similarly complex example is the regulation of the p53 tumor suppressor by the deubiquitinase USP7. USP7 is thought to directly stabilize p53 levels, but in addition, USP7 also stabilizes the levels of the p53-destabilizing E3 ubiquitin ligase, MDM2 in cells.[13,14] These two examples illustrate the importance of DUBs in regulating protein stability.

Several cell-signalling DUBs have further well-established links to cancer. Familial cylindromatosis, a rare benign skin cancer affecting hair follicles and sweat glands of skin and neck, has its genetic cause in truncation of the *cyld* tumor suppressor gene.[15]

Truncation ablates the function of the USP deubiquitinase domain of CYLD.[16-19] This domain has specificity for Lys63-linked and linear polyubiquitin chains[19,20] and has been implicated in regulation of nondegradative signalling pathways leading to the activation of the NF-κB transcription factor.[21] CYLD also has roles in numerous other Lys63-dependent processes and may serve as a general housekeeping enzyme regulating Lys63 ubiquitin linkages.[22] Other DUBs affecting primarily nondegradative ubiquitination events are the NF-κB regulator and tumor suppressor A20,[23,24] the TRABID enzyme involved in the Wnt/β-catenin pathway[25] and OTUD5 involved in the interferon response factor (IRF)-3 signalling pathway.[26]

Hence, DUBs have established roles in cancer, but also in inflammation and immune responses and in neurological disorders. This has led to an increasing interest to target these enzymes pharmacologically, for which a detailed mechanistic and structural insight is essential. This chapter provides an overview of the structural features of the deubiquitinases and discusses their mechanism and common concepts of specificity and regulation. For further information, readers are referred to recent reviews on the topic.[7,27,28]

MECHANISMS OF DEUBIQUITINATION

DUBs are proteases that hydrolyze the isopeptide bond between the ubiquitin C-terminus and the Lys ε-amino group. Four of the five human DUB families (USP, OTU, UCH, Josephin) are Cys proteases while the JAMM/MPN+ DUBs are zinc dependent metalloproteases.

Mechanism of Cys-Dependent DUBs

The Cys-dependent deubiquitinase families comprise a catalytic diad or triad and their mechanism is similar to that of the Cys protease papain.[29,30] A catalytic Cys performs a nucleophilic attack on the isopeptide linkage of a ubiquitinated Lys residue. This is facilitated by a nearby His side chain that lowers the pKa of the Cys. A third residue, usually Asp or Asn, aligns and polarizes the catalytic His. This is not always essential and some enzymes lack the third residue and polarize the His by other means. This mechanism has two additional features. A negatively charged transient reaction intermediate is stabilized by an oxyanion hole formed nearby by hydrogen-donating residues. A more stable acyl-intermediate is formed when the carboxyl-group is covalently bound to the enzyme, after the amino group has been hydrolyzed. The reaction cycle is completed by water-mediated hydrolysis of the acyl-Cys intermediate.

The mechanism of Cys-based deubiquitinases has been exploited by the generation of modified ubiquitin-derived probes that have reactive C-termini.[31,32] In the simplest molecule, ubiquitin aldehyde, the C-terminal carboxyl group of Gly76 is exchanged to an aldehyde group, which after binding to the catalytic Cys, is not hydrolyzed by water. This molecule acts as a potent and specific inhibitor of Cys-dependent deubiquitinases.[33] These ubiquitin probes have been improved since[34] and several probes are commercially available, including ubiquitin vinyl-sulfone (Ub-VS) and ubiquitin vinyl methyl ester (Ub-VME). Ubiquitin probes were instrumental in identifying novel deubiquitinases in cells[31] and to obtain the first ubiquitin-DUB complexes for structural characterisation.[35-37] However, different DUBs display different affinities for individual probes[38] and some enzymes cannot be modified by these reagents.

Mechanism of Metalloprotease DUBs

JAMM/MPN+ family deubiquitinases are zinc-dependent metalloproteases. Within their catalytic site, invariant His, Asp and Ser side chains coordinate two zinc ions.[39] The structure of the first JAMM/MPN+ domain revealed similarities to cytidine deaminase, suggesting that these families were evolutionarily related.[40] The catalytic mechanism was proposed to be similar between these two hydrolytic enzymes. The zinc ion in the catalytic site activates a water molecule to form a hydroxide ion, which is able to attack the carboxyl carbon in the isopeptide link. The transient tetrahedral intermediate collapses with elimination of the ε-amino group and replacement of the amine with a hydroxyl group from the activated water molecule. A nearby invariant Glu residue acts both as a proton acceptor and donor in this catalytic cycle.[40] These predictions were recently supported by crystal structures of the AMSH-LP JAMM/MPN+ domain in isolation and bound to diubiquitin (see below).[41]

CONSIDERATIONS FOR DEUBIQUITINASE SPECIFICITY

The 98 human DUBs are a diverse superfamily of enzymes. As will be discussed in detail below, the catalytic domains of the five DUB families share no sequence similarity and have distinct structural folds. Most DUBs however hydrolyze ubiquitin chains into monoubiquitin. Hence they can bind to two ubiquitin moieties, placing the isopeptide bond to be cleaved across their active site. In this arrangement, the 'distal' ubiquitin molecule presents its C-terminal Gly to the catalytic centre, while the 'proximal' ubiquitin is bound through its modified Lys. All DUBs analyzed to date bind ubiquitin through a significant distal binding site, while the proximal ubiquitin binding site is less extensive. The catalytic centre, bound to the flexible linker between ubiquitin moieties, rigidifies the linker region by tight interactions. While these general principles hold true for most DUBs, subtle differences in ubiquitin binding can change enzymatic properties significantly and contribute to DUB specificity.

It is important to comprehend the complexity of the ubiquitin system in order to discuss DUB specificity. In contrast to other modifications such as phosphorylation or acetylation, where a single modifying group is attached, ubiquitination is further organized by its polymeric nature. Ubiquitin chains are the principal outcome of ubiquitination and have different structural and topological features. By dealing with ubiquitin chains, DUBs face many additional layers where decisions regarding specificity have to be made. It is not yet clear whether all the ways to exercise specificity are employed in vivo, yet many observations suggest that DUBs exploit the system to its full potential. The following section outlines the emerging concepts in DUB specificity.

Ubiquitin versus Ubiquitin-Like Protein Cleavage

Ubiquitin is one of 17 small ubiquitin-like (UBL) modifiers in humans which all contain the characteristic ubiquitin fold.[42] Several UBLs, including SUMO, Nedd8, ISG15, FAT10 and ATG12 modify proteins using a similar mechanism compared to ubiquitin.[42,43] The result is a topologically similar modification (SUMO, Nedd8 and Atg12 are roughly the same size and shape as ubiquitin, while ISG15 and FAT10 resemble diubiquitin) yet

DUBs are able to distinguish between ubiquitin and UBLs. The key to this selectivity lies partly in the C-terminal four residues preceeding the Gly-Gly motif. SUMO, Atg12 and FAT10 share no sequence similarity with ubiquitin within these residues. However, Nedd8 has a similar sequence and ISG15 has an identical sequence compared to ubiquitin. It is therefore not surprising that both Nedd8 and ISG15 can also be hydrolyzed by some cross-reactive DUBs (see below for examples).

Isopeptide versus Peptide Bond Cleavage

Not all ubiquitin chains are linked to Lys residues via isopeptide bonds, but chains can also be linked through the α-amino group of the N-terminus (linear ubiquitin chains).[5] This chain type has nonproteolytic roles in NF-κB signaling[44] and linear chains are also the source of monoubiquitin in cells as ubiquitin is translated from linear polygenes. This requires DUBs to deal with this particular chain type and peptide bonds. Due to structural differences between the isopeptide (linked through an elongated, flexible side chain) and the peptide bond (bulky side chain of Met1, Ramachandran restraints), cleavage of linear chains requires a more spacious active site environment.[20] Recent data shows that USP enzymes can cleave linear chains, albeit with lower activity. Most other DUB families do not hydrolyze this chain type, although enzymes acting on linear chains may exist within these families.[20] Cleavage of peptide bonds by USPs may also allow them to hydrolyze non-ubiquitin sequences and was suggested to be used in the observed USP1 autoproteolysis within its USP domain.[45]

Linkage Specificity within a Ubiquitin Chain

The most striking layer of DUB specificity is the ability of many enzymes to select between different ubiquitin chain linkages.[20] Importantly, chain linkage specificity is not determined by DUB family. This is in contrast to e.g., phosphatases that utilize different enzyme families for removal of phosphates from Tyr, or Ser/Thr residues.[46] For example, OTU and USP family enzymes have evolved Lys48- and Lys63-specific members.[20] The JAMM/MPN+ family of DUBs may have intrinsic specificity for Lys63-linked chains (see below).

Currently, however, only three ubiquitin chain types (Lys63-, Lys48-linked and linear) are available for in vitro studies of DUB specificity. Hence the overall picture remains incomplete and requires development of new and better reagents and assays. As highly specific DUBs exist, it is possible that even new DUB families may be discovered once proper reagents are available.

Exo- vs. Endo Activity within a Ubiquitin Chain

Polymers of ubiquitin can be cleaved from the end (exo) or within a chain (endo) and both mechanisms have been described.[19,47] This mechanistic difference has profound consequences. An endo-DUB would be able to remove entire chains from substrates, reversing polyubiquitination most efficiently. It would however result in free chains and further DUB action (likely by distinct enzymes) is required to recycle monoubiquitin from the released chains. In contrast, exo-DUB activity seems inefficient if chains are long; such activity would be required though for recycling, e.g., proteasome-bound, DUBs.

Chain Cleavage versus Substrate Deubiquitination

Ubiquitination can often be divided into two independent steps, chain initiation and chain elongation. One mechanistic reason for this is that the sequence context of the 'first' ubiquitin on a substrate Lys is distinct from the (always equivalent) ubiquitin sequence used for elongating the chain. DUBs face the same problem. Some DUBs may only target ubiquitin-ubiquitin linkages, but their action might not remove the proximal ubiquitin, leaving the substrate monoubiquitinated. In fact, it is often not clear what the physiological end product of a deubiquitination reaction is. Ubiquitin chain editing,[48] i.e., the switch from one chain type (e.g., a 'signalling' Lys63-linked chain) to another type (e.g., 'degrading' Lys48-linked chain) may benefit from substrates not fully deubiquitinated. In such scenario, DUB action on a substrate leaves a platform, i.e., monoubiquitin, for subsequent ubiquitination with a different chain type. Enzymes that combine DUB and E3 ligase activity have been described[24,48] and many DUBs interact with E3 ligases.[49]

Sequence Specific Deubiquitination

There may be DUBs that act on monoubiquitinated targets, e.g., those left by prior chain deubiquitination (see above). These DUBs may specifically recognize a ubiquitinated sequence context in target proteins and hence hydrolyze monoubiquitin, or even entire ubiquitin chains en bloc. This would allow for a great level of specificity, yet such sequence specific DUBs have not been formally described yet. However, nonspecific DUBs such as USP family members, may be able to accommodate a wider range of sequences in their proximal binding site and hence may completely deubiquitinate substrates.

Substrate Recognition and Specificity

In order to function within a particular pathway, DUBs need to select their substrate proteins. Many DUBs contain additional protein interaction domains to facilitate direct substrate interaction, yet also indirect means, e.g., by localizing DUBs to specific places in the cell may aid such selectivity.[7] Localisation of a DUB via protein interaction domains may affect other layers of specificity, such as linkage preference. Formation of a DUB-substrate complex would significantly increase the local concentration of particular ubiquitin linkages, potentially overriding the intrinsic linkage preference of the DUB.

THE FIVE HUMAN DUB FAMILIES

A surge of data in the last years has revealed many aspects of DUB biology and in particular structural studies by X-ray crystallography and NMR have yielded important insights in DUB activity, specificity and regulation. In the following section, the five human DUB families are discussed individually and recurrent mechanisms of regulation and specificity are outlined.

USP Domain DUBs

USP family DUBs comprise the largest and most diverse family of deubiquitinases in mammalian cells with 56 distinct members. Another 12 USP17 (also known as DUB3)-like USP genes exist. USP domain DUBs are usually large proteins (between 350 and 3400 amino acids (Aa), average size ~1000 Aa) with a core catalytic domain of ~350 Aa. Outside of their catalytic core, USP enzymes comprise numerous other domains, including protein interaction domains that facilitate substrate binding, or domains determining subcellular localization. Only USP19, USP30 and USP48 contain predicted transmembrane regions. USP19 is anchored at the endoplasmic reticulum,[50] while USP30 is localized in the outer membrane of mitochondria.[51] In addition, ubiquitin binding domains (UBDs) such as zinc-finger ubiquitin specific protease (ZnF UBP), ubiquitin interacting motifs (UIM) and ubiquitin associated (UBA) domains are found in several enzymes.[6,7] Finally, ubiquitin-like (UBL) domains are found in at least 18 USP domain DUBs.[52] The presence of UBL domains might suggest a common autoregulatory mechanism that remains unstudied to date.

The USP domain itself consist of three sub-domains, Palm, Thumb and Fingers, resembling a right hand[35] (Fig. 1A). The catalytic centre lies at the interface between Palm and Thumb, while the Fingers domain grip the distal ubiquitin. Dramatic conformational changes are present in USP domains upon ubiquitin binding.[35,47] In USP7, the catalytic Cys shifts upon ubiquitin binding from a catalytically unproductive position to an active position where it interacts with the catalytic His residue[35] (Fig. 1B). In contrast, the catalytic machineries of USP14 and USP8 are properly aligned for catalysis in absence of ubiquitin, however ubiquitin-binding surface loops block the ubiquitin binding site[47,53] and these loops undergo conformational changes upon ubiquitin binding in USP14.[47] Furthermore, in USP8, which has so far only been crystallized without ubiquitin, the Fingers domain is tightened inward, additionally blocking the ubiquitin binding site (Fig. 1C).[53] Inactive conformations are not a global feature of USPs, as the CYLD USP domain was poised for catalysis and did not show a blocked active site cleft (Fig. 1D).[54]

Most of the analyzed USP family enzymes are nonspecific and will cleave any chain type,[20] yet some members show distinct specificities. USP14 preferentially cleaves Lys48-linked ubiquitin chains,[47] while CYLD specifically hydrolyzes Lys63-linked and linear chains.[20] The structures of USP14 and CYLD have given insights into their mechanism of action and specificity. The structure of the Lys63-specific enzyme CYLD has revealed that the proximal ubiquitin binding site and in particular an extended loop in this region, contribute to the observed linkage specificity (Fig. 1D).[19] USP domains can have endo- and exo-activity against polyubiquitin chains. The Fingers-subdomain of USP7 and USP14 wraps around the distal ubiquitin, restricting access to Lys48 and Lys63 (Fig. 1A). This allows these USPs to bind to the distal end of a chain only and consistently, USP14 acts primarily as an exo-DUB.[47] In contrast, CYLD lacks the Fingers subdomain, allowing Lys63 (and linear) chains to continue from the distal ubiquitin (Fig. 1D). Hence CYLD can interact with a ubiquitin chain at any point including at internal positions and has endo-activity.[19] Several USP domains are cross-reactive with other UBL modifiers.[55,56] These enzymes include USP18 and USP13 that interact with ISG15 suicide probes (ISG15-vinyl sulfone, similar to UbVS, see above) better than with ubiquitin probes and several other USP domains that bind to both ubiquitin and ISG15 probes.[55] Equivalent studies are important for other UBL modifiers with more elusive roles.

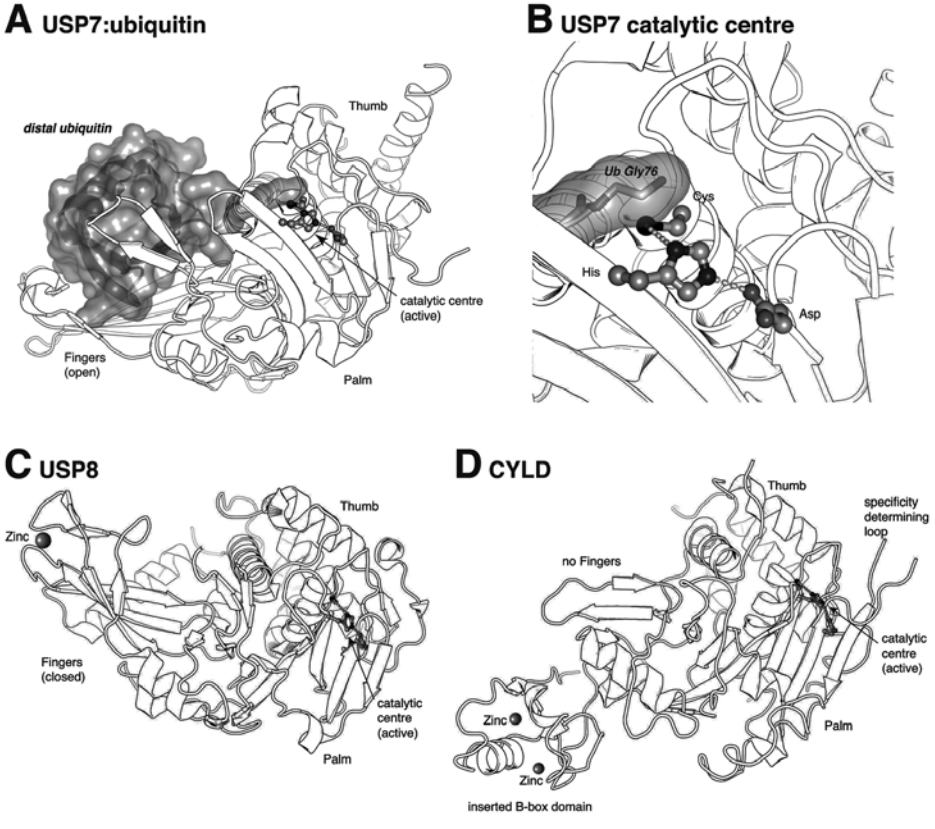

Figure 1. Structures of USP domain deubiquitinases. A) Structure of USP7 (also known as HAUSP) bound to ubiquitin (pdb-id 1nbf).[35] The USP domain (white) is shown in cartoon representation and the catalytic centre residues are shown as stick models in grey colors. Ubiquitin is shown under a grey semitransparent surface. Hydrogen bonds are indicated by dotted lines. The Fingers, Palm and Thumb domains are indicated. B) Close-up view of the active site of USP7 bound to ubiquitin. The catalytic triad residues and their interactions are shown. C) Structure of USP8 (pdb-id 2gfo).[53] In the absence of ubiquitin, the Fingers subdomain is closer to the Thumb/Palm preventing ubiquitin binding. The Fingers subdomains of 45 out of 56 USP domain DUBs including USP8 comprise a functional zinc-binding site (zinc indicated as a grey sphere).[57] D) Structure of CYLD (pdb-id 2vhf).[19] CYLD does not contain a Fingers subdomain, allowing it to act as an endo-deubiquitinase against Lys63-linked and linear chains. A specificity determining loop near the active site disfavors Lys48-chain binding. The CYLD USP domains contains a zinc-binding B-box domain inserted in its sequence.

An intriguing structural feature of USP domains is their disrupted catalytic domain. The catalytic core of USP domains comprises ~350 residues, yet more than half of the human USPs have catalytic domains of much larger sizes (400-850 Aa) annotated.[57] This is an artefact from the bioinformatic annotation, which defines USP domains as the region between the N-terminal Cys-box and C-terminal His- and Asp-boxes that contain the residues of the catalytic triad. More detailed analysis shows that the USP domain core can be subdivided into six conserved sequence boxes, spanning ~350-400 residues, in all human USP domains.[57] The five boundaries between boxes are points where large insertions occur. These inserted sequences contain additional independently

folded domains, including protein interaction domains (e.g., B-box in CYLD (Fig. 1D)[19] and MYND domain in USP19)[50] and ubiquitin binding domains (e.g., UBA domains in USP5,[58] or UIM motifs in USP37).[57] Seven USPs contain ubiquitin-like folds as an insertion.[52,57] Although not yet backed up by structural work, the UBL insertions are likely positioned near the distal ubiquitin binding site, where they may directly alter USP function.[52] Structures of USP domains containing an insertion will likely yield interesting insights regarding regulation of these enzymes.

Further regulation of USP domain DUBs is provided by interacting proteins, and more than 770 DUB interacting proteins have recently been revealed.[49] Many USP family members interact with WD40 repeat containing proteins. The WD40 protein UAF1 (USP1 associated factor, also known as WDR48) was shown previously to interact with USP1, USP12 and USP46 and more importantly to allosterically activate these USP enzymes.[59,60] Another commonly observed interaction exists between DUBs (not only USP domains, but also other classes) and E3 ubiquitin ligases.[7,49] DUB activity may prevent autoubiquitination, a common feature of E3 ligases, or alternatively, E3 ligases might down-regulate DUBs. This yet again illustrates intricate interplay between ubiquitination and deubiquitination.

To date, most USP domain containing enzymes remain poorly characterized and virtually no literature exists for more than 25% of the USP proteins. This is likely to change with new genome wide screens, which have proven highly successful in identifying new DUB functions (see ref. 61 for an example). Still, biochemical characterisation is important to understand more about this enzyme family.

OTU Domain DUBs

Human cells contain 15 OTU domain DUBs, only half of which have been studied to date. Several OTU enzymes are involved in cell signalling processes, regulating NF-κB signalling (A20, Cezanne1/2),[24,62] Wnt signalling (TRABID)[25] and IRF3 signalling (OTUD5, also known as DUBA).[26] Other OTU members have more elusive roles. OTU family proteins range in size from 230 Aa to 1222 Aa and like USP domains, often contain additional domains with links to the ubiquitin system, including UIM and UBA domains and UBL folds.[7]

The structure of the OTU domain does not resemble that of USP domains, yet the catalytic residues of the active enzymes superpose well (Fig. 2A,B).[54] The OTU domain core comprises ~150-200 residues,[63] however, a subclass of enzymes, (A20, Cezanne1/2, TRABID, VCIP135) contain an extended catalytic core of ~360 residues (Fig. 2D[54] and D.K., unpublished). Like some USP domains, the distal binding site of OTU domains undergoes a disorder-to-order transition upon ubiquitin binding.[37] At least in one case (OTUB1),[38] the active site is in an unproductive configuration and requires conformational changes prior to activation (Fig. 2C). The catalytically inactive resting state found in many DUBs, not only OTU members, may protect the catalytic Cys residue from oxidative stress. A low pKa Cys residue in the active site would be attacked by reactive oxygen species (ROS) and it has been suggested that high levels of ROS affect the function of the OTU DUB Cezanne.[64] ROS may also regulate other deubiquitinase classes.

OTU family enzymes display marked chain linkage specificity. TRABID and DUBA are Lys63-specific,[20,26] while OTUB1 is Lys48-specific.[38] The A20 OTU domain is Lys48-specific in vitro,[54,65] yet the substrates of A20 are modified with Lys63-linked chains. A20 was shown to act on Lys63-polyubiquitinated substrates

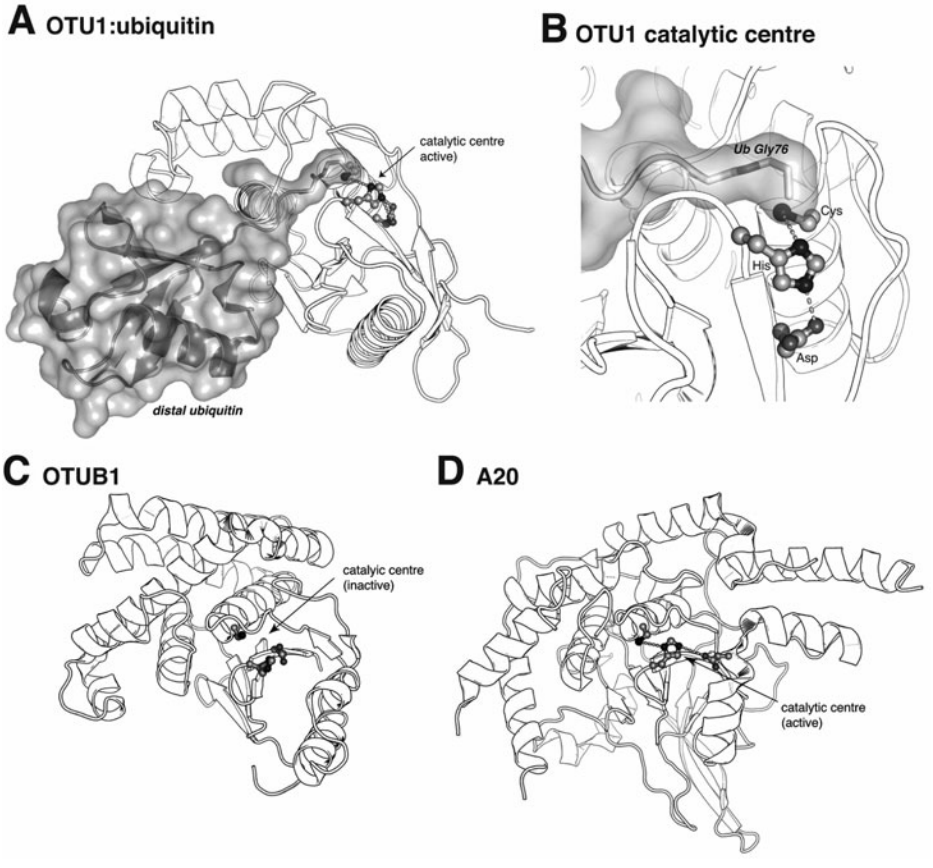

Figure 2. Structures of OTU domain deubiquitinases. A) Structure of OTU1 bound to ubiquitin (pdb-id 3by4).[37] The OTU domain (white) is shown in cartoon representation and the catalytic centre residues are shown as stick models in grey colors. Ubiquitin is shown under a grey semitransparent surface. Hydrogen bonds are indicated by dotted lines. B) Close-up view of the active site of OTU1 bound to ubiquitin. The catalytic triad residues and their interactions are shown. C) Structure of OTUB1 (pdb-id 2zfy).[38] The Otubains (OTUB1 and OTUB2) contain several additional helices. D) Structure of A20 (pdb-id 2vfj).[54] The A20 catalytic domain is ~150 residues longer and contains additional structural elements.

such as TRAF6, releasing whole chains from the proteins, potentially by cleaving the proximal ubiquitin.[65] Most OTU domains do not cleave linear chains efficiently and hence may be strict isopeptidases,[20] however, OTUB1 was suggested to cleave both ubiquitin and Nedd8 conjugates.[38]

UCH Domain DUBs

The UCH family of deubiquitinases contains four members, two of which consist of only a catalytic domain (UCHL1 and UCHL3, ~200 Aa).[6,7] UCHL1 and UCHL3 have roles in brain function[66-68] and the Ile93Met point mutant of UCHL1 is associated with familial Parkinson's disease.[69] A third member, UCHL5 (also known as UCH37) contains

a 100 Aa extension which is essential to bind to the proteasome subunit Rpn13.[70-72] Proteasome-bound UCHL5 is one of three DUBs that recycle ubiquitin chains from proteasome substrates.[10] The fourth human UCH enzyme, BAP1 (BRCA1 associated protein-1), contains a C-terminal extension of >500 Aa. BAP1 is a tumor suppressor and interacts with the BRCA1/BARD1 E3 ubiquitin ligase involved in DNA repair, yet its roles in the DNA damage response are debated.[73-75] Recent data shows that BAP1 also interacts with the cell cycle regulator host cell factor-1 (HCF1).[76,77] Human NCI-H226 squamous lung carcinoma cells harbor a deletion of BAP1 and overexpression of BAP1 in this cell line blocks their proliferation and tumor growth in mice.[77]

Structures of UCH domain reiterate common principles of DUB regulation and specificity. The catalytic residues in ubiquitin-free UCHL1 are in a nonproductive conformation[78] and need to undergo a conformational change upon binding to ubiquitin. In the active ubiquitin bound conformation of UCHL3 (Fig. 3A) or Yuh1 (the single yeast UCH enzyme), the catalytic triad residues superpose well with other DUB classes and several loops are remodelled upon ubiquitin binding (Fig. 3B).[36,79] The most striking

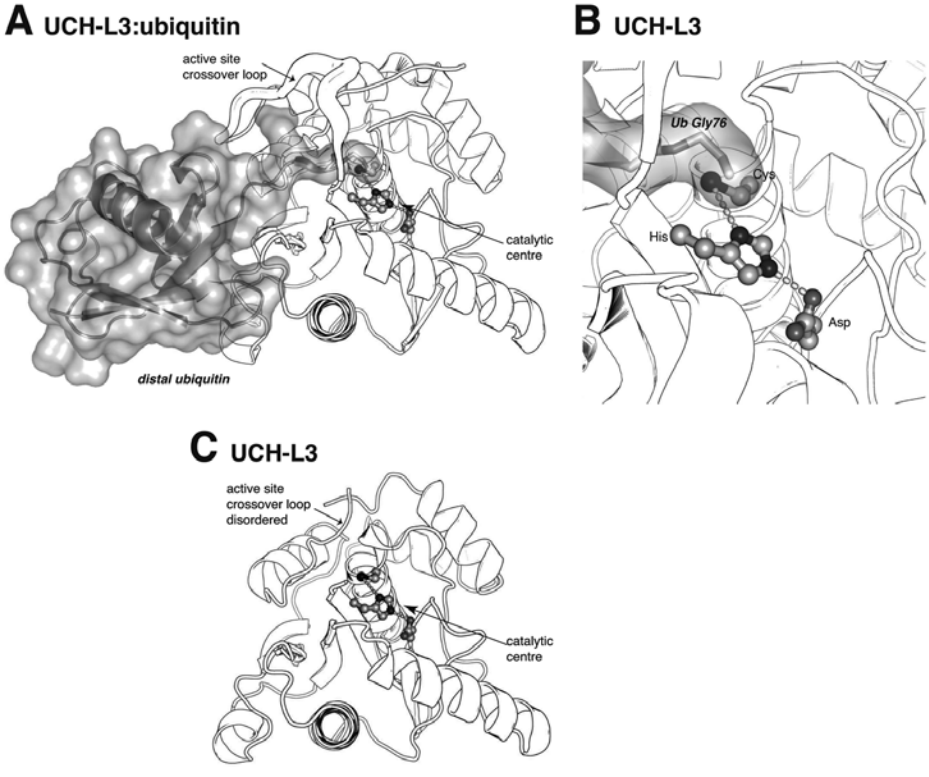

Figure 3. Structure of a UCH domain deubiquitinase. A) Structure of UCHL3 bound to ubiquitin (pdb-id 1xd3).[79] The UCH domain (white) is shown in cartoon representation and the catalytic centre residues are shown as stick models in grey colors. Ubiquitin is shown under a grey semitransparent surface. Hydrogen bonds are indicated by dotted lines. The active site crossover loop forming across the ubiquitin C-terminus at the active site is indicated. B) Close-up view of the active site of UCHL3 bound to ubiquitin. The catalytic triad residues and their interactions are shown. C) Structure of UCHL3 in the apo form without ubiquitin (pdb-id 1uch).[30] The active site crossover loop is disordered.

feature of UCH enzymes is a large surface loop, the active site crossover loop, which forms upon ubiquitin binding (Fig. 3A,C).[36,79] The ubiquitin C-terminus has to thread through this loop in order to reach the active site. This poses a significant steric constraint and does not allow binding of folded ubiquitinated proteins of more than approximately 10 Å in diameter. This structural feature excludes ubiquitin chains, which would be too big to enter through the crossover loop. Indeed, UCH enzymes have negligible activity against ubiquitin polymers of any linkage type in vitro.[20,80] Only significant extension of the crossover loop allows polyubiquitin cleavage.[80] Hence, UCH enzymes with their restricted accessibility to the active site, can act on ubiquitination sites in unfolded regions of proteins (and maybe perform chain amputation) and on ubiquitin-peptide conjugates which may be a by-product of proteasomal degradation. Interestingly, proteasome-bound UCHL5 can act against polyubiquitin chains, despite a predicted analogous active-site crossover loop.[81] Hence, either proteasome interaction induces a conformational change in UCHL5 to remodel the obstructing loop, or the proteasome unfolds ubiquitin polymers significantly so they can enter through the cross-over loop. UCHL3 but not UCHL1 is inhibited by diubiquitin[82] and UCHL5 also does not hydrolyze diubiquitin efficiently.[70] The molecular basis for this inhibition is not clear at the moment.

Josephin Domain DUBs

Four human DUBs contain a catalytic Josephin domain, which was identified by bioinformatics[83] and subsequently validated to be catalytically active.[84] The most prominent member of Josephin DUBs is Ataxin-3. Ataxin-3 is the protein mutated in Machado-Joseph disease (MJD, SCA3), the most common form of spinocerebellar ataxias.[85] Ataxin-3 contains a stretch of Gln residues (polyQ), which is significantly extended in the disease state as the consequence of amplification of an unstable CAG triplet repeat. The resulting polyQ stretch leads to protein aggregation in the form of intracellular inclusion bodies.[86]

Josephin domains have been studied by nuclear magnetic resonance (NMR) techniques and currently several inactive structures are available, where the catalytic triad is in nonproductive conformations (Fig. 4).[87-90] The key feature of Josephin domains is a large helical lever that restricts access to the active site in absence of ubiquitin (Fig. 4A, C, D).[87,88] NMR-based docking analyzes of diubiquitin onto Ataxin-3 suggest that ubiquitin binding stabilizes an active conformation of Ataxin-3.[90] Interestingly, Ataxin-3 catalytic activity is activated by ubiquitination of the Josephin domain itself by an unknown E3 ligase.[91] It is tempting to speculate that ubiquitination stabilizes the helical lever in an open conformation.

Ataxin-3 contains three UIM motifs in its C-terminal part.[92] The two Josephin-proximal UIMs were recently shown to preferentially interact with Lys48-linked ubiquitin chains,[93] however, Ataxin-3 was also suggested to edit Lys63-linkages in mixed linkage chains.[94] The substrates of Ataxin-3 and the roles of the remaining Josephin domain proteins are currently unclear.

JAMM/MPN+ Domain DUBs

Eight human DUBs contain a JAMM/MPN+ metalloprotease domain and these proteins often operate as part of multi-subunit protein complexes. A JAMM/MPN+ DUB in the proteasome, POH1, contributes to recycling ubiquitin chains,[10] while AMSH and

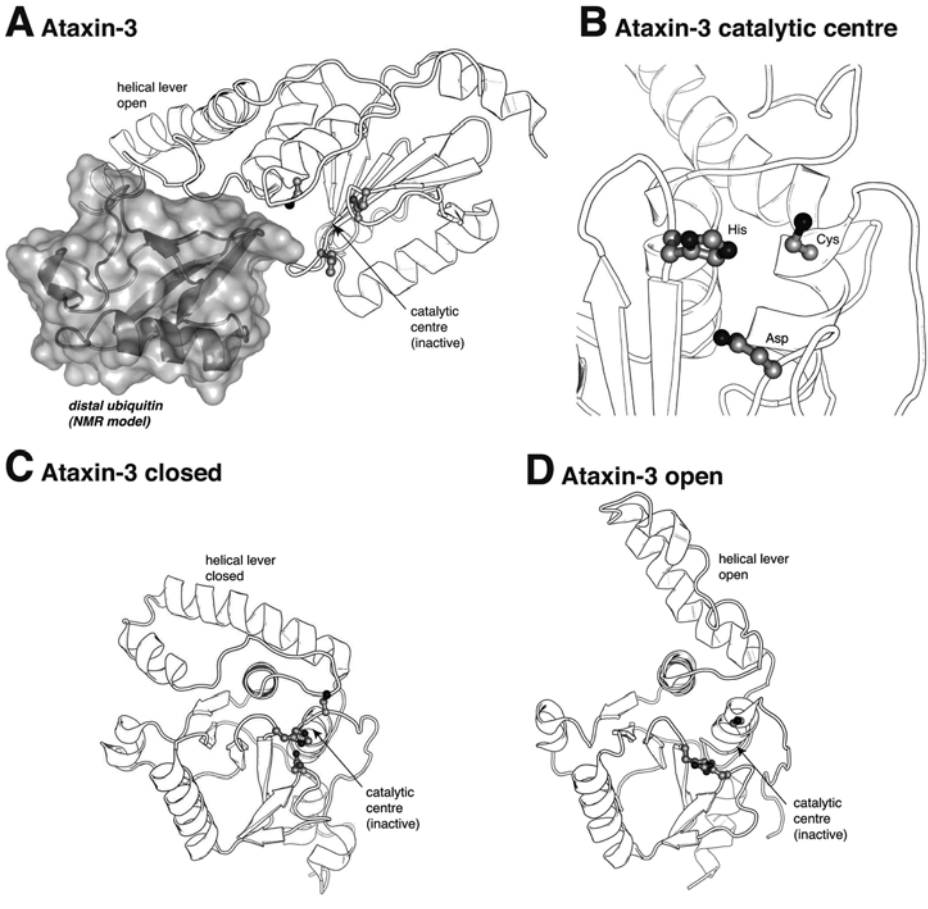

Figure 4. Structure of a Josephin domain deubiquitinase. A) Structure of Ataxin-3 bound to ubiquitin (pdb-id 1jri).[90] The Josephin domain (white) is shown in cartoon representation and the catalytic centre residues are shown as stick models in grey colors. Ubiquitin is shown under a grey semitransparent surface. The helical lever regulating access to the active site is labelled. B) Close-up view of the active site of Ataxin-3. The invariant catalytic residues have been verified by mutagenesis, but are in an unproductive conformation in all structures. C,D) Structure of Ataxin-3 in absence of ubiquitin (pdb-id 2aga,)[87] and in presence of diubiquitin (2jri).[90] Several NMR models have indicated high flexibility of the helical lever that moves between closed (C) and open (D) conformations. Diubiquitin has been omitted from D for clarity.

AMSH-LP are associated with the ESCRT machinery and are involved in membrane receptor trafficking.[95] BRCC3 has been found in two DNA repair complexes, the BRISC complex and the BRCA1 A complex.[96-99] CSN5 is a component of the COP9 signalosome and acts as deneddylating enzyme to remove the activating Nedd8 modification from Cullin E3 ligases.[100] MYSM1 is part of a histone deubiquitinase complex.[101] PRPF8, a splicing factor, contains an impaired metal binding site and hence may have lost DUB activity.[102] The remaining enzyme, MPND has not been studied to date.

Most JAMM/MPN+ DUBs cleave Lys63 ubiquitin chains and some (AMSH, AMSH-LP, BRCC3) with exquisite specificity.[96,103] The molecular basis for this linkage

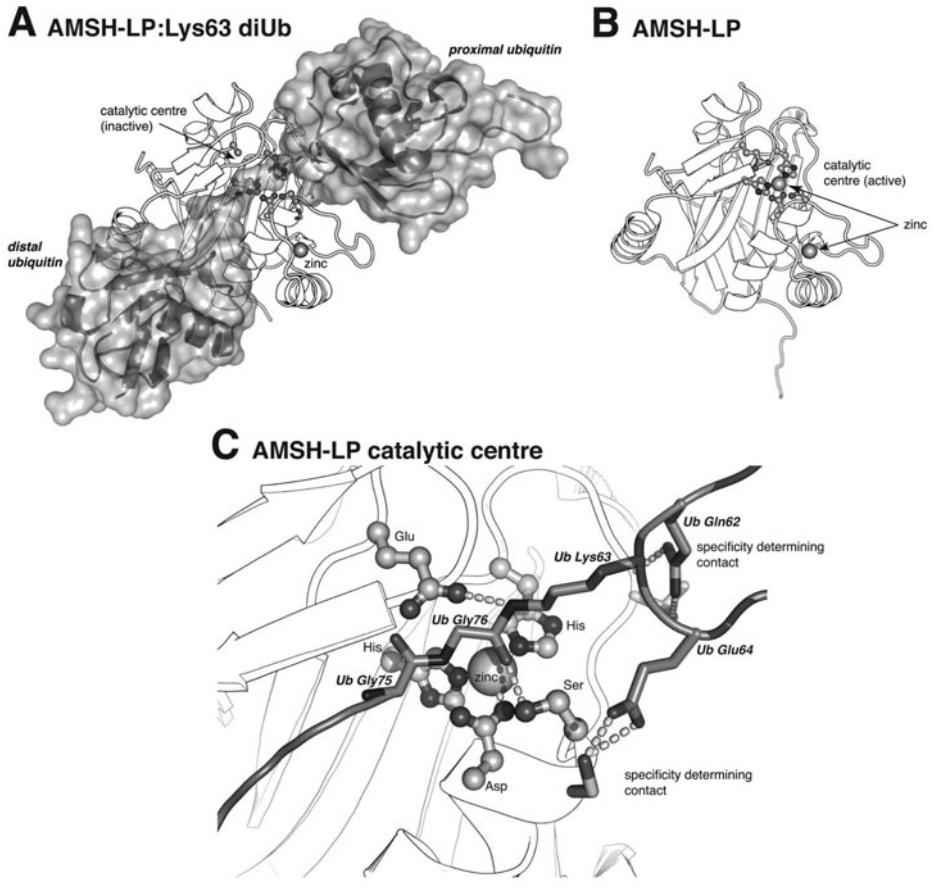

Figure 5. Structure of a JAMM/MPN+ domain deubiquitinase. A) Structure of AMSH-LP bound to Lys63-linked diubiquitin (pdb-id 2znv).[41] The JAMM/MPN+ domain (white) is shown in cartoon representation and the catalytic centre residues are shown as stick models in grey and zinc ions as grey spheres. The Lys63-linked diubiquitin is shown under a semitransparent surface and binds across the active site. The complex was obtained by disrupting the primary zinc binding site and mutation of the catalytic Glu residue. B) Structure of the AMSH-LP JAMM/MPN+ domain without ubiquitin (pdb-id 2znv).[41] The enzyme is in an active configuration with two zinc ions. C) Catalytic centre of the AMSH-LP enzyme. The active zinc-bound form is superposed onto the ubiquitin complex. The catalytic residues and their interactions are shown. Also the Lys63-adjacent residues Gln62 and Glu64 are shown in the proximal ubiquitin, which make specificity-determining contacts to the AMSH-LP protein.

specificity was revealed in the crystal structure of AMSH-LP bound to Lys63-linked diubiquitin (Fig. 5).[41] Apart from representing the first DUB structure with a substrate chain bound across the active site, this structure also gave important insights into Lys63 specificity of DUBs. Lys63-linked polyubiquitin chains show an extended conformation[20] and AMSH-LP exploits this, by stretching the Lys63-linkage maximally (Fig. 5A,C).[41] The linker residues are contacted by the protein and furthermore, the sequence context of the Lys63 residue, Gln62 and Glu64, are specifically contacted

by the AMSH-LP JAMM/MPN+ core (Fig. 5C).[41] Hence similarly to CYLD,[19] the proximal ubiquitin containing the Lys residue of the linkage plays an important part in determining the linkage specificity of the DUB. The molecular details for Nedd8 cleavage by the CSN5 JAMM/MPN+ domain, or for the activity of POH1 in the proteasome are less clear.

CONCLUSION AND FUTURE PERSPECTIVES

Protein deubiquitination is being recognized as a key instrument to understand the complex ubiquitin system. The systematic analysis of DUB involvement in biological processes, facilitated by powerful siRNA screening methods[16,26,61] and by the recent comprehensive analysis of DUB interacting proteins,[49] allowed deep insights into ubiquitin mediated regulatory cascades. The prevalent idea that ubiquitination is primarily a degradation signal has been challenged by identification of DUBs such as TRABID[25] and DUBA,[26] which are specific for nondegradative Lys63-chains. This chain type was not known to be involved in the pathways regulated by these DUBs (Wnt- and IRF signalling, respectively) opening new avenues for understanding of, but also for interfering with, these pathways.

Chain linkage specificity will be a hot topic in the years to come, as the abundance of atypical chain types has just been realized through powerful developments in proteomics.[104,105] However, in order to gain further insight, novel tools have to be developed. Most importantly, chain synthesis of the remaining chain types has to be achieved. DUBs will undoubtedly play a major role to unravel the roles of novel ubiquitin modification and furthermore, the specific members have great potential to become important tools in ubiquitin research.

Despite much progress to understand the deubiquitinases at a structural level, more work lies ahead. The key to understanding DUB specificity is to obtain further structures of DUBs bound to ubiquitin polymers of different linkages. Also the recent identifications of allosteric DUB activators require further studies. Most DUBs are poor enzymes and hydrolyze ubiquitin polymers with slow kinetics. The reasons for this may be non-ideal substrates, or general allosteric mechanisms regulating DUB activity that have not been uncovered. With more DUB structures available, the subtle differences will become apparent.

Numerous DUBs have tight links with human disease. As proteases were in the focus of pharmaceutical intervention for a long time, it is surprising that there has been relatively little progress on the development of DUB inhibitors (for a recent review see ref. 32).The potential of DUBs as drug targets is being realized, but requires careful biochemical and genetic analysis, as well as better assay technologies.[106] This area of research promises to yield exciting and interesting insights in the years to come.

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CHAPTER 7

UBIQUITIN CONJUGATION AND DECONJUGATION IN NF-κB SIGNALING

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Abstract: Transcription factor NF-κB regulates the physiological response to a variety of stimuli. The NF-κB pathway has served as a paradigm for analyzing the impact of the covalent protein modifier ubiquitin on signal transduction. The discovery in the early 1990s that degradation of cytosolic NF-κB inhibitors (IκBs) is mediated by the ubiquitin proteasome system (UPS) was the first example for a direct involvement of ubiquitination in cellular signaling. By now it has become clear that the role of the ubiquitin system in the NF-κB pathway extends far beyond triggering IκB destruction. The IκB kinase (IKK) complex is the key regulator of NF-κB. Attachment of ubiquitin chains to the IKK complex and to further upstream components drives NF-κB signaling pathways by promoting the clustering of the signaling network. Whereas ubiquitin conjugation serves a positive function in the NF-κB pathway, ubiquitin deconjugation acts as a negative regulatory feedback mechanism that is critically involved in balancing the strength and the duration of the NF-κB response. Moreover, inactivation of deconjugating enzymes can cause sustained NF-κB activity under pathological conditions like chronic inflammation or cancer. Here we review the impact of the ubiquitin system on the NF-κB signaling network by putting a focus on the enzymes that help to shape the plasticity of the NF-κB response.

INTRODUCTION

The NF-κB family of transcription factors consists of five members: p65/RelA, c-Rel, RelB, p50 and p52. NF-κB proteins share an amino-terminal REL homology domain (RHD) which confers dimerization, DNA binding and interaction with inhibitors of NF-κB, the IκB proteins. In resting cells, interaction of the RHD with the ankyrin

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repeat domain of the cytosolic IκB proteins IκBα, IκBβ and IκBε prevents NF-κB from entering the nucleus. In addition, p105 and p100 precursors, which generate the active NF-κB subunits p50 and p52 after an internal processing reaction, function as IκBs.[1] NF-κB activation involves its nuclear translocation that is initiated by multiple stimuli, such as pro-inflammatory cytokines, pathogens, antigenic peptides, developmental signals or environmental stressors. Activated NF-κB binds to regulatory elements on the DNA and induces expression of target genes involved in immune response, inflammation, survival, proliferation, differentiation or development.[1-3] The IκB kinase (IKK) complex is the central regulator of NF-κB signaling on which all upstream signaling pathways converge. The core IKK complex consists of the two catalytic subunits IKKα and IKKβ and the adaptor subunit IKKγ/NEMO (NF-κB essential modulator). NEMO deficiency completely abrogates NF-κB activation in response to stimulation.[4] Two distinct signaling pathways exist downstream of IKKs on the route to NF-κB, the so called canonical and noncanonical NF-κB pathways.[1] In this chapter we focus on the impact of the ubiquitin system on the canonical NF-κB pathway.

The 76 amino acid polypeptide ubiquitin functions as a pleiotropic signal that regulates protein stability, activity and localization in virtually all cellular processes. Ubiquitin is covalently attached to lysine residues in target proteins. The process is catalyzed by a three step enzymatic reaction that involves an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2) and an ubiquitin ligase (E3). The critical enzymes that confer specificity to the system are the E3 ligases, which recognize the substrate proteins. More than 500 E3 ligases exist in the human genome. E3s catalyze substrate modification either by attaching a single ubiquitin (mono-ubiquitination) or an ubiquitin chain that is generated by recurrent conjugation to one of the seven lysine residues in ubiquitin (poly-ubiquitination). All forms of ubiquitin linkage exist, but best studied are lysine 48 (K48) and lysine 63 (K63) linked ubiquitin chains.[5] While K48-linked ubiquitin chains are mostly tagging proteins for proteasomal degradation, K63-linked chains are often regulating proteolysis independent functions. Ubiquitination is a highly dynamic modification that can be reversed by ubiquitin deconjugating enzymes (DUBs). DUBs are specialized proteases that cleave mono- or poly-ubiquitin moieties from substrate proteins.[6] Approximately 90 different DUBs have been identified in the human genome, which underscores the versatility of the ubiquitin system and its suitability for regulating dynamic cellular processes.

UBIQUITIN CONJUGATION ACTIVATES NF-κB SIGNALING

Ubiquitin conjugation takes place at every step of the NF-κB pathway: Ubiquitin triggers degradation of cytosolic IκB proteins, it directly promotes activation of the IKK complex and it facilitates the recruitment of the IKK complex to the upstream receptor complexes.

The Ubiquitin Proteasome System Dictates Nuclear Entry of NF-κB

The canonical NF-κB pathway, which operates in most cells, generates a rapid response to extracellular stimuli and involves activation of the IKK core complex. IKKs phosphorylate IκB proteins in an amino-terminal signal destruction domain. Phosphorylated IκBs are recruited to a large E3 ligase complex, the SCF (SKP1-cullin-F-Box). In this complex,

phospho-IκBs are directly associating with the F-Box protein β-TRCP, which in turn is recruited via SKP1 to cullin1 (Cul1) and ROC1/RBX1. ROC1 acts as a RING E3 ligase that mediates the assembly of K48-linked ubiquitin chains to IκBs.[7] Poly-ubiquitinated IκBs are recognized and degraded by the 26S proteasome. As a consequence, NF-κB is released to enter the nucleus and to activate its target genes. Post-inductive shut-down of canonical NF-κB signaling is granted by de novo synthesis of IκBα that mediates nuclear export of NF-κB after removal of the stimulus.[8]

Ubiquitin Chains Mediate IKK Activation

Canonical NF-κB signaling initiated by interleukin-1 receptor (IL-1R)/Toll-like receptor-4 (TLR4), tumor necrosis factor receptor (TNFR) or T-cell receptor (TCR) are prototypic examples of distinct upstream signaling pathways that converge at the IKK complex (Fig. 1). In vitro reconstitution experiments have yielded first clues that a proteasome-independent ubiquitination may provide a signal for IKK activation.[9] As a basic concept, the IKK activation involves K63-linked poly-ubiquitination of the regulatory subunit NEMO. At the same time NEMO associates through its ubiquitin binding domain (UBD) with K63-linked ubiquitin chains in response to various stimuli.[10] More recent data suggest that the LUBAC E3 ligase complex triggers IKK activation by modifying NEMO with linear head-to-tail ubiquitin chains, where the amino-terminus of ubiquitin is fused to the carboxyl-terminus of the preceding ubiquitin in the chain.[11] Moreover, structure-function analysis of the NEMO UBD in complex with K63-linked or linear di-ubiquitin suggests that NEMO binds with high preference to linear di-ubiquitin.[12-14] However, the carboxyl-terminal zinc finger (ZF) of NEMO may enhance the affinity towards K63-linked ubiquitin chains.[15] In principle, independent of the respective linkage, this dual mechanism of ubiquitin modification and ubiquitin sensing of NEMO could promote a juxtaposition of IKK complexes and auto-activation by induced proximity.

Besides the possibility of auto-activation, ubiquitinated IKK complexes recruit another kinase complex consisting of the TGFβ-activated kinase1 (TAK1) and the K63 ubiquitin chain sensor TAK-binding proteins TAB2-TAB3 (Fig. 1).[16-18] There is clear biochemical and genetic evidence that support a role of TAK1 as the IKK activating kinase in response to diverse stimuli.[19-22] However, possibly due to redundancy, the role of the ubiquitin sensor TAB2-TAB3 has not yet been resolved.[20] Finally, recent data even suggest that so called unanchored K63-linked ubiquitin chains, which are not attached to any substrate, directly activate TAK1 and IKK kinase activity.[23] Importantly, while all of these models attempt to elucidate the molecular trigger of IKK activation, i.e., the activating events at the IKK complex itself, they fail to explain how upstream signaling processes reach the complex and promote IKK activation.

Ubiquitin Conjugation Bridges Upstream Receptors to the IKK Complex

First indications that upstream ubiquitination channels receptor complexes to canonical NF-κB signaling came from studies on IL-1β and lipopolysaccharide (LPS) signaling. IL-1β and LPS utilize the same signaling adaptors downstream of the homologous IL-1R and TLR4.[24] Using a shRNA/reconstitution strategy, which replaces endogenous ubiquitin with a K63R mutant of ubiquitin, an essential role of K63-linked ubiquitin chains for IKK activation was demonstrated.[25] Biochemical

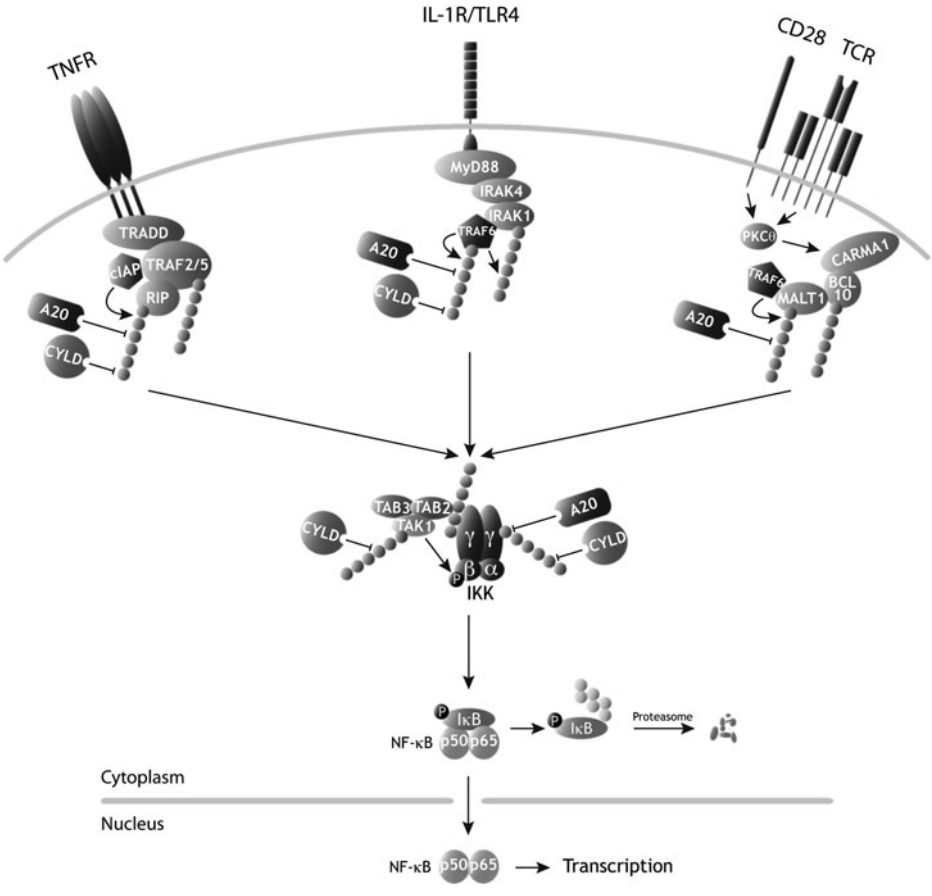

Figure 1. Role of ubiquitination and deubiquitination in NF-κB signaling pathways. The NF-κB pathways mediated by the TNF, IL-1/TLR4 and TCR are schematically depicted. Activation of NF-κB depends on initial formation of receptor proximal protein complexes for each signaling pathway, i.e., TRADD-TRAF2/5-RIP-cIAP in the TNFR pathway, MyD88-IRAK4-IRAK1-TRAF6 in the IL-1/TLR4 receptor pathway and CARMA1-BCL10-MALT1-TRAF6 in the TCR pathway. Subsequently, the E3 ligases cIAP1/2 or TRAF6 poly-ubiquitinate RIP (TNFR), TRAF6/IRAK1 (IL-1/TLR4) and MALT1/BCL10 (TCR), respectively. In a second step, the poly-ubiquitin chains (displayed as a separate chain exemplarily for all poly-ubiquitinated proteins) serve as a binding platform for IKKγ/NEMO of the IKK complex and the TAB/TAK complex. The latter activates the IKK complex, which in turn mediates the degradation of IκB, the inhibitor of NF-κB. The DUBs A20 and CYLD counteract the signaling pathway at various stages as illustrated in the figure.

analysis and knock-out studies further demonstrated that the RING domain E3 ligase TRAF6 is an essential component in IL-1β/LPS induced IKK activation.[26,27] Induction of TRAF6 E3 ligase activity involves the recruitment to the receptor via the adaptor proteins MyD88 and IRAK.[28] Oligomerization of TRAF6 is critical for its function and trimerization of the conserved coiled-coil and TRAF-C domain as well as dimerization of the RING domain has been reported (Fig. 2).[29,30] TRAF6 does not have a catalytic activity per se, but the RING domain binds to the E2 enzyme complex UBC13/UEV1a, which assembles specifically K63-linked ubiquitin chains on the substrates.[30] Thereby

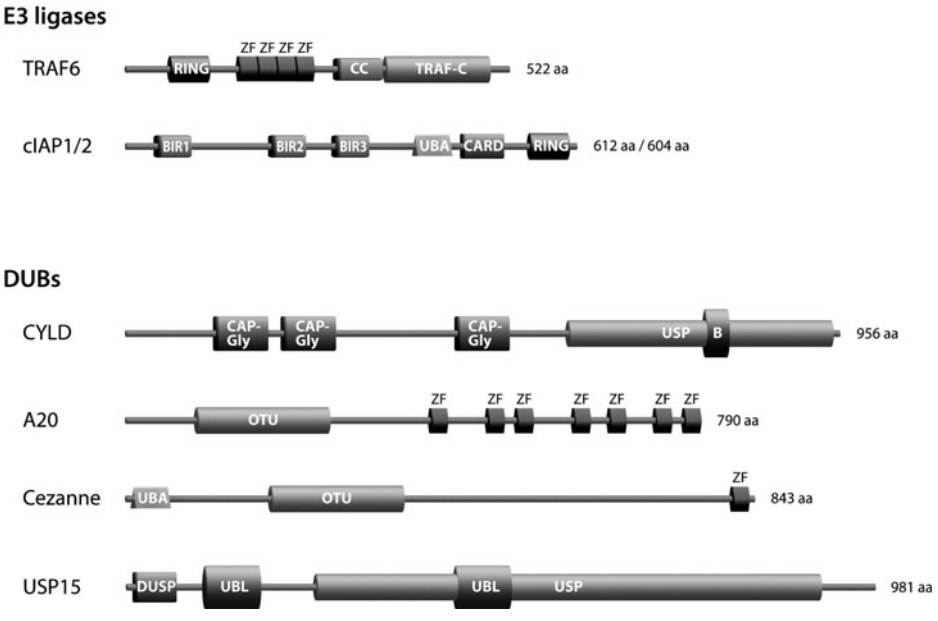

Figure 2. Domain structure of different E3 ligases and deubiquitinases (DUBs). Schematic organization of functional domains of the E3 ligases cIAP1/2 and TRAF6 and the DUBs CYLD, A20, Cezanne and USP15. Ring finger domain (RING), Zinc Finger domain (ZF), Coiled-Coil domain (CC), TRAF-C domain, Baculoviral Inhibition of apoptosis protein Repeat domain (BIR), Ubiquitin Associated Domain (UBA), Caspase Recruitment Domain (CARD), Cytoskeleton-Associated Protein Glycin-rich domain (CAP-Gly), Ubiquitin Specific Protease domain (USP), B-Box domain (B), Ovarian Tumor Protease domain (OTU), Domain in USPs (DUSP), Ubiquitin-like domain (UBL).

TRAF6 induces K63-linked poly-ubiquitination of itself as well as of IRAK1 and IKKγ, which in turn recruits ubiquitin sensing TAK1 and IKK complexes to the receptor.[18,31,32] However, it needs to be noted that even though shRNA studies support a role of UBC13 in this process[25], genetic ablation of UBC13 does not affect IL-1β- or LPS-induced NF-κB signaling.[33] It awaits further investigation whether other E2s, e.g., UBCH7, can compensate for the loss of UBC13.

Biochemical evidence also supports a role for TRAF6 in mediating TCR stimulation to the canonical NF-κB pathway. TCR/CD28 costimulation induces the formation of the large CARMA1-BCL10-MALT1 (CBM) complex and TRAF6 associates with the CBM either by directly binding to MALT1 or indirectly through binding to MALT1 associated CASPASE8.[34-36] Upon TCR stimulation, K63-linked ubiquitin chains are rapidly added to MALT1 by TRAF6 and poly-ubiquitinated MALT1 promotes the recruitment of TAB2/3-TAK1 complexes as well as IKK complexes to the CBM (Fig. 1).[35] Whereas T-cell specific ablation of UBC13 impairs TCR induced NF-κB signaling,[37] TRAF6 deficiency does not alter NF-κB activation in T-cells.[38] Interestingly, BCL10 is also ubiquitinated in activated T-cells and IKK complexes may also bind to poly-ubiquitinated BCL10.[39] Sequence analysis predicts that the IAP E3 ligases (Fig. 2) can interact with UBC13 and cIAP2 has been shown to induce BCL10 ubiquitination, suggesting that IAPs could compensate for the loss of TRAF6 in T-cells.[30,40] The IKK activating kinase TAK1 is found to be constitutively ubiquitinated in T-cells and the

ubiquitination is a prerequisite for TAK1 activity.[41] TRAF6 was identified as a potential E3 ligase for TAK1 in TGFβ signaling, but it remains to be determined if that holds true for T-cells.[42]

Binding of TNFα induces trimerization of the TNFR and association of the adaptor proteins TRADD, TRAF2, TRAF5 and RIP1 to the receptor complex (Fig. 1). RIP1 is poly-ubiquitinated after TNFα stimulation and ubiquitinated RIP1 recruits NEMO via its UBD and thereby the IKK complex to the TNFR.[43-45] In contrast to IL-1R/TLR4 and TCR mediated signaling, TNFR clustering does not promote association of TRAF6, which actually has a negative regulatory function in TNFα induced NF-κB signaling.[46] Originally, based on knock-out mice, it was assumed that the RING domain-containing TRAF6 homologues TRAF2 and TRAF5 act as E3 ligases for RIP1 in TNFα signaling.[47] However, structural comparison of TRAF6 and TRAF2 RING domains reveal that TRAF2 is unable to associate with UBC13 and thus cannot promote attachment of K63-linked ubiquitin chains.[48] TRAF2 can interact with the E3 ligases cIAP1 and cIAP2, which are crucial for TNFα induced IKK activation.[42,49] Instead of UBC13, IKK activation by TNFα requires the E2 enzyme UBCH5, which can collaborate with cIAPs in poly-ubiquitination of RIP1.[25,50] Interestingly, association of NEMO to ubiquitinated RIP1 does not strictly require K63-linked ubiquitin chains suggesting that mono-ubiquitin or K48-linked chains or even an alternatively linked ubiquitin chain could positively regulate signaling to NF-κB.[25]

UBIQUITIN DECONJUGATION TERMINATES NF-κB SIGNALING

Most evidence for a direct involvement of ubiquitin chains in IKK activation were originally derived from biochemical analysis and RNA interference. The concept for a positive function of nondegradative ubiquitination in NF-κB signaling was strengthened by the identification of CYLD and A20, two ubiquitin deconjugating enzymes (DUBs) that balance NF-κB signaling (Fig. 2).

CYLD Acts as a Pleiotropic DUB in NF-κB Signaling

The ubiquitin protease CYLD (Cylindromatosis) was originally identified as a human tumor suppressor gene that is mutated in hair follicle tumors.[51] Mutations occur preferentially in the carboxyl-terminus DUB domain of CYLD (Fig. 2). By a systemic screen for DUBs that interfere with NF-κB and by its interaction with NEMO, CYLD was shown to act as a negative regulator of NF-κB signaling and this activity requires a functional DUB domain.[52-54] CYLD belongs to the USP (ubiquitin-specific proteases) family, which constitutes the largest family of DUBs.[6] CYLD catalyzes ubiquitin deconjugation of multiple signaling effectors in the NF-κB pathway, including NEMO, TRAF6, TRAF2, RIP1 and TAK1 (Fig. 1).[41,52-57] In vitro CYLD is highly selective for K63- versus K48-linked ubiquitin chains, which correlates with its preference for cleaving K63-linked chains attached to NF-κB mediators in vivo.[58,59] In contrast to most other DUBs tested, CYLD is also efficiently cleaving linear ubiquitin chains, suggesting that CYLD could also balance NF-κB activation controlled by the LUBAC E3 ligase complex.[11]

Much less is known about the regulation of CYLD. TNFα stimulation induces CYLD expression in an NF-κB dependent manner, which provides an auto-regulatory feedback

mechanism that terminates upstream signaling by increasing the amount of CYLD.[60] Further, CYLD is posttranslationally regulated by phosphorylation. Upon stimulation, CYLD undergoes rapid and transient phosphorylation which is catalyzed either by IKKα/β or by the IKK-related kinase IKKε.[61,62] Phosphorylation of CYLD at serine 418 decreases its deubiquitinase activity and is required for efficient NF-κB activation in response to stimulation.

CYLD deficient mice have no apparent phenotype, but they are more susceptible to chemically induced skin tumors.[63] Chemical tumor promoters induce hyperproliferation of CYLD-/- keratinocytes, which is caused by an elevated cyclinD1 level. CYLD DUB activity is regulating nuclear accumulation of the IκB protein BCL3 that activates cyclinD1 expression together with NF-κB p50 or p52, which indicates a quite unexpected mechanism how CYLD inhibits tumor growth.[63] In the immune system, CYLD can act as a positive regulator of T-lymphocyte development, because it cleaves inactivating poly-ubiquitin chains from TCR signaling mediator LCK.[64] However, CYLD also counteracts constitutive NF-κB activity by inactivating TAK1, suggesting that it is involved in the homeostatic control of NF-κB signaling in lymphocytes.[41] These studies exemplify that CYLD is a multifunctional DUB whose activity is not restricted to a negative regulatory role in the NF-κB signaling pathway. It still needs to be ruled out whether other DUBs can compensate for the loss of CYLD, which could explain the very mild effect on NF-κB signaling in CYLD deficient mice.

A20 Balances NF-κB Signaling by an Ubiquitin Editing Mechanism

Genetic ablation in mice revealed that A20 is a negative regulator of TNFR- and TLR-mediated NF-κB activation. Loss of A20 abrogates homeostatic NF-κB inhibition and results in a systemic inflammation, which is primarily caused by commensal bacteria.[65-67]

Mechanistically, A20 can modulate cellular signaling via a dual mechanism that involves successive ubiquitin deconjugation and conjugation by a process that was coined as the ubiquitin-editing function of A20. Whereas the amino-terminus of A20 encodes an ovarian tumor (OTU) domain that possesses DUB activity, the carboxyl-terminal part is composed of seven ZFs and functions as an E3 ligase (Fig. 2).[68] Upon TNFα stimulation, A20 is recruited to the TNFR complex, where it removes K63-linked ubiquitin chains from active RIP1. Subsequently, ZF4 of A20 in conjunction with the E2 UBCH5 catalyzes K48-linked poly-ubiquitination and thereby A20 targets RIP1 for degradation by the UPS. It should be noted that the catalytic activity of the ZF4 in A20 constitutes a novel class of E3 ligases. However, the switch from K63 to K48 ubiquitin linkage of RIP1 and the resulting RIP1 inactivation requires the A20 adaptor protein TAX1BP1 and the two E3 ligases ITCH and RNF11.[69,70] Thus, it remains to be seen whether ubiquitin conjugation is an A20 intrinsic function or utilizes associated E3 ligases.

Much work has shed light on the function of A20 DUB activity in signaling. Since the negative regulatory function of A20 is not restricted to TNFR signaling, other A20 DUB substrates where eventually identified. These include TRAF6 and NEMO as well as MALT1 in activated T-cells.[65,67,71] In vivo A20 seems to be highly specific towards the cleavage of K63-linked ubiquitin chains. This is in sharp discrepancy to in vitro data, where the A20 OTU domain displays a high specificity towards K48-linked ubiquitin chains.[72,73] The molecular basis for this discrepancy is currently unknown, but in vitro

studies were confined to the DUB domain and interactions with adaptor proteins in vivo may be important for controlling specificity. In addition, A20 was shown to remove the entire K63-linked poly-ubiquitin chains from TRAF6 without disassembling the ubiquitin within the chains.[73]

With the exception of T-lymphocytes, A20 is barely expressed under resting conditions but highly induced upon NF-κB activation. Thus, A20 activity is largely controlled on the transcriptional level, which correlates with its function as a negative feedback regulator of NF-κB signaling.[74] A20 is also controlled at the level of localization, because A20 mutations in the carboxyl-terminal ZF region causing mislocalization show a reduced potency to inhibit NF-κB.[75] Furthermore, phosphorylation of A20 by IKKβ increases its DUB activity and thus increases A20 mediated NF-κB inhibition.[76] As mentioned above, CYLD activity is decreased by IKK phosphorylation.[62] Importantly, whereas CYLD is generally constitutively expressed at high levels, A20 is not expressed in the absence of stimulation, which may provide a physiological rationale for the opposing effects of IKK phosphorylation on both DUBs. In contrast to most other cells, A20 is highly expressed in T-lymphocytes in the absence of any stimulus and TCR/CD28 ligation induces an initial removal of A20 by two distinct mechanisms. (i) A20 is cleaved by its own substrate, the MALT1 paracaspase, that contains a novel type of caspase-like cleavage activity.[77,78] (ii) A20 is prone to proteasomal degradation in activated T-cells.[78] Thus, stimulus-dependent inactivation of CYLD or A20 by either phosphorylation, cleavage or degradation is apparently a common mechanism to release the cells from a negative regulatory activity and to promote signal propagation.

Congruent with the strong inflammatory phenotype of A20 knock-out mice, A20 inactivation is associated with several chronic inflammatory and autoimmune diseases.[79] Moreover, somatic, bi-allelic inactivating mutations of A20 are recurrently found in human lymphomas that exhibit constitutive NF-κB activation, indicating that A20 possesses tumor suppressor activity.[80,81] Interestingly, constitutive A20 cleavage by MALT1 paracaspase was recently found in some lymphoma cells, indicating that alternative mechanisms exist to inactivate A20 in tumor cells that do not carry A20 mutations.[82]

Other DUBs Implicated in the NF-κB Pathway

In particular studies on CYLD and A20 deficient mice reveal that certainly other DUBs are involved in balancing NF-κB signaling. Based on sequence homology, Cezanne was identified as an OTU-domain containing DUB enzyme (Fig. 2).[83] Cezanne expression is induced by TNFα stimulation and overexpression as well as siRNA experiments indicate that Cezanne acts as a negative regulator of NF-κB signaling upstream or at the level of the IKK complex.[84] Upon TNFα stimulation, Cezanne is recruited to the TNFR, where it can deconjugate K63-linked ubiquitin chains from RIP1. However, genetic evidence for a role of Cezanne in controlling NF-κB signaling is still missing.

Another set of data indicates that DUBs are also controlling post-inductive stabilization of IκBα. USP15 negatively regulates the SCF-dependent assembly of K48-linked ubiquitin chains on resynthesized IκBα after sustained TNFα stimulation.[85] Interestingly, USP15 is associated with the COP9 signalosome that deconjugates NEDD8 from cullin proteins and thereby interferes with SCF E3 ligase activity that is responsible for IκBα degradation. This result suggests an interesting novel cross-talk of ubiquitin and NEDD8 deconjugating activities in the regulation of NF-κB activity.

CONCLUSION AND FUTURE PERSPECTIVES

By combining biochemical, structural and genetic approaches it is now well established that ubiquitin conjugation and deconjugation are key events in the regulation of NF-κB signaling. The findings include the identification of critical E3 enzymes, the mapping of ubiquitin acceptor sites on many substrates, the determination of different ubiquitin linkages and the characterization of counteracting DUB enzymes. However, we are still lacking a complete picture how the ubiquitin system is specifically affecting the roadmap to NF-κB. This becomes more relevant, because largely due to much more sensitive methodology, such as better anti-ubiquitin antibodies or mass spectrometry, we are faced with the fact that virtually every single component of the NF-κB signaling system can be prone to ubiquitination. It remains to be seen how specificity is achieved and how distinct UBDs are directed to a specific ubiquitin chain. Furthermore, when analyzed in cell culture E3 ligases and DUBs are quite promiscuous in targeting many signaling mediators. However, the phenotypes of gene deficient mice often indicate very distinct functions in vivo. At present it is unclear if these discrepancies are merely explained by redundancy or if we are missing important determinants for specificity. Finally, the issue of ubiquitin linkage and the impact of the different types of ubiquitin chains on NF-κB signaling are far from being resolved. Two approaches seem to be most promising to specifically dissect the role of the ubiquitin system on NF-κB signaling. First, the great amount of biochemical and structural information should be applied to generate sophisticated mouse models to determine the exact in vivo functions of the different reactions. Second, identification of small molecules that selectively influence different stages in the ubiquitin system, e.g., inhibition of E2s or DUBs or disruption of UBD-ubiquitin interactions, would yield valuable tools for further analysis and could help to explore the drugability of the process.

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CHAPTER 8

FUNCTIONS OF LINEAR UBIQUITIN CHAINS IN THE NF-κB PATHWAY

Linear Polyubiquitin in NF-κB Signaling

Kazuhiro Iwai*

Abstract: The ubiquitin conjugation system regulates a wide variety of biological phenomena, in most cases, by modulating protein function via polyubiquitin conjugation. Several types of polyubiquitin chains exist in cells and the type of chain conjugated to a protein seems to determine how the protein is regulated. The polyubiquitin chains that have been reported thus far are generated by conjugation via Lys residues of ubiquitin. We have identified a novel linear polyubiquitin chain, in which the C-terminal Gly of one ubiquitin is conjugated to the α-amino group of the N-terminal Met of another ubiquitin and the ubiquitin ligase complex mediating these reactions specifically generates linear chains. We have shown that linear polyubiquitination is involved in activation of the canonical NF-κB pathway. The regulatory roles of Lys63-linked ubiquitin chains in the NF-κB pathway have been extensively studied. In this chapter, we will discuss the distinct roles of linear and K63-linked ubiquitin chains in TNF-α mediated NF-κB activation and the future directions for linear ubiquitin chain research.

Ubiquitin is a highly conserved small globular protein in eukaryotic organisms that is conjugated to proteins by a cascade of reactions catalyzed by three enzymes, E1, E2 and E3. Although the ubiquitin conjugation system was first identified as part of an energy dependent protein degradation system, the system is now recognized to be involved in a vast array of biological phenomena and to regulate protein function in various ways.[1] Conjugation of polyubiquitin chains, which are polymers of ubiquitin, is crucial for regulating protein function, although mono-ubiquitination has also been shown to have signaling functions in the endocytic pathway.[1,2] Recent reports now indicate that there exist several kinds of polyubiquitin chains in cells and that the mode

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of regulation of substrate proteins may depend on the type of conjugated polyubiquitin chain. For example, polyubiquitin chains linked via the ubiquitin K48 residue function as signals for degradation of the conjugated protein by the proteasome.[3] In contrast, polyubiquitin chains linking via the ubiquitin K63 residue mediate DNA repair and signal transduction without functioning as a degradation signal.[4] Polyubiquitin chains are thought to be generated by conjugation of the C-terminal Gly of one ubiquitin to a Lys residue in the next ubiquitin. The presence of isopeptide linkages via all seven Lys residues of ubiquitin has been revealed by mass spectrometric analyses[5] and further broadens the scope of ubiquitin mediated regulation of cellular function. Indeed, a recent report by Jin et al has indicated that K11-linked polyubiquitin chains are specifically generated by UbcH10 (E2) and APC/C (anaphase promoting complex/cyclosome, an E3) and target the conjugated substrate for degradation by the proteasome.[6]

IDENTIFICATION OF A UBIQUITIN LIGASE COMPLEX THAT SPECIFICALLY GENERATES HEAD-TO-TAIL LINEAR POLYUBIQUITIN CHAINS

We have identified a new, linear type of polyubiquitin chain, in which the C-terminal Gly of one ubiquitin is conjugated to the α-amino group of the N-terminal Met of another ubiquitin. The linear polyubiquitin chain is generated by a unique ubiquitin ligase complex that we have named LUBAC (linear ubiquitin chain assembly complex).[7] LUBAC is composed of two RING-IBR-RING proteins, HOIL-1L and HOIP, which have molecular masses of 58 kD and 120 kD, respectively (Fig. 1). We have hypothesized that the complex is composed of two or three molecules of each protein since the molecular mass of the complex, estimated by gel filtration, is approximately 600 kD.[7] Among the domains that are present in HOIL-1L and HOIP, the UBA domain of HOIP and the UBL domain of HOIL-1L are involved in LUBAC formation. LUBAC can generate linear chains in concert with several E2s, including UbcH5s, E2-25K and UbcH7. Furthermore, LUBAC cannot generate polyubiquitin from N-terminally tagged wild type ubiquitin, indicating that it only generates linear (and not Lys-linked) chains. Notably, E2-25K exclusively generates K48-linked chains in the absence of an E3 in vitro,[8] but, generates linear chains in the presence of LUBAC in vitro. Therefore, LUBAC, not the E2 enzyme, appears to be predominantly responsible for determining the linkage specificity.[9] This situation differs from the generation of K63- and K11-linked chains, since the specificity of both these types of chains is determined by E2s (K63: a Ubc13-containing E2 complex, K11: UbcH10).[6,10]

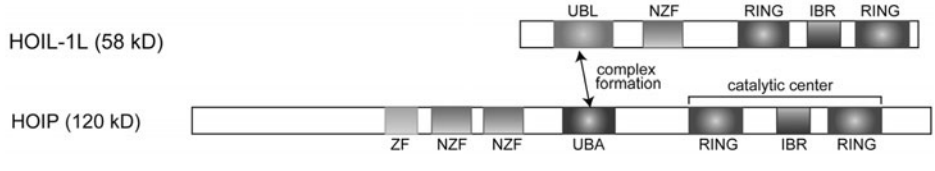

Figure 1. Schematic structure of the LUBAC subunits HOIL-1L and HOIP. The NZF motifs of LUBAC possess both ubiquitin binding activity and NF-κB activation, although they are dispensible for linear polyubiquitin chain formation.

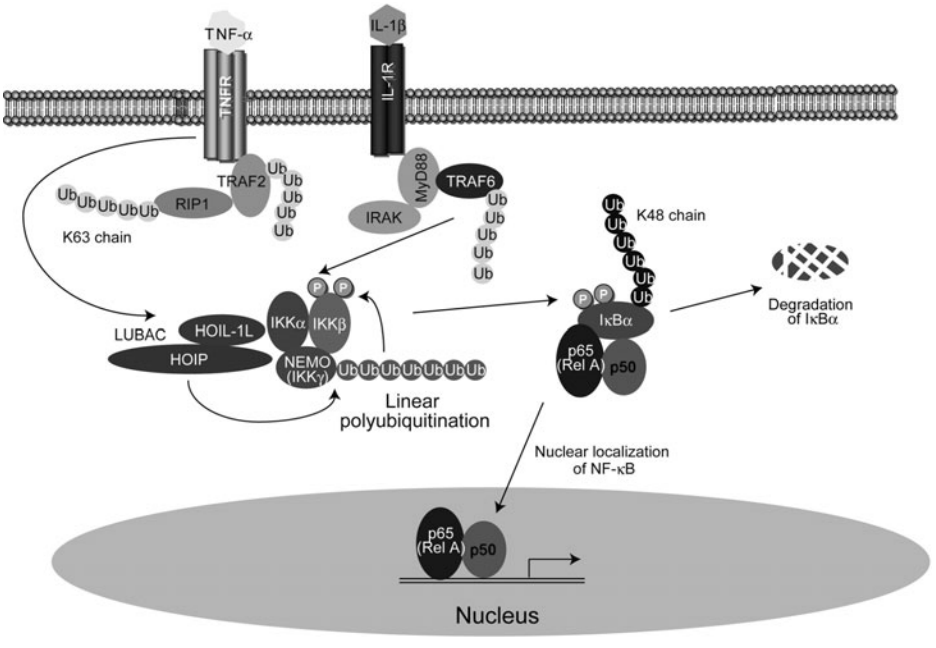

Figure 2. Possible relationships between K63-linked and linear polyubiquitin chains in NF-κB activation. Upon activation by various stimuli, IκBα is phosphorylated by the IKK complex. K48-linked polyubiquitination of phosphorylated IκBα leads to its degradation. Subsequently, free NF-κB translocates into the nucleus and induces the expression of target genes. LUBAC-mediated linear polyubiquitination of NEMO, but not K63-linked polyubiquitination, appears to be indispensable for TNF-α induced NF-κB activation. However, both linear and K63-linked chains appear to be involved in IL-1β induced NF-κB activation.

LUBAC SPECIFICALLY ACTIVATES NF-κB

Since LUBAC is involved in NF-κB activation,[9] I will briefly introduce the NF-κB activation pathway (Fig. 2). NF-κB is a dimeric transcription factor that is activated via multiple signals and induces expression of genes involved in a broad array of biological phenomena, including inflammation and cell survival.[11] In the resting state, most NF-κB resides in the cytoplasm complexed with inhibitory proteins known as IκBs. Upon activation by various stimuli, specific serine residues of IκBs (ex. Ser 32 and 36 of IκBα) are phosphorylated by the IKK complex. Phosphorylated IκBs are specifically recognized by the $SCF^{\beta TrCP}$ ubiquitin ligase and are conjugated with K48-linked ubiquitin chains which targets them for degradation by the proteasome. The liberated NF-κB then translocates into the nucleus and induces expression of target genes. Thus, signal induced activation of the IKK complex plays a key regulatory function in NF-κB activation. The IKK complex is composed of three proteins, IKKα, IKKβ and NEMO (IKKγ). NEMO is an important integrator of upstream signals for IKK activation although it does not possess kinase activity, as do the other two proteins in the IKK complex. Activation of IKKs can also be mediated by phosphorylation of specific serine residues of the kinase subunits.[11,12]

Introduction of LUBAC into 293T cells induces IKK and NF-κB activation. LUBAC binds to NEMO in the IKK complex after stimulation with TNF-α and conjugates linear chains onto the K285 and/or K309 residues of NEMO in vitro. NEMO, but not the NEMO mutant K285, 309R, was linearly ubiquitinated in cells after stimulation. More importantly, TNF-α mediated activation of NF-κB was severely impaired in primary hepatocytes and embryonic fibroblasts (MEFs) from mice with a knockout of HOIL-1L, a component of LUBAC. Hepatocytes in HOIL-1L KO mice, as well as HOIL-1L null MEFs, undergo apoptosis in response to TNF-α administration due to impaired NF-κB activation. These results strongly indicate that LUBAC-mediated linear ubiquitination of NEMO is specifically involved in TNF-α-induced NF-κB activation.[13]

Polyubiquitin chains are thought to exert their function upon recognition by proteins containing chain-specific ubiquitin-binding motifs. Two groups have reported that the UBAN motif of NEMO, which is also called a NUB or CoZi domain, binds preferentially to linear di-ubiquitin.[14,15] Mutation of the amino acids specifically required for linear di-ubiquitin recognition by NEMO drastically reduced TNF-α induced NF-κB activation.[15] Moreover, anhidrotic ectodermal dysplasia and immunodeficiency (EDA-ID) is caused by hypomorphic mutations of NEMO[16,17] and some of the mutations found in EDA-ID (D311N, E315A and R319Q) are residues that are critical for ubiquitin binding by the UBAN motif.[15] Thus, linear polyubiquitination and the linear polyubiquitin binding activity of NEMO play essential roles in NF-κB activation.[9]

DISTINCT ROLES OF LINEAR POLYUBIQUITIN AND K63-LINKED POLYUBIQUITIN CHAINS IN TNF-α SIGNALING

The role of K63-linked polyubiquitination in NF-κB activation has been extensively characterized.[18] Therefore, determining whether the roles of linear polyubiquitin chains in NF-κB activation differ from the role of K63-linked chains is of great importance. Since data regarding the functions of linear polyubiquitin in NF-κB activation is limited in TNF-α signaling,[13] I will focus on NF-κB activation in response to TNF-α signaling in this section. K63-linked polyubiquitin chains conjugated to RIP1, which is induced by TNF-α, have been hypothesized to recruit NEMO in the IKK complex and TAB2 in the TAK1-TAB1-TAB2 complex to TNFR1 at the plasma membrane. TAK1 then phosphorylates and activates IKKβ leading to activation of NF-κB.[19] NEMO and TAB2 both have been suggested to possess K63-linked ubiquitin binding activity.[19] The K63-specific binding activity of the proteins seems to be crucial for K63-chain induced activation of NF-κB, which is consistent with the crucial role of other polyubiquitin chain specific binding motifs in decoding ubiquitin signaling.[20] However, as mentioned above, two groups have shown that the binding affinity of the NEMO UBAN motif, which is the main ubiquitin binding motif in the protein, to K63-di-ubiquitin is approximately 100 times weaker than UBAN binding to linear di-ubiquitin.[14,15] Moreover, deletion of Ubc13 in mice does not overtly affect TNF-α-induced activation of NF-κB, although the E2 complexes containing Ubc13 were thought to be crucial E2s for K63-linked ubiquitin chain generation.[21] We have shown that deletion of a component of LUBAC drastically suppressed TNF-α induced NF-κB activation in primary hepatocytes and MEFs.[13] Moreover, NF-κB was activated by LUBAC when the complex was introduced into Ubc13 null MEFs and siRNA mediated suppression of HOIP severely attenuated TNF-α-induced NF-κB activation in these same cells.[13] Chen et al showed that K63-linked

ubiquitin chains are dispensable for TNF-α-mediated NF-κB activation by replacing the endogenous wild-type ubiquitin with a mutant ubiquitin with a K63R substitution using an elegant small-interfering RNA-depletion/rescue strategy.[22] Moreover, their study also showed that activation of NF-κB by TNF-α depends on another E2 enzyme, UbcH5C. Since UbcH5C can generate linear ubiquitin chains in concert with LUBAC,[7] this E2 may activate NF-κB by conjugating linear chains onto NEMO. These observations strongly indicate that the linear ubiquitin chain plays a role distinct from K63-linked chains in TNF-α induced NF-κB activation. Linear polyubiquitination may be indispensable for TNF-α-induced NF-κB activation, whereas K63-linked ubiquitination is dispensable.[9]

What role, then, do the K63-linked chains play in TNF-α-signaling? Although deletion of Ubc13 did not affect TNF-α induced NF-κB activation, TNF-α induced JNK activation was strongly suppressed.[21] Conversely, linear polyubiquitination is necessary for TNF-α-induced NF-κB activation, but not JNK activation.[13] Rahighi et al observed that mutations of amino acid residues in the UBAN motif of NEMO, which are critical for the recognition of linear chains, drastically reduced TNF-α induced NF-κB activation, but activation of JNK or p38 was not affected.[15] Thus, K63-linked polyubiquitination may be involved in activation of JNK and p38 MAP kinases in TNF-α signaling.[9]

THE MECHANISM UNDERLYING LUBAC-MEDIATED NF-κB ACTIVATION

Activation of IKK is the key event in signal-induced NF-κB activation. However, the precise mechanism underlying IKK activation, namely, signal-induced site-specific phosphorylation of IKKβ has not yet been clarified. Two mechanisms have been proposed: trans-autophosphorylation and IKK-kinase mediated phosphorylation.[12] TAK1 and MEKK3 have both been proposed to be the upstream kinases that phosphorylate IKK. The recruitment of TAK1 by K63-linked polyubiquitin conjugation suggests that the TAK1 phosphorylation of IKK is a plausible model.[18] However, observation of LUBAC-mediated NF-κB activation has provided new insights into IKK activation. We have identified K285 and K309 as possible linear polyubiquitinated residues in NEMO.[13] Although K309 is inside the UBAN motif and linear polyubiquitin conjugation to K309 disrupts the linear ubiquitin binding activity of conjugated NEMO,[15] linear polyubiquitination of K285 does not affect linear ubiquitin binding by UBAN. Since the UBAN motif would not be likely to recognize a linear ubiquitin chain in cis, if the length of the chain is short, linear polyubiquitin conjugated NEMO may be recognized by other NEMO proteins which then induce multimerization of the IKK complex and trans-autophosphorylation of IKKs. However, it is also possible that linear polyubiquitination of NEMO may function as a scaffold for upstream IKK-kinases. Alternatively, binding of linear ubiquitin to the UBAN motif of NEMO may induce a conformational change in NEMO, which affects the spatial positioning of IKKs and leads to trans-phosphorylation of the kinases.[15]

ROLES OF K63-LINKED AND LINEAR UBIQUITIN CHAINS IN NF-κB ACTIVATION IN CELLS STIMULATED BY SIGNALS OTHER THAN TNF-α

We have identified linear polyubiquitination as a crucial component mediating TNF-α-induced NF-κB activation. However, in response to signals other than TNF-α, K63-linked chains may play critical roles in NF-κB activation. Indeed, Ubc13-catalyzed

K63-linked ubiquitin chain conjugation is thought to be involved in NF-κB activation in T-cell receptor signaling.[23] Chen et al also demonstrated that K63-linked chains are essential for the activation of IKK after stimulation of cells with IL-1β using a small-interfering RNA-depletion/rescue strategy, as described above.[22] Since we have observed that linear polyubiquitination is involved in IL-1β-induced NF-κB activation,[13] both K63-linked and linear ubiquitin chains appear to be necessary to activate NF-κB. We suspect that K63-linked chains may function upstream of linear chains in some signaling cascades. In those situations, K63-linked chains may be indispensable for recruitment of LUBAC and NZFs of LUBAC, which possess ubiquitin binding activity,[7] may be involved in the recruitment. Further analyses will be definitely needed to dissect the distinct roles of K63-linked and linear polyubiquitin chains in signaling.

CONCLUSION AND FUTURE PERSPECTIVES

Linear polyubiquitin genes have been found to exist in the genome.[24] Therefore, genetically encoded "free" linear polyubiquitin may compete with posttranslationally generated linear chains. However, such a competition may be unlikely because translated linear polyubiquitins are cleaved into ubiquitin monomers cotranslationally by de-ubiquitinating enzymes,[25] which enables enzymatically generated linear polyubiquitin chains conjugated to specific substrates to work as modulators of protein function.

Moreover, linear chain dependent NF-κB activation may provide a valuable target for drug discovery. Since NF-κB is involved in many diseases including cancer, autoimmune and allergic diseases, agents that specifically inhibit NF-κB are regarded to be good candidates for drugs to treat such disorders. Either a drug that specifically suppresses LUBAC-mediated linear ubiquitination or inhibits binding between linear ubiquitin chains and the NEMO UBAN motif may supply a target for NF-κB specific inhibitors.

Identification of an E3 complex which generates linear polyubiquitin chains has opened up a new field in ubiquitin biology. Of course, much work will be needed to clarify the roles of these linear chains and the mechanisms regulating linear vs. branched chain formation. We have recently shown that the E3 activity of LUBAC is attenuated by PKC signaling[26] and I believe that novel and unexpected functions of linear polyubiquitin will be revealed in the near future.

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CHAPTER 9

ASSEMBLY OF K11-LINKED UBIQUITIN CHAINS BY THE ANAPHASE-PROMOTING COMPLEX

Michael Rape*

Abstract: Ubiquitin chains are assembled, when a ubiquitin is connected to one of the seven Lys residues or the amino-terminus of a ubiquitin molecule already attached to a substrate. K48-linked ubiquitin chains target proteins for degradation by the 26S proteasome, while those chains connected through K63 regulate intracellular signaling cascades independently of protein degradation. Although all other linkages are detected in cells, their function is not well understood. Here, we review recent progress in delineating substrates, enzymes and functions of K11-linked ubiquitin chains. In particular, we discuss the mechanism of assembly for K11-linked chains by the human anaphase-promoting complex and its physiological E2s UbcH10 and Ube2S and we speculate on the particularities of these noncanonical chains in cells.

INTRODUCTION

Ubiquitination controls cellular signaling by changing the activity, localization, or stability of multiple proteins (reviewed in ref. 1). Ubiquitin is covalently linked to its targets through an isopeptide bond between its C-terminus and an ε-amino group in a substrate lysine. The attachment of a single ubiquitin, referred to as monoubiquitination, has various functions in regulating chromatin structure, transcription, or endocytosis.[2] In addition, ubiquitin itself has seven Lys residues and an amino-terminal NH_2-group, which can serve as acceptor sites for the attachment of further ubiquitin molecules during the assembly of ubiquitin chains (Fig. 1). Depending on which type of linkage is preferred during chain formation, these ubiquitin chains differ in structure and function.[1,3]

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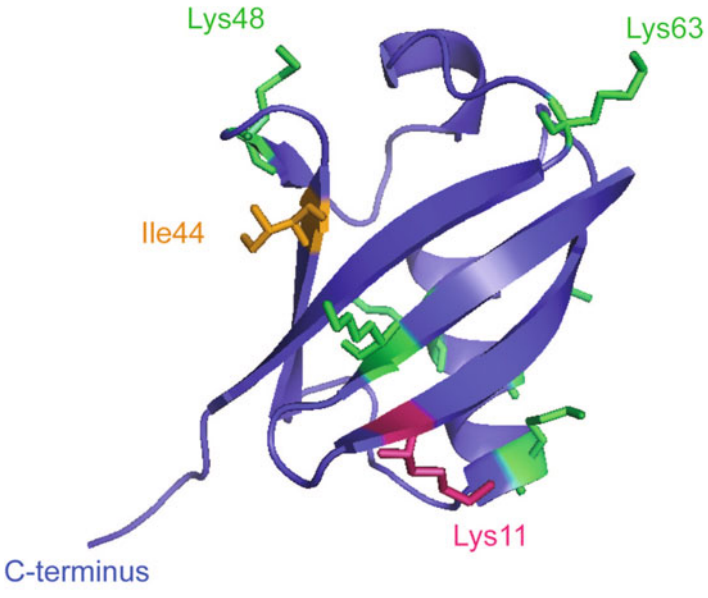

Figure 1. Location of Lys11 on ubiquitin. Lys11 is shown in red, while the sox other lysine residues on ubiquitin are depicted in green. The lysine residues used most frequently in chain assembly[11,48,63] are marked and the relative position of the hydrophobic patch around Ile44 is shown in orange.

Quantitative proteomics in yeast and human cells revealed that most chains are linked through K11, K48 and K63 of ubiquitin, but all other possible linkages are also detected.[4,5] Early work established the role of "canonical" chains linked through Lys48 and Lys63 of ubiquitin: K48-linked ubiquitin chains are essential in yeast and target the modified proteins for degradation by the 26S proteasome.[1,6] By contrast, K63-linked chains usually do not result in degradation, but instead promote the association of a modified target with proteins containing specific ubiquitin-binding domains.[7] In this manner, K63-linked ubiquitin chains help organize signaling complexes, thereby playing key roles during DNA repair or transcription factor activation.[8]

Despite their prevalence in yeast and humans, the functions of ubiquitin chains with alternative or "noncanonical" topology are less well understood. For most linkages other than K48 or K63, only in vitro data is available and consequences of such chains for the modified proteins remain unknown. For example, chains linked through Lys6 of ubiquitin have been observed in autoubiquitination reactions of the yeast E2 Rad6 and the human E3 Brca1/Bard1, but substrates modified with K6-linked chains in cells have not been identified.[9,10] However, progress has recently been made for ubiquitin chains linked through Lys11 and for linear chains, which are connected through the C-terminus of one ubiquitin and the amino-terminal NH_2-group of another.[5,11-13] Especially for K11-linked chains, we now know substrates, enzymes, acceptors and functions, ranging from cell cycle control to ER-associated degradation. Here, we discuss the mechanism of assembly of K11-linked chains by their first known E3, the human anaphase-promoting complex (APC/C) and we speculate on reasons for using alternative ubiquitin chains as degradation signals in higher eukaryotes.

K11-LINKED UBIQUITIN CHAINS TARGET PROTEINS FOR DEGRADATION BY THE PROTEASOME

Ubiquitin chains linked through Lys11 were first detected in yeast, where they are almost as abundant as K48-linked chains during normal cell division.[5,14] In this organism, the levels of K11- and K48-linked chains increase to a similar extent upon inhibition of the yeast proteasome.[5] Likewise, K11-linked chains accumulate in diseases, such as Alzheimer's or Huntington's, in which the function of the proteasome has been impaired[4] and blocking the formation of K11-linked chains severely impairs bulk protein degradation in reticulocyte lysate.[9] These findings suggested that noncanonical K11-linked chains target proteins for degradation by the 26S proteasome.

A more detailed analysis of the cellular role of K11-linked chains had to await the discovery of their physiological substrates, which was achieved only recently for regulators of the cell cycle and ER-associated degradation.[5,11,15] With known substrates at hand it could be demonstrated that K11-linked chains efficiently target proteins for degradation by the 26S proteasome, which was observed in purified systems in vitro, in more complex extracts and in cells. As expected from these observations, K11-linked chains are recognized by the proteasomal substrate-receptors Rad23 and Rpn10[9,11] and mutation of Rad23 in yeast leads to an accumulation of K11-linked chains in cells.[5] K11-linked chains even appear to be preferentially recognized by some substrate delivery factors, such as p97, which escorts ubiquitinated proteins to the proteasome.[16,17] Together, these experiments provide firm evidence that K11-linked ubiquitin chains efficiently target proteins for degradation by the 26S proteasome.

ASSEMBLY OF K11-LINKED UBIQUITIN CHAINS BY THE APC/C

The first ubiquitin ligase shown to assemble K11-linked ubiquitin chains was the human E3 anaphase-promoting complex (APC/C), which is essential for cell division in all eukaryotes.[11,18] By modifying a large family of substrates with ubiquitin chains, the human APC/C triggers the sequential proteasomal degradation of cell cycle regulators to orchestrate progression through mitosis,[18-20] but it can also promote the disassembly of protein complexes independently of degradation.[21,22] The inhibition of the APC/C in cells stabilizes mitotic regulators, such as cyclin B1, arrests cells in metaphase and causes defects in spindle assembly.[15,23,24] Importantly, the effects of APC/C-inhibition are largely phenocopied by injection of a ubiquitin mutant lacking Lys11 (ubi-R11) into embryos of *X. tropicalis*, or by downregulation of the K11-specific E2 module of the APC/C in human and fly cells.[11,15] Moreover, the overexpression of ubi-R11 stabilizes APC/C-substrates in human tissue culture cells and addition of recombinant ubi-R11 to extracts abrogates APC/C-activity also in this system.[11,15] These observations led to the conclusion that the APC/C in higher eukaryotes functions by assembling K11-linked ubiquitin chains.

Initiation of K11-Linked Ubiquitin Chain Formation

The human APC/C employs two E2 enzymes, UbcH10 and Ube2S, to catalyze the formation K11-linked chains;[15,25] (Fig. 2). Among these E2s, the highly conserved UbcH10 is responsible for initiating chain formation.[11] UbcH10 is found in all eukaryotes, except

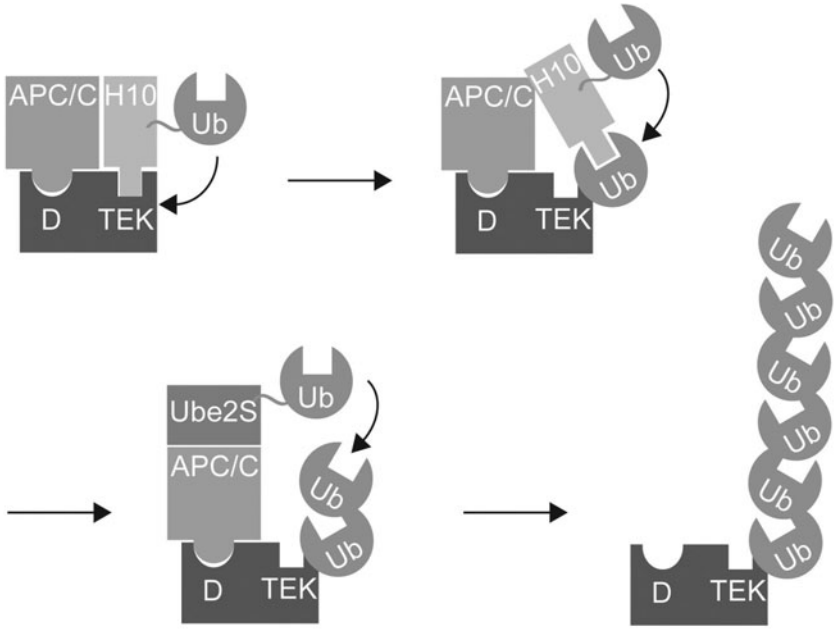

Figure 2. Mechanism of K11-linked chain assembly by the human APC/C. APC/C-substrates (red) contain a signature APC/C-binding motives, such as D-box or KEN-boxes (not shown). They also contain a secondary site, the TEK-box, which is important for ubiquitin chain initiation by UbcH10 (light orange). Ubiquitin (blue) also contains a TEK-box, allowing UbcH10 to initiate and elongate K11-linked ubiquitin chains. Ube2S (dark orange) binds to APC/C at a different site as UbcH10 and further extends K11-linked ubiquitin chains. A color version of this figure is available at www.landesbioscience.com/curie.

for yeast, but yeast APC/C does not assemble K11-linked chains.[26,27] The depletion of UbcH10 by siRNA causes a mitotic delay and the mutation of its *D. melanogaster* homolog, Vihar, results in mitotic arrest, indicating that UbcH10 is an essential regulator of the APC/C.[15,28,29] Conversely, the overexpression of UbcH10 leads to premature activation of the APC/C during mitosis, which can result in genetic instability.[21,30] As expected for its crucial role in driving cell cycle progression, the abundance of UbcH10 is tightly regulated by APC/C-dependent autoubiquitination and proteasomal degradation.[28,29] The degradation of UbcH10 occurs after most, if not all, APC/C-substrates have been depleted from cells.[29] Loosing this control mechanism likely has pathophysiological consequences, as overexpression of UbcH10 and amplification of its genomic locus are widely observed in cancer.[31-33]

UbcH10 initiates the formation of K11-linked ubiquitin chains by recognizing a short motif in APC/C-substrates, the TEK-box;[11] (Fig. 2). The TEK-box is a stretch of amino acids rich in Lys residues, which is found ~20 residues downstream of D- or KEN-boxes, the initial APC/C-binding sites in substrates.[11,34] If all TEK-boxes are deleted, chain initiation is impaired and substrates are stabilized. Consistent with a role in chain initiation, the requirement for a TEK-box can be bypassed by fusing a single ubiquitin directly to an APC/C-substrate.[11] Intriguingly, residues with high similarity to the TEK-box are found in the proximity of Lys11 in ubiquitin, which allows UbcH10

to extend short K11-linked ubiquitin chains.[11,25,35] By recognizing similar TEK-boxes in substrates and ubiquitin, UbcH10 is very powerful in initiating the formation of K11-linked ubiquitin chains.

Are other E2 enzymes able to initiate chain formation by the human APC/C? In vitro, the nonspecific E2 UbcH5 is able to support APC/C-dependent ubiquitination and its homolog Ubc4 functions as a chain initiating E2 for the yeast APC/C.[11,26] Mass spectrometry experiments showed that UbcH5 preferentially modifies Lys residues in substrates, but not ubiquitin, which might suggest chain initiating-activity.[35] However, it is unlikely that UbcH5 has an essential role in APC/C-dependent ubiquitination in humans. The depletion of all four UbcH5 homologs from human cells does not result in any obvious cell cycle phenotype[15] and addition of UbcH5 to extracts does not promote the degradation of APC/C-substrates at normal ubiquitin concentrations.[11,30] Moreover, as UbcH5 promotes ubiquitination by most E3 enzymes in vitro,[36] its concentration available for the APC/C in cells is likely to be very low. It is possible that UbcH5 might promote chain initiation during quiescence, when the APC/C is active but UbcH10 is absent,[37] but this hypothesis has not yet been supported by experimental evidence obtained from cells.

Elongation of K11-Linked Ubiquitin Chains

Following the UbcH10-dependent transfer of the first ubiquitin molecules, the K11-linked chains are then elongated by the E2 Ube2S. *UBE2S* was cloned more than a decade ago as a gene encoding autoantigens in the disease endemic pemphigus foliaceus (EPF).[38] There are striking similarities between UbcH10 and Ube2S: Like UbcH10, Ube2S is highly conserved in eukaryotes, but absent from yeast.[27] It is overexpressed in several cancers, including breast, colon and ovarian tumors.[39] The overexpression of Ube2S transforms cells in culture and high levels of Ube2S lead to tumorigenesis in mice.[40] Intriguingly, just like UbcH10, the abundance of Ube2S is tightly regulated by APC/C-dependent autoubiquitination and proteasomal degradation.[15,39] As a consequence of their parallel degradation, the chain initiating E2 UbcH10 and the elongating E2 Ube2S are strictly coregulated in cells.[15]

Ube2S is recruited to the APC/C by binding to the APC/C-activators Cdc20 and Cdh1.[15] Ube2S binds Cdc20 only in early mitosis and Cdh1 only in late mitosis or early G1, when these proteins activate the APC/C.[15,41,42] The interaction between Ube2S and Cdh1 requires a C-terminal extension in Ube2S and the WD40-repeat domain in Cdh1. Thus, different to most E2 enzymes, Ube2S does not associate with the RING-domain of an E3 by using loops in its UBC domain.[36,43] This allows Ube2S to bind to the APC/C at the same time as UbcH10, which does recognize the RING-domain in Apc11.[30,42,44] In this manner, Ube2S and UbcH10 are able to cooperate most efficiently in catalyzing the assembly of K11-linked ubiquitin chains and indeed, the depletion of both UbcH10 and Ube2S in cells leads to an almost complete inhibition of the APC/C during mitosis.[15] Thus, UbcH10 and Ube2S together form the K11-specific E2 module for human APC/C (Fig. 2).

Ube2S elongates ubiquitin chains with a striking specificity for Lys11-linkages.[9,15] The mechanism underlying this specificity is not yet understood, but might be similar to the mode of action of the heterodimeric K63-specific chain-elongating E2 Ubc13/Mms2. As shown by crystallography, Mms2 binds the acceptor ubiquitin using a noncovalent interaction surface on the backside of its UBC-domain.[45] This orients Lys63 of the acceptor ubiquitin towards the catalytic site of Ubc13, which has been charged with the donor ubiquitin and thereby leads to the exclusive assembly of K63-linked chains. Ube2S can

promote the specific formation of K11-linkages between two ubiquitin molecules in the absence of the APC/C, showing that its linkage specificity is determined independently of an E3.[9,15] This suggests that Ube2S also has a noncovalent ubiquitin interaction surface to orient K11 of the acceptor ubiquitin relative to the active site of a charged Ube2S molecule.

Does Ube2S cooperate with E3 enzymes other than the APC/C? Ubc13/Mms2 functions as K63-specific chain-elongating E2 for a large family of different E3s[36] and Ube2S might accordingly engage with more E3s. Indeed, one study has found an interaction between Ube2S and the substrate-targeting factor of Cul2-dependent E3s, VHL.[40] $Cul2^{VHL}$ triggers the ubiquitination and degradation of the transcription factor HIF1α.[46] The degradation of Hif1α also depends on the ubiquitin-selective segregase p97,[17] which efficiently binds K11-linked ubiquitin chains, but a role of Ube2S in this process has not yet been shown. In yeast, which lack Ube2S, K11-linked chains are important for ER-associated degradation,[5] but a similar role in human cells has not been reported. It therefore remains an open question, whether Ube2S functions only with the APC/C or whether it is a more general K11-specific chain-elongating factor.

WHAT IS SPECIAL ABOUT K11-LINKED CHAINS?

The identification of K11-linked chains as critical proteasomal targeting signals in human cells was surprising, given that only K48-linked ubiquitin chains are essential for cell division in yeast.[6] This suggests that K11-linked chains have become more important later in evolution, which is supported by the conservation of the K11-specific E2s UbcH10 and Ube2S in higher eukaryotes.[27] Why do higher eukaryotes need to assemble this alternative chain type? No experiments have directly addressed this question, forcing us to speculate on the particularities of K11-linked ubiquitin chains.

K11-linkages could set their substrates apart from the bulk of proteins modified with other chain types to allow for differential regulation by deubiquitinating enzymes (DUBs). DUBs are known to exert control over the APC/C in human cells by stabilizing the APC/C-inhibitory spindle checkpoint and by fine-tuning the rate of APC/C-substrate degradation.[20,22] The specificity of DUBs often results from recognizing ubiquitin chains of a certain linkage.[47-49] Thus, by modifying a class of mitotic regulators with K11-linked ubiquitin chains, these substrates could be channeled towards specific DUBs and regulated independently of proteins modified with K48- or K63-linked chains. However, DUBs with specificity for K11-linked chains remain to be identified.

K11-linked chains could also affect the efficiency of proteasomal degradation. The 26S proteasome associates with the DUBs Ubp6 and Uch37, which chew ubiquitin chains off proteasome-bound substrates to limit the residence time of substrates on the proteasome and to avoid clogging of this cellular machine.[50,51] Because K11-linked ubiquitin chains are disassembled by proteasomal DUBs more slowly that K48-linked chains,[5] substrates modified with K11-linked chains should have more time to be degraded. In this scenario, K11-linked chains could facilitate the degradation of proteins difficult to unfold, which is consistent with their role in promoting the degradation of aggregation-prone proteins emerging from the ER.[5,17]

K11-linkages could also affect the ubiquitination of proteins already captured by ubiquitin-binding domains. Most proteasomal substrate-targeting factors associate with their cargo by recognizing the hydrophobic patch on ubiquitin comprised of Leu8,

Ile44 and Val70, which is close to Lys48;[1,7,52] (Fig. 1). The binding of these proteins usually interferes with further ubiquitination and indeed, the substrate-targeting factor Rad23 was initially described as an "anti-E4".[53] This interaction, thus, leads to size-restriction of ubiquitin chains and thereby prevent excessive use of ubiquitin,[16] but it also decreases the residence time of the substrate on proteasome. Lys11, which is on the opposite site of ubiquitin as Lys48 (Fig. 1), might still be available for chain elongation in complexes with proteasomal targeting factors, which appears to be the case for p97-bound proteins.[17] As discussed above, the longer ubiquitin chains resulting from this additional ubiquitination might increase the likelihood of proteasomal degradation of particular substrates.

If K11- and K48-linked chains do not differ substantially in their ability to trigger proteasomal degradation, it might be the enzymatic machinery assembling K11-linked chains, which provides the selective advantage justifying the evolutionary conservation and high abundance of this nonconventional chain type.[27,36] Both UbcH10 and Ube2S are first observed in higher eukaryotes, which disassemble their nuclear envelope during mitosis.[27] Consequentially, the spatial regulation of processes such as spindle assembly or sister chromatid separation becomes more complex and UbcH10 and Ube2S possibly provide a mechanism to allow localized protein degradation under these conditions. This hypothesis is supported by the fact that Ube2S has been shown to interact with the APC/C and VHL, both of which regulate microtubule dynamics during mitosis.[15,40,54] As demonstrated in this last chapter, many experiments remain to be done to unravel the mysteries underlying K11-linked ubiquitin chains.

CONCLUSION

We found K11-linked ubiquitin chains as novel regulators of cell division in higher eukaryotes. K11-linked chains are assembled by the E3 APC/C, which is essential for cell division in all eukaryotes. Their assembly depends on a chain-initiating E2, UbcH10 and a specific chain-elongating E2 Ube2S. K11-linked chains are recognized by proteasomal substrate acceptors and sufficient to trigger proteasomal degradation in purified systems, extracts and cells. K11-linked chains increase in their abundance if the proteasome is inhibited. Future work will have to address specific functions of K11-linked ubiquitin chains, which will require the identification of substrates modified with this novel and conserved chain type.

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CHAPTER 10

UBIQUITIN FAMILY MEMBERS IN THE REGULATION OF THE TUMOR SUPPRESSOR p53

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Abstract: It is commonly assumed that the p53 tumor suppressor pathway is deregulated in most if not all human cancers. Thus, the past two decades have witnessed intense efforts to identify and characterize the growth-suppressive properties of p53 as well as the proteins and mechanisms involved in regulating p53 activity. In retrospect, it may therefore not be surprising that p53 was one of the very first mammalian proteins that were identified as physiologically relevant substrate proteins of the ubiquitin-proteasome system. Since then, plenty of evidence has been accumulated that p53 is in part controlled by canonical (i.e., resulting in proteasome-mediated degradation) and noncanonical (i.e., nonproteolytic) ubiquitination and by modification with the ubiquitin family members SUMO-1 and NEDD8. In this chapter, we will largely neglect the plethora of mechanisms that have been reported to be involved in the regulation of p53 ubiquitination but will focus on the enzymes and components of the respective conjugation systems that have been implicated in p53 modification and how the respective modifications (ubiquitin, SUMO-1, NEDD8) may impinge on p53 activity.

INTRODUCTION

In 1979, p53 was discovered as a protein that interacts with the large tumor antigen of SV40 and that is present at elevated levels in SV40-transformed cells as well as in tumors/transformed cells derived from mice.[1-3] While subsequent characterization of p53 initially indicated that p53 functions as a cellular proto-oncoprotein (mainly because p53 cDNAs encoding p53 molecules with point mutations were studied), it

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Figure 1. Scheme of p53. Human p53 consists of 393 amino acids that can be roughly divided into three functional domains: The central conformational domain that binds sequence-specifically to DNA and comprises amino acids 100-290; the N-terminal 100 amino acids harboring the transcriptional transactivation function and a nuclear export sequence (amino acids 11-27;[182] not indicated); the C-terminal 100 amino acids containing the main nuclear localization sequence (NLS; amino acids 315-321), the oligomerization domain (OD; amino acids 324-355) and a nuclear export sequence (NES; amino acids 340-351). The region spanning amino acids 14-27 represent the primary interaction site for Mdm2 and MdmX, respectively. In addition, amino acids 260-270 represent a second interaction site for Mdm2 (not indicated).[183] Lysine residues reported to serve as attachment sites for ubiquitin (Ub), NEDD8 (N8) and/or SUMO-1 (S1) are indicated.

was in 1989 when it was reported that p53 has the properties of a tumor suppressor and that the p53 gene is frequently mutated in colorectal carcinomas.[4,5] Since then, p53 has become one of the most intensely studied genes/proteins and it is now common knowledge that the p53 gene is mutated in approx. 50% of all cancers resulting either in the expression of a mutant p53 that has lost the tumor suppressive properties of the wild-type (wt) protein or, less frequently, in loss of p53 expression. Furthermore, in the remaining ~50% of cancers, the p53 pathway is impaired by alternative mechanisms including interaction with cellular or viral oncoproteins (e.g., Mdm2, human papillomavirus E6), aberrant cellular localization, or alterations in regulatory factors up- or down-stream of p53.[6-11]

The best characterized biochemical function of p53 is that of a transcriptional modulator that depending on the respective conditions, affects various cellular processes including cell cycle, apoptosis, senescence, metabolic pathways and autophagy (for reviews see 6,9-13).It should be noted, however, that p53 has also transcription-independent growth-suppressive functions (see below). Human p53 consists of 393 amino acid residues and can be roughly divided into three functional domains (Fig. 1). The central domain comprises amino acids 100-290 and represents a sequence-specific DNA binding domain.[14,15] The importance of this domain for p53 function is impressively illustrated by the fact that approx. 90% of the missense mutations in the p53 gene identified in cancers map to this region resulting in compromised DNA binding properties and as recently reported, also other growth-suppressive properties of the respective mutant p53 proteins.[6,9,11-13,16,17] The N-terminal 100 amino acids harbor the transcriptional transactivation function and have been shown to act as interaction sites for basic components of the transcriptional machinery including subunits of TFIID and TFIIH[18-20] and the transcriptional coactivators CBP/p300.[21,22] The C-terminal 100 amino acids can be further subdivided into at least three regions, the main nuclear localization sequence (amino acids 315-321), the oligomerization domain (amino acids 324-355) and amino acids 363-393, which have the property to bind to nucleic acids (DNA, RNA) in a sequence-independent manner and may be involved in regulating the sequence-specific DNA binding properties of p53.[23-25] Furthermore, it should be noted that besides full-length p53, expression of the human p53

gene can result in the production of p53 variants via the use of an alternative promoter and the generation of multiple splice variants in a tissue-dependent manner. Intriguingly, some of these bind differentially to promoters and modulate the expression of p53 target genes in a promoter-dependent manner, while others act in a dominant-negative fashion by forming complexes with full-length p53.[26] If these variant forms of p53 are subject to modification by ubiquitin family members and/or if they affect modification of full-length p53, is currently not known.

In a simplistic view, p53 surveils the (genomic) integrity of a cell and is activated by various stress stimuli including genotoxic agents (e.g., UV, ionizing radiation, chemotherapeutic drugs including cisplatin and etoposide)[27-29] and nongenotoxic insults (e.g., hypoxia, nutrient stress, depletion of NTPs).[30,31] Thus, key to understanding the role of p53 in tumor suppression is not only the identification of genes and pathways affected by p53 but also identification of pathways and mechanisms involved in the regulation of p53 activity. As with other regulatory proteins, p53 activity can be posttranslationally regulated by numerous means including noncovalent interaction with other proteins or biomolecules, subcellular localization and covalent modification (e.g., phosphorylation, methylation, acetylation, glycosylation, ADP-ribosylation, ubiquitination) (for reviews see 10,11). Although the number of mechanisms involved in p53 regulation is ever increasing, covalent modification by ubiquitin family members has become a central theme in the regulation of p53 activities. In fact, p53 represents a good example to illustrate various aspects and concepts of modification by ubiquitin and ubiquitin-like proteins.

p53 AND UBIQUITIN I: TARGETING p53 FOR DEGRADATION

p53 is a short-lived protein with a reported half-life ranging from 10-15 minutes (rodent fibroblasts) to 2-4 hours (human keratinocytes).[32,33] Already in 1984, it was shown that degradation of p53 is ATP-dependent,[27] a characteristic feature of ubiquitin-proteasome-dependent degradation and that the increase in p53 levels observed upon treatment of cells with UV light is due to an extended half-life of the protein. However, these observations were not further pursued until the early 1990s, when it became clear that the E6 oncoprotein of cancer-associated human papillomaviruses (HPVs) targets p53 for degradation via the ubiquitin-proteasome system (see below) and that in response to stress stimuli, p53 stabilization and, thus, accumulation contributes to the activation of its growth-suppressive properties.[28,34] Subsequently, it was shown that p53 is stabilized in a cell line harboring a temperature-sensitive ubiquitin-activating enzyme, when grown at the nonpermissive temperature[35] and that p53 is ubiquitinated within cells and represents a substrate of the proteasome.[36] This "early" era of ubiquitin-related p53 research culminated in the findings that the proto-oncoprotein Mdm2 targets p53 for proteasome-mediated degradation and that Mdm2 has the activity of a (RING type) ubiquitin ligase.[37-41] Since then, a still increasing number of ubiquitin ligases have been reported to be involved in p53 ubiquitination (Table 1).

Table 1. Ligases involved in modification of p53 by ubiquitin family members (UbF). For refs see text.

Ligase	UbF	Effect on p53
Mdm2	Ubiquitin NEDD8	Degradation; Nuclear export, mitochondrial translocation inhibition of transcriptional activity
COP1	Ubiquitin	Degradation
Pirh2	Ubiquitin	Degradation
ARFBP1/MULE/ HECTH9	Ubiquitin	Degradation
CARPs	Ubiquitin	Degradation
Synoviolin	Ubiquitin	Degradation of cytoplasmic p53
MKRN1	Ubiquitin	Degradation
TRIM24	Ubiquitin	Degradation
β-TrCP (SCF complex)	Ubiquitin	Degradation of S362 and S366 phosphorylated p53
JFK (SCF complex)	Ubiquitin	Degradation
CHIP	Ubiquitin	Degradation of mutant p53 and not properly folded/assembled forms (?) of p53
Topors	Ubiquitin SUMO-1	Degradation accumulation (via stabilization?)
E6/E6AP	ubiquitin	Degradation in HPV transformed cells
E1B55K/E4orf6 (SCF-like complex)	Ubiquitin	Degradation in adenovirus infected and transformed cells
E1B55K	SUMO	???
ICP0	Ubiquitin	Degradation in HSV-1 infected cells
LANA (SCF-like complex)	Ubiquitin	Degradation in KSHV infected cells
BZLF1 (SCF-like complex)	Ubiquitin	Degradation in EBV infected cells
WWP1	Ubiquitin	Cytoplasmic localization, stabilization
E4F1	Ubiquitin	Chromatin binding, activation of transcriptional activity
MSL2	Ubiquitin	Cytoplasmic localization
FBXO11 (SCF complex)	NEDD8	inhibition of transcriptional activity
PIAS family members	SUMO-1	Modulation of transcriptional activity; inhibition of DNA-binding activity

p53 AND MDM2

Most importantly with respect to the physiological relevance of the p53-Mdm2 interaction, Mdm2 null mice die early in embryonic development, while mice deficient for both Mdm2 and p53 are viable but similar to mice with a knockout of only the p53 gene develop tumors.[42,43] This and data obtained more recently with tissue-specific transgenic mice[44-49] clearly show that Mdm2 is a major regulator of p53 activity in every tissue studied making the p53-Mdm2 interaction a very attractive target for the development of molecular anti-cancer therapies. The major interaction site for Mdm2 is contained within the N-terminal 40 amino acids of p53 (amino acids 14-27). As a proof of principle, small peptides that disrupt the p53-Mdm2 interaction and small molecules called nutlins that bind in the hydrophobic Mdm2 pocket (amino acids 25-108), where the p53 N-terminus is buried, stabilize and activate wt p53 in cells.[50-54]

Mdm2 was originally (i.e., before its ubiquitin ligase activity was discovered) shown to inhibit the transcriptional activity of p53 by competing with factors of the basal transcriptional machinery for p53 binding,[19,55,56] indicating that binding per se is sufficient to interfere with at least some p53 activities. Nonetheless, it is commonly assumed that Mdm2-mediated degradation of p53 is the major mechanism, by which Mdm2 interferes with p53 activity, for several reasons including the notions that degradation provides a catalytic mechanism of inactivation (in contrast, binding is stoichiometric) and that degradation also inactivates nontranscriptional activities of p53. How and where in a cell does Mdm2-mediated degradation of p53 occur? The latter issue is still a matter of debate and there is evidence that p53 can be degraded in both the nucleus and the cytosol (see below). Concerning the conjugation machinery, in vitro reconstitution assays and RNA interference analyses indicate that UbcH5b and UbcH5c function as the cognate E2 ubiquitin-conjugating enzymes of Mdm2 in p53 ubiquitination.[57] Although these E2 enzymes have the potential to form K48-linked ubiquitin chains,[58] there is evidence to indicate that at least within cells, additional factors are required for polyubiquitination of p53. Indeed, the transcriptional co-activator p300 that binds to the so-called acidic domain of Mdm2 (amino acids 209-275) appears to act as a polyubiquitin chain assembly factor E4 in Mdm2-mediated p53 ubiquitination.[59] Furthermore, degradation of polyubiquitinated substrates frequently involves proteins that connect or shuttle ubiquitinated proteins to the 26S proteasome. In the case of p53, hHR23A and/or hHR23B (human orthologs of *S. cerevisiae* Rad23) may serve such a function.[60-62] The hHR23 proteins contain both UBA domains (bind to ubiquitin chains) and a UBL domain (binds to subunits of the proteasome) thereby transferring polyubiquitinated p53 to the proteasome (Fig. 2).

Unambiguous identification of lysine residues of a substrate protein that serve as attachment sites for ubiquitin within cells can be achieved by mass spectrometric analysis. However, such endeavors are in many cases hampered by the lack of sufficient material (i.e., ubiquitinated proteins) and by the notion that no tools are yet available to selectively enrich respective samples for ubiquitinated peptides. Also in the case of p53, all the lysine residues reported to be ubiquitinated were identified by mutational analysis and, thus, when interpreting such data, one should keep in mind that some of these lysine residues may not represent actual attachments sites but rather affect the efficiency of p53 ubiquitination by other means (e.g., by altering the conformation of certain regions of p53 or by serving as attachment sites for other modifications including acetylation and methylation, which in turn may facilitate ubiquitination). In addition, the notion that

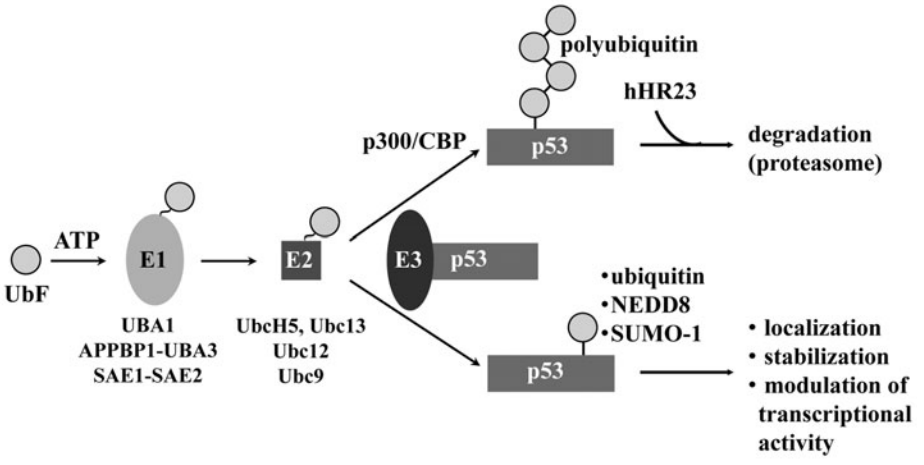

Figure 2. Enzymes and proteins involved in modification of p53 by ubiquitin family members. Ubiquitin family members (UbF; for p53: ubiquitin, NEDD8 and SUMO-1) are activated by the respective E1 activating enzymes (ubiquitin, UBA1; NEDD8, APPBP1-UBA3; SUMO-1, SAE1-SAE2) at the expense of ATP. The activated UbF is then transferred to the respective E2 conjugating enzymes (for p53: ubiquitin, UbcH5b/c or Ubc13; NEDD8, Ubc12; SUMO-1, Ubc9). Finally, E2s in concert with respective E3 UbF ligases (for E3s involved in p53 modification, see Table 1) catalyze the covalent attachment of the respective UbF to p53. For example, UbcH5b and UbcH5c, respectively are involved in Mdm2-mediated ubiquitination of p53, which results in monoubiquitination or polyubiquitination (presumably K48-linked ubiquitin chains) of p53. Furthermore, modification of p53 with K48-linked ubiquitin chains may require the presence of p300/CBP, which act as E4 ubiquitin chain assembly factors. While monoubiquitination affects the localization of p53 (nuclear export, mitochondrial translocation), modification of p53 with ubiquitin chains induces either p53 degradation (K48-linked chains) or cytoplasmic sequestration (Ubc13-mediated modification by K63-linked chains). Furthermore, transfer of polyubiquitinated p53 to the proteasome may require the activity of "shuttle factors" including hHR23 that interact with both polyubiquitinated substrates and the proteasome. Neddylation and sumoylation of p53 affect the transcriptional properties of p53. Finally, it should be noted that modification of p53 by UbFs is a highly dynamic process, as UbF modification of p53 is a reversible process by the action of demodifying enzymes (not indicated). For further details, see text.

p53 is subject to ubiquitination that does not serve as signal for degradation (see below) further complicates the identification of lysine residues that upon ubiquitination, target p53 to the proteasome. In fact, overexpression studies clearly showed that mutation of 6 lysine residues located in the C-terminal 30 amino acids of p53 (K370, K372, K373, K381, K382, K386) results in a p53 mutant that is only very inefficiently ubiquitinated by Mdm2.[63,64] However, knock-in mice or mouse embryonic stem cells expressing a p53 mutant, in which the respective lysine residues are substituted by arginine, develop/grow normally, are viable and the half-life of the p53 mutant is very similar to the one of wt p53.[65,66] The only reported difference is that the p53 mutant mice are somewhat defective in the activation of certain p53 target genes.[66] However, since lysine residues are subject to several types of posttranslational modification (see above), it remains unclear, if the phenotype is in any way related to a defect in ubiquitination. In the meantime, several lysine residues in the DNA binding domain (K101, K120, K132, K139) and in the C terminus (K291, K292, K319, K320, K321, K351, K357) of p53 were reported to serve as ubiquitination sites.[67-70] However, if ubiquitination of any of these is involved in determining the turnover rate of p53, remains to be determined.

Mdm2-facilitated degradation of p53 is probably required at two distinct phases in a cell. Firstly, it contributes to keep p53 at low levels and, thus, in an inactive or latent state under normal growth conditions. Secondly, the *mdm2* gene is a target gene of p53 and, accordingly, upon activation of p53 by various stress stimuli, Mdm2 levels increase in cells after a certain lag phase.[71] This negative feedback loop presumably contributes to the inactivation of p53 and allows a cell to resume its normal state, when the respective stress stimulus and/or the damage evoked by it have been cleared. This begs the question as to how Mdm2-mediated degradation of p53 is controlled/regulated under these three circumstances (i.e., normal growth, stress-mediated activation and stabilization of p53, resuming normal growth). As indicated above, an ever increasing number of mechanisms has been proposed to affect Mdm2-mediated degradation of p53 including several proteins that bind to the acidic domain of Mdm2, which is required for p53 degradation,[72,73] multiple posttranslational modifications of p53 and Mdm2, respectively, that may affect their interaction and/or activity and deubiquitinating enzymes that control the ubiquitination status of p53 and Mdm2 (Mdm2 itself is a substrate of the ubiquitin-proteasome system) (see below). However, the amount of data that would need to be discussed to do justice to this important issue (for reviews see refs. 6-13) would easily fill another chapter and, thus, we will only briefly discuss two proteins, p14ARF and MdmX, that play a major role in the control of the activity of Mdm2 and p53, respectively.

MdmX is a RING domain protein and shares significant structural and functional similarity with Mdm2.[74,75] It binds to the N-terminal region of p53 thereby interfering with the transcriptional transactivation properties of p53.[76,77] Similar to Mdm2, knockout of the *mdm4* gene (which encodes MdmX) in mice causes embryonic lethality and this is rescued by concomitant deletion of the p53 gene.[78,79] Further analysis of tissue-specific MdmX null mice indicate that the presence of both MdmX and Mdm2 is required to keep p53 in check in most but not all tissues[44-48,80] However, despite the presence of a C-terminal RING domain, MdmX has no or only little ubiquitin ligase activity but rather acts as a stimulator of the ubiquitin ligase activity of Mdm2 via heterocomplex formation which is mediated by the respective RING domains of Mdm2 and MdmX.[58,81] Indeed, di- or multimeric forms of Mdm2 rather than Mdm2 monomers have ubiquitin ligase activity and Mdm2-MdmX heteromers appear to be more stable (thermodynamically) than Mdm2 homomers providing a possible explanation for the observation that MdmX stimulates Mdm2 activity.[82-84] Furthermore, Mdmx localizes predominantly in the cytosol in the absence of Mdm2, while complex formation with Mdm2 recruits MdmX into the nucleus and there is evidence to indicate that Mdm2 controls the levels of MdmX and vice versa.[58,85-88] Taken together, the available data indicate that Mdm2 and MdmX co-operate to control the turnover rate of each other as well as that of p53.

The tumor suppressor $p14^{ARF}$ (p19 in mice), or briefly ARF, was identified as an alternative transcript of the Ink4a/ARF locus that was originally shown to encode the cyclin-dependent kinases inhibitor $p16^{Ink4a}$.[89] ARF directly interacts with the acidic domain of Mdm2 and protects p53 from Mdm2-mediated degradation.[90-92] Furthermore, cell culture studies revealed that ARF blocks proteasomal degradation of both p53 and Mdm2, while it interfered with ubiquitination of p53 but not with Mdm2 auto-ubiquitination indicating that substrate ubiquitination and auto-ubiquitination of Mdm2 are differentially modulated by ARF.[93] Mdm2 contains a cryptic nucleolar sequence in the RING domain (amino acids 466-473) that is exposed upon interaction with ARF.[94] ARF itself localizes predominantly to the nucleolus and sequesters Mdm2 to the same compartment thereby disrupting the interaction with p53 or inhibiting nuclear export of the p53-Mdm2 complex (nuclear export

may at least under certain instances represent an intermediate step in p53 degradation and may occur via the nucleolus).[95-97] However, protection of p53 from Mdm2-mediated degradation by nucleolar sequestration does not seem to be the only mechanism, by which ARF interferes with Mdm2 activity, as ARF mutants that do not localize in the nucleolus were reported to stabilize and activate p53.[98] A possible explanation for this observation is provided by the finding that in vitro, ARF peptides that bind to Mdm2 can inhibit the ubiquitin ligase activity of Mdm2.[99,100]

p53 AND OTHER UBIQUITIN LIGASES

Although the available genetic evidence clearly indicates that Mdm2 represents a major ubiquitin ligase for p53 and that Mdm2 activity is crucial to keep the growth-suppressive properties of p53 under control, results obtained in a transgenic mouse model indicate that even in the absence of Mdm2, p53 can be targeted for proteasome-mediated degradation.[101] This suggests that p53 can be degraded either in a ubiquitin-independent manner or maybe more likely that additional ubiquitin ligases for p53 exist. Indeed, a still increasing number of ubiquitin ligases have been reported to recognize p53 as substrate for ubiquitination and degradation (see Table 1). In many of these cases, the results were exclusively obtained in cell culture studies (overexpression, RNA interference analysis) and in part supported by in vitro ubiquitination assays. Since genetic evidence is largely missing, the actual importance of the individual ubiquitin ligases for p53 ubiquitination and degradation remains to be determined. Thus, we will only briefly review the respective literature.

The first two ubiquitin ligases that after the discovery of Mdm2-mediated ubiquitination of p53, were reported to target p53 for degradation are Cop1 and Pirh2. Both are members of the RING type ubiquitin ligase family and, similar to Mdm2, the respective genes are targets for p53-induced transcriptional transactivation.[102,103] Thus, at least three different ubiquitin ligases are potentially involved in a negative feedback loop upon activation of p53 by stress. Furthermore, Mdm2 is frequently overexpressed in sarcomas, while increased expression of Pirh2 and Cop1 is found in lung and breast cancers, respectively.[104-107] Importantly with respect to the physiological relevance of the ability of these ubiquitin ligases to target p53 for degradation, cancers overexpressing Mdm2/Cop1/Pirh2 express wt p53. This suggests that in these cancers, the tumor-suppressive functions of p53 are negated by overexpression of the respective ligases.

ARF-BP1/Mule/HectH9 represents a giant HECT type ubiquitin ligase that has also been implicated in cancerogenesis.[108,109] It interacts with p14ARF and p53 and targets p53 for ubiquitination and degradation.[110] ARF-BP1/Mule/HectH9 does not appear to be a p53 target gene and it was shown to have both p53-dependent and p53-independent pro-proliferative functions.[110-112] Thus, to appreciate the importance of the interaction of ARF-BP1/Mule/HectH9 (as well as of other ubiquitin ligases) with p53, it will be important to functionally separate the ability of ARF-BP1/Mule/HectH9 to target p53 from its p53-independent functions.

Other (RING type) ubiquitin ligases reported to target p53 for ubiquitin-dependent degradation include Topors, which has also been reported to promote p53 sumoylation,[113-115] the CARP (caspase-8 and -10 associated RING proteins) family of apoptotic inhibitors,[116] Synoviolin/Hrd1, which plays a role in ERAD and may be involved in cytoplasmic ubiquitination of p53,[117] the F-box proteins β-TrCP and JFK (in the form SCF ubiquitin

ligase complexes, with SCF defined as Skp1-Cul1-F-box protein),[118,119] TRIM24[120] and MKRN1[70] (summarized in Table 1). Finally, the RING type ubiquitin ligase CHIP that via its ability to bind to Hsc70 and Hsp90 facilitates ubiquitination and degradation of chaperone associated proteins also targets p53 for degradation.[121] Accordingly, it is tempting to speculate that CHIP preferentially targets aberrantly folded or misassembled p53 for degradation. Indeed, it was reported that p53 mutants represent preferred substrates for CHIP.[122,123]

p53 AND VIRUSES

In view of the notion that p53 is activated in response to numerous stress stimuli, it is not surprising that viruses have evolved various strategies to cope with p53 function. In fact, the first ubiquitin ligase reported to target p53 for degradation is the HECT ubiquitin ligase E6AP (E6 associated protein) that is recruited to p53 by the E6 oncoprotein of HPVs etiologically associated with cervical cancer.[34,124,125] The importance of the ability of the HPV E6 oncoprotein to utilize E6AP to target p53 and other cellular proteins for degradation for HPV-induced cellular transformation is illustrated by the findings that cervical cancer is one of the very few tumor types, in which the p53 gene is very rarely mutated and that both ablation of p53 activity and the interaction with PDZ domain proteins is critical for HPV-induced tumorigenesis in transgenic mouse models.[8,126-128] Furthermore, downregulation of E6AP expression by antisense RNA-based approaches or by RNA interference induces p53 accumulation and activation of its growth-suppressive properties.[129-132] These data indicate that E6-E6AP-induced degradation of p53 is at least in part functionally equivalent to inactivation of p53 by mutation of the p53 gene, although the situation in HPV-positive cancers may be more complicated.

Similar to E6 and E6AP, the E1B55K and E4orf6 proteins of oncogenic adenoviruses target p53 for ubiquitin-mediated degradation by functioning as recognition components of an SCF-like ubiquitin ligase complex consisting of Cul5, Elongins B and C and the RING protein Rbx1/Roc1.[133] In addition, E1B55K induces modification of p53 with SUMO.[134] However, while it is assumed that E1B55K-E4orf6-mediated degradation of p53 is required to ensure proliferation of infected cells and, thus, viral propagation or persistence, the relevance of E1B55K-induced sumoylation of p53 for the viral life cycle remains to be determined.

Other human pathogenic viruses targeting p53 stability include the herpesvirus family members Herpes simplex virus type 1 (HSV-1), Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV), all of which have apparently evolved several strategies to affect p53 ubiquitination. The HSV-1 regulatory protein ICP0 contains a RING domain and acts as a ubiquitin ligase for p53.[135] In addition, ICP0 and EBNA1 of EBV may indirectly affect p53 stability by binding to the ubiquitin-specific protease USP7/HAUSP (Herpesvirus associated ubiquitin-specific protease).[135,136] Similar to E1B55K-E4orf6, the BZLF1 protein of EBV and the LANA protein of KSHV have been reported to function as recognition components of Cul2/5-containing ubiquitin ligase complexes and to target p53 for degradation.[137,138] This property is assumed to play an important role during lytic infection of EBV and tumorigenic progression of KSHV-infected cells, respectively. Finally, EBNA3C, which is involved in primary B-cell transformation and the vIRF4 protein of KSHV may affect p53 degradation indirectly by

inducing Mdm2 stabilization (and, thus, accumulation).[139,140] Interestingly, in the case of EBNA3C, the effect on Mdm2 stability may be achieved by an intrinsic deubiquitinating activity of EBNA3C.

p53 AND UBIQUITIN II: NONPROTEOLYTIC UBIQUITINATION

In the late 1990s, studies with the nuclear export inhibitor leptomycin B and heterokaryon experiments revealed that the interaction with Mdm2 can result in nuclear export of p53 and that degradation of p53 can occur in the cytoplasm.[141-143] Furthermore, a ubiquitin ligase-deficient mutant of Mdm2 (C464A) is severely impaired in facilitating nuclear export of p53 indicating that ubiquitination of p53 not only affects its turnover rate but also its subcellular localization.[144] This assumption is supported by experiments with p53-ubiquitin fusion proteins (possibly mimicking mono-ubiquitinated p53) showing that such fusion proteins preferentially localize to the cytoplasm and that this effect is specific to ubiquitin fusions, since similar p53 fusion proteins with NEDD8 or SUMO do not detectably affect p53 localization.[145-147] Intriguingly, low levels of Mdm2 were reported to promote monoubiquitination of p53 resulting in p53 nuclear export (Fig. 2), whereas high Mdm2 levels promote polyubiquitination of p53 and proteasomal degradation in the nucleus.[145] In addition, evidence was provided that monoubiquitination and Mdm2, respectively, facilitate the interaction of p53 with the SUMO ligase PIASy promoting sumoylation and nuclear export of p53 and that monoubiquitination targets p53 to mitochondria.[13,147,148] Taken together, these data suggest a model, in which p53 is exported to the cytoplasm for degradation in unstressed cells (i.e., low levels of Mdm2) or to exert transcription-independent growth-suppressive functions, such as induction of apoptosis through interaction with mitochondria, during the initial phase of a stress response. In contrast, at later stages of the p53 response, where due to the negative feedback loop (see above) Mdm2 levels are high or in malignancies with amplification of the Mdm2 gene, p53 is polyubiquitinated and degraded in the nucleus. An additional mechanism, by which p53 modification may switch from mono- to polyubiquitination, has recently been provided by the observation that inhibition of the peptidyl-prolyl cis/trans isomerase Pin1, which is known to interact with p53, alters the modification status of p53 from mono- or oligoubiquitination to polyubiquitination.[149-151]

Although Mdm2-mediated monoubiquitination is assumed to play a major role in the modulation of the cellular localization of p53, the observation that a mutant p53, which cannot interact with Mdm2, can be localized to the cytoplasm indicates that additional pathways must exist. Indeed, the ubiquitin ligase MSL2 has recently been reported to mediate ubiquitination of p53 in an Mdm2-independent manner and this ubiquitination results in cytoplasmic localization of p53 but does not affect its half-life.[69] Furthermore, the ubiquitin-conjugating enzyme Ubc13, an E2 enzyme that forms complexes with catalytically inactive ubiquitin-conjugating enzyme variants and specifically catalyzes the modification of proteins with K63-linked ubiquitin chains, also affects subcellular localization of p53.[152] Ubc13 preferentially interacts with monomeric forms of p53 on translationally active polysomes and targets these for K63-dependent ubiquitination. This interferes with p53 oligomerization and Mdm2-mediated polyubiquitination and sequesters p53 in the cytoplasm. Furthermore, the interaction of p53 and Ubc13 can be controlled

by DNA damage and by JNK-facilitated phosphorylation of p53, thereby contributing to the regulation of the oligomerization status, cellular localization and ultimately the activity status of p53.[153]

Finally, the atypical ubiquitin ligase E4F1 and the HECT ubiquitin ligase WWP1 were shown to ubiquitinate p53.[68,154] p53 ubiquitinated by E4F1 is associated with chromatin and appears to be involved in the induction of p53 target genes that mediate cell cycle arrest but not apoptosis. In contrast, WWP1-mediated ubiquitination results in increased accumulation of p53 in the cytoplasm and, concomitantly, in reduction of p53 transcriptional activity.

p53 AND UBIQUITIN III: DEUBIQUITINATION

Similar to ubiquitin ligases, the human genome encodes for a large number of deubiquitinating enzymes indicating that modification of proteins with ubiquitin is a highly dynamic process and providing an additional level, by which the ubiquitination status and the activity of p53 can be controlled. Besides EBNA3C (see above), five USPs (USP2a, USP5/Isopeptidase T, USP7/HAUSP, USP11, USP28) have been reported to affect the p53 pathway[139,155-160] and two of these (USP2a, USP7/HAUSP) directly act on p53 and/or Mdm2.

USP7/HAUSP was first identified as a p53 interacting protein and was subsequently shown to bind also to Mdm2.[157,158] Accordingly, USP7/HAUSP has the potential to block or reverse Mdm2-mediated ubiquitination of p53 thereby activating p53 but also to induce Mdm2 accumulation (by reversing Mdm2 autoubiquitination) thereby enhancing p53 degradation. Indeed, in cells derived from USP7/HAUSP-deficient mice, Mdm2 levels are significantly decreased with a simultaneous increase in p53 levels,[161] while a partial decrease of HAUSP/USP7 levels by RNA interference results in decreased p53 levels but does not affect Mdm2 levels.[158] These data indicate that the actual effect of USP7/HAUSP on the p53 pathway depends on the relative amount of active USP7/HAUSP and that the activity of USP7/HAUSP must be finely controlled. The death associated protein DAXX was shown to interact with both USP7/HAUSP and Mdm2 in a ternary complex facilitating deubiquitination and stabilization of Mdm2 and, in consequence, promoting Mdm2-mediated degradation of p53. Upon DNA damage, however, DAXX dissociates from Mdm2 inducing destabilization of Mdm2 and activation of the p53 response.[162] The disruption of the DAXX-Mdm2 complex may be mediated by the RASSF1A tumor suppressor protein, which was shown to bind both Mdm2 and DAXX but in a mutually exclusive manner.[163] Similarly, USP7/HAUSP was shown to control MdmX levels and also in this case, deubiquitination is impaired in response DNA damage. Thus, inactivation of USP7/HAUSP may in part account for the rapid and transient destabilization of Mdm2/Mdmx observed upon DNA damage.[164-166]

In a bacterial two-hybrid screen, a peptide with significant sequence similarity to USP2a was identified as an Mdm2 interactor.[155] Subsequent characterization of USP2a revealed that USP2a indeed binds to Mdm2 within cells and can deubiquitinate Mdm2, while it is inactive towards ubiquitinated p53. Furthermore, ectopic expression of USP2a results in accumulation of Mdm2 and concomitant degradation of p53, while downregulation of endogenous USP2a expression has the opposite effect (i.e., increased Mdm2 degradation and p53 accumulation). The significance of the USP2a-Mdm2 interaction is indicated

by the finding that the USP2 gene (encoding USP2a and USP2b) is found amplified in approx 50% of prostate cancers.[155] As many of these express wt p53, this may indicate that USP2 gene amplification is functionally equivalent to Mdm2 gene amplification (i.e., both are expected to result in increased Mdm2 levels) suppressing the growth-inhibitory properties of wt p53.

p53 AND NEDD8

Amongst ubiquitin family members, NEDD8 is the most similar to ubiquitin at the amino acid sequence level. Nonetheless, covalent attachment of NEDD8 to substrate proteins requires a distinct set of enzymes including a heterodimeric NEDD8-activating enzyme and the NEDD8-conjugating enzymes Ubc12 and Nce2/Ube2F (Fig. 2).[167] Furthermore, NEDD8 modification does not appear to target proteins for destruction but similar to nonproteolytic ubiquitination, affects the biochemical/physiological properties of a protein. Until a few years ago, members of the Cullin protein family were the only described substrates for neddylation and in these cases, neddylation appears to activate the activity of the respective SCF and SCF-like ubiquitin ligase complexes.[168] In fact, p53 and Mdm2 represent two of the first identified nonCullin substrates for NEDD8.[169] Moreover, Mdm2 acts as a RING type ligase in the neddylation of p53. Neddylation of p53 requires the presence of 3 lysine residues in the C terminus (K370, K372, K373) indicating that at least one of these represents the major attachment site for NEDD8. To obtain insight into the possible effect of neddylation on p53 function, CHO-TS41 cells[170] that encode a temperature-sensitive APP-BP1 protein (subunit of the heterodimeric NEDD8 activating enzyme; Fig. 2) were used. When these cells are grown at the restrictive temperature (i.e., neddylation is switched off), the transcriptional activity of p53 is increased but also the ability of Mdm2 to interfere with p53 activity. While the latter observation cannot be readily explained, the former indicates that neddylation of p53 negatively affects the transcriptional properties of p53, though by a yet unknown mechanism.

FBXO11, an F-box protein acting as the substrate recognition component of a respective SCF complex, represents another NEDD8 ligase for p53.[171] FBXO11 promotes conjugation of NEDD8 to the abovementioned C-terminal lysine residues and in addition to K320 and K321 of p53 and inhibits the transcriptional activity of p53 supporting the notion that neddylation interferes with p53 function. K320 and K321 are part of the major nuclear localization sequence (NLS) of p53 (Fig. 1). The observation that K320 and K321 may represent neddylation sites of p53 suggests therefore the intriguing but purely speculative hypothesis that neddylation of these residues may control the subcellular localization of p53, for instance by masking the NLS.

Although FBXO11 is a subunit of an SCF complex, which normally act as ubiquitin ligases, FBXO11 was reported to promote neddylation but not ubiquitination of p53.[171] Thus, it will be interesting to determine if FBXO11-containing SCF complexes in general act as NEDD8 ligases rather than ubiquitin ligases or if this (FBXO11 promotes neddylation) represents a peculiarity of p53. Similarly, since Mdm2 can function as both ubiquitin ligase and NEDD8 ligase for p53, an important issue is how it is decided if p53 is neddylated or ubiquitinated by Mdm2. Finally, since recently a second NEDD8-conjugating enzyme has been discovered (Nce2/Ube2f),[167] it remains to be determined which of these enzymes is preferentially involved in p53 neddylation.

p53 AND SUMO-1

Similar to NEDD8, SUMO modification does not act as a direct recognition signal for the 26S proteasome but rather affects the ability of the modified proteins to interact with other proteins or molecules (in this context, it should be noted that sumoylated proteins have recently been shown to be recognized as substrates for ubiquitination and degradation by specific ubiquitin ligases).[172] All available data indicate that p53 is modified by SUMO-1 in a Ubc9-dependent manner providing an explanation for the observation that p53 is modified by a single SUMO moiety rather than by SUMO chains (unlike SUMO-2/3, SUMO-1 does not form chains). Furthermore, K386 of p53, which is presented in the context of the sumoylation consensus sequence ψKXD/E, represents the major attachment site for SUMO-1.[173,174] Members of the PIAS family of SUMO ligases were identified as interaction partners of p53 in yeast two-hybrid screens and subsequent biochemical and cell biological studies demonstrated that PIASy, PIAS1 and PIASxβ, respectively, can act as SUMO ligases for p53.[175-178]

Although it is commonly accepted that sumoylation affects the transcriptional properties of p53, it is still controversial if modification with SUMO stimulates or represses p53-mediated transcriptional transactivation. In the original studies reporting on p53 sumoylation, ectopic expression of Ubc9 or SUMO-1 resulted in increased p53 transcriptional activity.[173,174] In line with these findings, PIASy stimulates sumoylation of p53 and activates p53 transcriptional activity.[178] In contrast, expression of PIAS1 and PIASxβ, respectively, enhanced p53 sumoylation but resulted in repression of p53-mediated transcriptional activation.[177] A possible explanation for these apparent contradictory results, has recently been provided by a report studying the effect of sumoylation on the ability of p53 to bind to chromatin and activate transcription in a defined in vitro system.[179] The data obtained indicate that sumoylated p53 cannot bind to chromatin, unless it is acetylated by p300 prior to sumoylation. Furthermore, p53 that is bound to chromatin prior to sumoylation, can still be sumoylated but in this case, sumoylation does not dissociate the p53-DNA complex (i.e., only sumoylation of free forms of p53 interferes with chromatin binding). Thus, an attractive but purely speculative hypothesis is that the different PIAS proteins recognize different subpopulations of p53 as substrates and, thus, have different effects on the transcriptional activities of p53.

Analysis of the effect of the SUMO pathway on p53 function, however, is likely to be more complicated than discussed above, since Mdm2 and MdmX are also modified by SUMO and this modification may affect their ability to interfere with p53 function. For instance, the SUMO protease SUSP4 was reported to remove SUMO-1 from Mdm2 resulting in increased ubiquitination/degradation of Mdm2 and, in consequence, p53 accumulation.[180] This (loss of sumoylation results in Mdm2 destabilization and p53 activation) may also provide an explanation for the recent finding that in zebrafish, genetic inactivation of all SUMO genes results in p53 activation.[181] Finally, SUSP4 levels appear to increase upon UV-induced DNA damage, providing an example for the potential cooperation of ubiquitin family members in the activation of the p53 pathway.[180]

CONCLUSION

In the past two decades, p53 has become one of the most intensely studied proteins. In consequence of this interest, the factors and pathways reported either to be affected by p53 or to be involved in the regulation of p53 have become so numerous that one of the major challenges is to put all the available information into a comprehensive biological context. With respect to the modification of p53 by ubiquitin, a prominent issue is the startling finding that p53 can be potentially recognized as substrate by more than 10 different ubiquitin ligases. Are all of these interactions physiologically relevant? Although this may well be (e.g., at least some of these interactions will turn out to be specific for certain tissues and/or stages of development or differentiation or will be relevant only in response to certain environmental stimuli), it may not be possible to satisfactorily address this issue, at least with the tools and technologies currently available. Another pressing issue that will also be difficult to address for technical reasons is the delineation of the actual contribution of modification of certain lysine residues by ubiquitin, SUMO-1, or NEDD8 to the regulation of p53 activity. As discussed above, the dilemma in this respect is that the ε-amino group of a lysine residue does not only serve as attachment site for ubiquitin family proteins but also for other modifications including methylation and acetylation. Indeed, many of the lysines of p53 assumed to be ubiquitinated/neddylated/sumoylated represent also acetylation and/or methylation sites.[10] Finally, another interesting but probably more amenable aspect of modification of p53 by ubiquitin family proteins is the observation that Mdm2 can function as both ubiquitin ligase and NEDD8 ligase for p53. Thus, it will be interesting to see if mechanisms/factors exist that determine whether Mdm2 acts as a ubiquitin ligase or as a NEDD8 ligase for p53.

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CHAPTER 11

UBIQUITYLATION IN THE ERAD PATHWAY

Frederik Eisele, Antje Schäfer and Dieter H. Wolf*

Abstract: Ubiquitylation is a protein modification mechanism, which is found in a multitude of cellular processes like DNA repair and replication, cell signaling, intracellular trafficking and also, very prominently, in selective protein degradation. One specific protein degradation event in the cell concerns the elimination of misfolded proteins to prevent disastrous malfunctioning of cellular pathways. The most complex of these ubiquitylation dependent elimination pathways of misfolded proteins is associated with the endoplasmic reticulum (ER). Proteins, which enter the endoplasmic reticulum for secretion, are folded in this organelle and transported to their site of action. A rigid protein quality control check retains proteins in the endoplasmic reticulum, which fail to fold properly and sends them back to the cytosol for elimination by the proteasome. This requires crossing of the misfolded protein of the endoplasmic reticulum membrane and polyubiquitylation in the cytosol by the ubiquitin-activating, ubiquitin-conjugating and ubiquitin-ligating enzyme machinery.

Ubiquitylation is required for different steps of the ER-associated degradation process (ERAD). It facilitates efficient extraction of the ubiquitylated misfolded proteins from and out of the ER membrane by the Cdc48-Ufd1-Npl4 complex and thereby triggers their retro translocation to the cytosol. In addition, the modification with ubiquitin chains guarantees guidance, recognition and binding of the misfolded proteins to the proteasome in the cytosol for efficient degradation.

About 30% of all cellular proteins are secretory proteins, which enter the endoplasmatic reticulum (ER) for further distribution to their site of action. They pass the ER membrane in an unfolded state via a channel, the Sec61 translocon.

Upon entry into the ER the proteins are folded and undergo modifications as are glycosylation and disulfide bridge formation. After reaching their native conformation the proteins are allowed to leave the ER for further transport to their cellular location. The proper folding state of a protein is monitored by quality control systems of the ER,

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which finally recognize misfolded proteins and retain them in the ER. Subsequently they are retro-translocated out of the ER membrane via a channel comprising in some cases Sec61. After poly-ubiquitylation and removal from the ER membrane the misfolded proteins are guided to the proteasome where they are degraded.[1-7]

PROTEIN FOLDING, QUALITY CONTROL IN THE ER AND THE ERAD DEGRADATION SIGNAL

Directly after import of the polypeptide chain into the ER through the Sec61 translocon the Hsp70 chaperone Kar2/BiP (yeast/mammals; Table 1) binds to hydrophobic patches of the protein and the oligosaccharyl transferase (OST) complex links glycans of the structure $Glc_3Man_9GlcNAc_2$ covalently to asparagine residues located within an Asn-X-Ser/Thr motif (N-glycosylation) (Fig. 1). Glycans increase the hydrophilicity of proteins. At the same time the glycans play an important role in the folding process of proteins in the ER lumen.[8,9] During the Kar2/BiP assisted folding of the polypeptide chain, trimming of the carbohydrate chain occurs. One glucose residue is rapidly removed from the glycan chains by glucosidase I (Gls1) followed by removal of the second glucose residue by glucosidase II (Gls2) (Fig. 1). In mammalian cells the $Glc_1Man_9GlcNAc_2$ carrying protein then associates with ER resident lectin chaperones, the membrane bound calnexin and the soluble calreticulin. Upon release of the folding polypeptide from these chaperones, glucosidase II removes the innermost glucose, generating the $Man_9GlcNAc_2$ structure, which prevents association with both chaperones. Successfully folded proteins are allowed to leave the ER. However, some proteins require more time for folding. For this purpose an UDP-glucose: glycoprotein glucosyltransferase (UGGT) inspects the folding state of the $Man_9GlcNAc_2$ carrying protein and reglucosylates the terminal mannose of not yet properly folded proteins. Regeneration of the $Glc_1Man_9GlcNAc_2$ oligosaccharide leads to re-association with calnexin/calreticulin for an additional round of folding. Repeated calnexin-calreticulin cycles with the counteracting actions of UGGT and glucosidase II generates off-phases where the N-glycan is exposed to ER-resident α-1,2-mannosidases. At first trimming of the α-1,2 bounded mannose of the central oligosaccharide branch (B-branch) by the slow acting ER α-mannosidase I (Mns1) occurs.[10,11] Subsequently an α-1,2 bounded mannose residue of the C-branch is cleaved off by Htm1/Mnl1 (yeast) or EDEM (mammals) generating an α-1,6 terminal mannose providing the N-glycan degradation signal[12-14] (Fig. 1).

In yeast reglucosylation by UGGT and the calnexin/calreticulin cycle of binding of a folding protein does not exist, leaving only the time frame for protein folding until α-1,2 mannosidase cleaves off the mannose of the central, B-branch followed by removal of a mannose of the C-branch.

In addition, ER localized protein disulfide isomerase (PDI) activity or/and its chaperone function is required for retrotranslocation and degradation of misfolded proteins of the ER.[15] Of the five PDI family members in yeast, Pdi1 has been found to form an intermolecular disulfide bounded complex with Htm1/Mnl1.[12,16] Also one of the 19 PDI orthologs of mammals, the DnaJ domain containing oxido-reductase ERdj5 was found to interact with EDEM1, the mammalian ortholog of Htm1/Mnl1. Interestingly the DnaJ domain of ERdj5 contacts the ER lumenal Hsp70 chaperone BiP. Both, ERdj5 reductase activity and interaction between ERdj5, BiP and EDEM are required for efficient degradation of disulfide-bond containing ERAD substrates.[17]

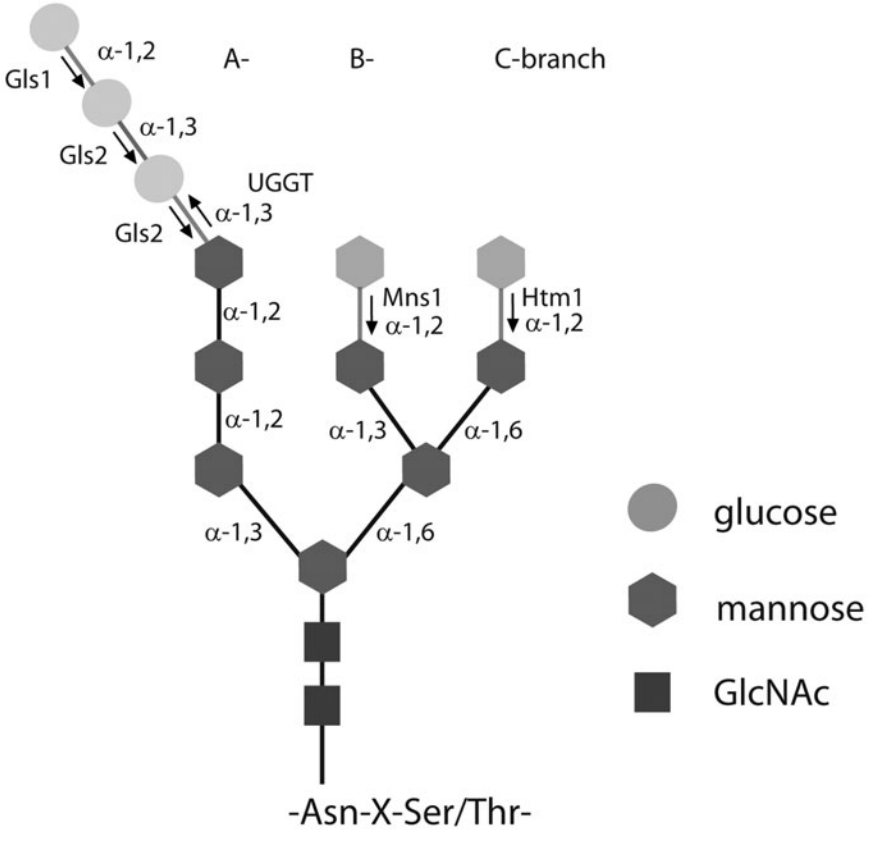

Figure 1. The N-linked core oligosaccharide structure of secretory proteins. Cleavage of the three glucose residues followed by trimming of the terminal mannose residue in the B-branch (indicated in orange) and subsequently the terminal mannose residue in the C-branch (indicated in orange) offers an α-1,6 linked mannose for recognition of the misfolded protein for elimination. A color version of this image is available at www.landesbioscience.com/curie.

The trimmed mannose glycan signal is interpreted by the glycan binding lectins Yos9 in yeast[18-20] and OS-9 and XTP-3B in mammalian cells.[21-23] These proteins have lectin-like domains with homology to the mannose-6-phosphate receptor family. Yeast Yos9 is linked to the Hrd3 protein, a Type 1 transmembrane protein with a large lumenal domain, which itself is connected to the Hrd1/Der3 ubiquitin ligase. Mammalian OS-9 and XTP-3B were found in complexes containing SEL1 and the E3 ligase HRD1, the orthologs of yeast Hrd3 and Hrd1/Der3, respectively.[21-25]

Earlier work had shown that the positioning of a carbohydrate chain on the misfolded protein is important for degradation.[26,27] This led to the detection of a bipartite signal for degradation of a misfolded protein: the trimmed carbohydrate and an exposed hydrophobic amino acid patch close to this carbohydrate chain.[28] This hydrophobic amino acid patch may be decoded by the Hrd3 (yeast)/Sel1 (mammals) proteins and/or the Hsp70 chaperone Kar2/BiP to initiate the elimination process.

UBIQUITYLATION AND DEGRADATION OF ER-LUMENAL SUBSTRATES: THE HRD-DER LIGASE COMPLEX

The detailed mechanism of ER associated ubiquitin-proteasome dependent degradation of a lumenal misfolded protein was first discovered by virtue of a mutated vacuolar (lysosomal) enzyme of yeast, carboxypeptidase yscY (CPY*).[29] The protein carries a Gly-Arg mutation at a highly conserved site of serine proteases,[30] is fully imported into the ER lumen, N-glycosylated, discovered as being misfolded, retrograde transported out of the ER, polyubiquitylated and degraded by the proteasome[29,31](Fig. 2). Polyubiquitylation occurs to a minor part by the soluble cytosolic ubiquitin-conjugating enzyme Ubc1 and by the ubiquitin-conjugating enzyme Ubc6, a tail anchored ER membrane protein with its active site reaching into the cytosol.[32] The main ubiquitin-conjugating enzyme of the ubiquitylation process of CPY* is represented by the ubiquitin-conjugating enzyme Ubc7.[29] Ubc7 is recruited to the ER membrane by the membrane anchor protein Cue1 which leads to its activation.[33]

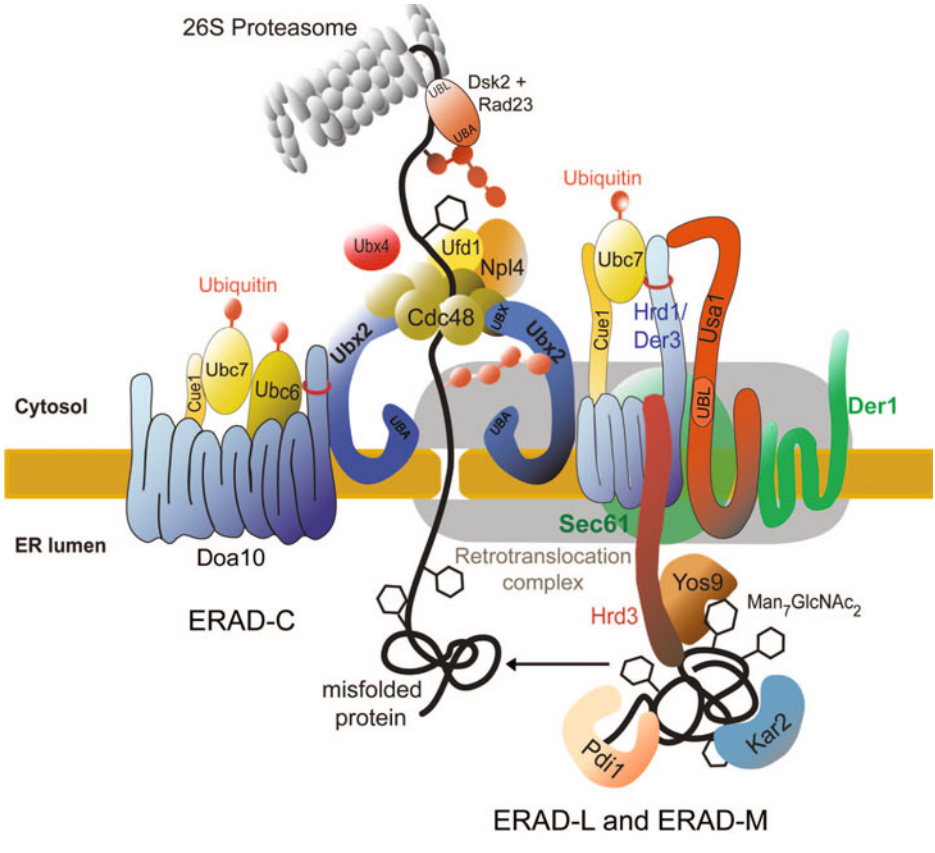

Figure 2. The ubiquitylation machineries of the ER for misfolded secretory proteins. The two ubiquitin ligation machineries of yeast consisting of the Hrd1/Der3 ligase and the Doa10 ligase merge with their polyubiquitylation activity at the AAA-ATPase complex Cdc48-Ufd1-Npl4, which initiates delivery of the polyubiquitylated ERAD substrates to the proteasome.

The ubiquitin ligase responsible for the polyubiquitylation process of CPY* turned out to be Der3,[34] a six times the ER membrane spanning protein.[35] Der3 exposes a RING (Really Interesting New Gene) finger motif into the cytoplasm, which is necessary for its activity.[35-37] The same enzyme was also found as Hrd1 in the regulated degradation process of the integral ER membrane protein HMG-CoA reductase[38] (Fig. 2). The Hrd1/Der3 ligase is also involved in the degradation of the misfolded ER membrane protein, Pdr5*, carrying a mutation in its ER lumenal domain[39] as well as in the degradation of Sec61-2,[34] a mutated translocation channel protein with a defect in an ER membrane segment.[40] Hrd1/Der3 is linked to Hrd3, a Type I transmembrane protein composed of a large N-terminal ER lumenal domain, a single transmembrane span and a short C-terminal cytosolic region.[41,42] Together with Yos9 acting as a gatekeeper, Hrd3 is thought to be responsible for handing over mannose trimmed Man_7-$GlcNAc_2$ containing misfolded proteins to the Hrd1/Der3 ligase for polyubiquitylation[12,13,18,20] (Fig. 2). Interestingly, an Hrd1/Der3-Hrd3 ligase complex without Yos9 was found which might be responsible for the delivery of lumenal, nonglycosylated proteins to degradation.[20] Cells lacking Hrd3 cannot degrade CPY*. A *HRD3* deletion leads to rapid digestion of the Hrd1/Der3 ligase. Most interestingly, however, over-expression of the Hrd1/Der3 ligase in the absence of Hrd3 leads to the recovery of CPY* degradation.[41] An Hrd-Der ligase complex of the ER membrane has been defined which is composed of the Hrd1/Der3 ubiquitin ligase with its interaction partner Hrd3, as well as Usa1, which connects the four transmembrane domain protein Der1 with the ligase[20,40,43,44] (Fig. 2). Usa1 has been described as a double spanning ER membrane protein with cytosolic N- and C-termini. At the N-terminus it possesses an ubiquitin-like (UBL) domain. A recent study uncovered that the C-terminus of Usa1 interacts with Der1, while the N-terminus directly contacts the Hrd1/Der3 ligase at its very C-terminus while the ligase itself stays in contact with Hrd3 and through this also with Yos9[45] (Fig. 2). Another study shows direct interaction of Usa1 to both Hrd3 and Hrd1/Der3.[46] Bridging of Der1 to the Hrd1/Der3 ligase via Usa1 is essential for the degradation of ER lumenal misfolded proteins. The N-terminus of Usa1 induces oligomerization of the Hrd1/Der3 ligase, necessary for the degradation of some misfolded ER membrane proteins, but not required for elimination of misfolded ER- lumenal proteins.[45] Interestingly, the mammalian orthologs of Der1, Derlin-1, Derlin-2 and Derlin-3 are required for efficient proteolysis of both, soluble and transmembrane ERAD substrates.[47-50]

Recently the translocon Sec61 has been shown to interact with central components of the Hrd-Der ligase complex indicating its participation in retrotranslocation of misfolded ERAD substrates with a lumenal misfolded domain.[51] This extends the Hrd-Der ligase complex to a retrotranslocation complex (RTC) (Fig. 2): The RTC connects retrotranslocation with polyubiquitylation. The finding of Sec61 biochemically interacting with components of the Hrd-Der ligase complex complements previous genetic studies which assigned a function of the translocon to degradation of ER-lumenal proteins.[2,41,51-53]

While the core components of the ligase complex Hrd1/Der3, Hrd3, Usa1 and Der1 are required for degradation of all soluble substrates with an ER lumenal misfolded domain (ERAD-L substrates, see above), both proteins Usa1 and Der1 were found to be dispensable for the elimination of Pdr5*, Sec61-2 and HMG-CoA reductase. All these proteins are characterized as ERAD-M substrates.[40] The polytopic membrane

Table 1. Mammalian orthologs of yeast proteins involved in ERAD

Yeast	Mammals	References
Kar2	BiP/Grp78	97-99
Yos9	OS-9, XTP-3B	18-23
Htm1/Mnl1	EDEM1, EDEM2, EDEM3	12,13,100
Der1	Derlin-1, Derlin-2, Derlin-3	43,44,48-50,101
Usa1	HERP	40,45
Hrd3	SEL1L	24,25,38
Hrd1/Der3	HRD1 (Synoviolin), Gp78	34,35,38,75,76,81,82
Doa10	TEB4 (MARCH-IV)	70,71
Ubc6	Ube2g1	29,102,103
Ubc7	Ube2g2	29,33,76,82,86
Ubx2/Sel1	KIAA0887?	61,62
Ubx4	TUG (ASPCR1/UBXD9)?	63,104
Cdc48	P97/VCP	56-60,101,105
Ufd1	UFD1	56,106,107
Npl4	NPL4	56,106,107
Dsk2	PLIC-1, PLIC-2	64,108
Rad23	hHR23A and B	64,109

substrate Pdr5* has a misfolded lumenal domain, which may extend into the membrane. Sec61-2 carries most likely a misfolded membrane section and HMG-CoA reductase undergoes intramembrane domain misfolding upon regulation by farnesol.[54] The fact that Usa1 was not required for degradation of these membrane substrates as published in Carvalho et al, 2006[40] is in contrast to the results of Horn et al, 2009.[45] The latter authors attribute the necessity of oligomerization of the Hrd1/Der3 ligase by Usa1 to its potential to degrade membrane substrates. For the recognition of misfolded ERAD-M substrates specific hydrophilic amino acid residues within the multi-membrane spans of the Hrd1/Der3 ubiquitin ligase are required.[55]

In the cytosol, the homohexameric AAA-ATPase Cdc48p (p97 in mammals) and its substrate recruiting factors Ufd1 and Npl4 provide the driving force for final extraction of polyubiquitylated misfolded proteins from the ER membrane.[56-60] Ubx2, an ER membrane protein with two membrane spans enables the binding of the Cdc48 complex to the retrotranslocation complex. Its cytoplasmic N-terminal ubiquitin associated (UBA) domain is important for binding to ubiquitylated ERAD substrates while a C-terminal UBX (ubiquitin-regulatory X) domain is necessary for recruiting the Cdc48 complex to the ER membrane.[61,62] The UBX domain containing protein Ubx4 modulates the Cdc48-Ufd1-Npl4 complex loaded with polyubiquitylated proteins to guarantee its correct function.[63] In the cytosol the two UBA-UBL domain ubiquitin receptor proteins Dsk2 and Rad23 are required for further delivery of polyubiquitylated proteins to the proteasome[64,65] (Fig. 2).

A proteasome bound E4 ligase, Hul5[66] was found to be required for degradation of the ERAD substrate CTL*, a CPY* fusion protein spanning the ER membrane and containing the enzyme isopropylmalate dehydrogenase at the cytoplasmic side of the ER. It may be involved in the extension of the ubiquitin chain of the substrate.[67]

UBIQUITYLATION AND DEGRADATION OF ER SUBSTRATES CONTAINING A MISFOLDED CYTOPLASMIC DOMAIN: THE UBIQUITIN LIGASE DOA10

ER proteins carrying cytosolic misfolded domains as degradation signals (ERAD-C substrates in yeast) are degraded by the ER membrane located ubiquitin ligase Doa10[40,68,69] (Fig. 2). Doa10 (degradation of alpha2) was found in a screen for factors involved in degradation of proteins containing the Deg1 domain of the soluble short-lived transcriptional repressor Matα2.[70] Doa10 is a 151 kDa ER/nuclear envelope protein with 14 transmembrane domains and a N-terminal RING-finger.[71] No additional helper proteins of this ubiquitin ligase are known. The enzyme works together with the E2 enzymes Ubc6 and Ubc7. While Ubc6 contains a transmembrane domain and is therefore linked to the ER, the enzyme Ubc7 is recruited to the ER membrane by Cue1[33] (see chapter by Xirodimas and Scheffner in this volume). The requirement of membrane substrates for polyubiquitylation by Doa10 is often not absolute: In addition to Doa10 also the Hrd1/Der3 ligase is often involved in the degradation process.[68,72] The ubiquitylation function of Doa10 is not only limited to ERAD substrates.[69,70] Also mutated nuclear envelope proteins, soluble nuclear proteins, as well as synthetic cytoplasmic proteins fused to the Deg1 domain[73] or to another degron called CL1[74] are substrates of Doa10. The ERAD-C pathway using the ubiquitin ligase Doa10 and the ERAD-L and ERAD-M pathways, which make use of the Hrd1/Der3 ubiquitin ligase, merge at the Cdc48-Ufd1-Npl4 complex segregating the polyubiquitylated substrates from the ER membrane for further delivery to the proteasome (see previous paragraph; Fig. 2).

MAMMALIAN E3S INVOLVED IN ERAD

Due to the easy amenability to biochemical, genetic and molecular biological methods the yeast *Saccharomyces cerevisiae* has been the model and a pacemaker in the elucidation of the mechanisms of polyubiquitylation in the ERAD pathway. Several E3 ligases being involved in ERAD have been described in mammalian cells but in many cases little is known about their substrates and their reaction mechanism.

Two structural orthologs of the yeast Der3/Hrd1 ligases are known: HRD1 (or Synoviolin) and gp78 (also known as RNF45 or AMFR; Table 1).

HRD1 has been described as an ortholog of yeast Hrd1/Der3.[75] The enzyme is known to function together with the E2 Ube2g2 in vitro but no conjugating enzyme working together with HRD1 in vivo has been described yet.[76] It is involved in the degradation process of the ERAD substrates TCR-α, CD3-δ,[76] unassembled Igμ chains[77] and a nonglycosylated variant of the Igκ light chain.[78] Also cytosolic proteins like serum- and glucocorticoid-induced kinase 1 (Sgk1)[79] or tumor suppressor gene p53[80] were shown to be ubiquitylated via HRD1.

gp78 was the first E3 ligase found in the ER membrane of mammals.[81] In comparison to HRD1 it possesses a G2BR (UBE2G2-binding region) that enables the enzyme to recruit the ubiquitin-conjugating enzyme UBE2G2.[82] As in the case of HRD1, substrates of gp78 are the unassembled subunits of the T-cell receptor TCR-α and CD3-δ.[81,82] In addition, gp78 seems to be the mammalian E3 that is able to ubiquitylate HMG-CoA reductase in a sterol regulated fashion.[83] Two recent studies showed that HRD1 targets gp78 for

ubiquitin-proteasome dependent degradation.[84,85] Fang and coworkers also proposed a role of gp78 in the degradation of the mutant form of cystic fibrosis transmembrane conductance regulator (CFTRΔF508). Silencing of gp78 leads to accumulation of CFTRΔF508.[85]

TEB4 (or MARCH VI) is a mammalian protein with homology to yeast Doa10.[71,86] It is a multi membrane spanning protein of the ER with a RING finger domain. TEB4 was shown to be able to auto-ubiquitylate with the help of Ube2g2, by this inducing its own degradation.[86] A recent study revealed that TEB4 is involved in ubiquitylation of Type 2 iodotyronine deiodinase (D2), which is the key thyroid hormone-acivating deiodinase.[87] This enzyme was also shown to be ubiquitylated by a SOCS-box containing ligase called WSB-1,[88] suggesting tissue specific and expression dependent parallel pathways of ubiquitylation.

Trc8 is another ER membrane RING finger containing ubiquitin ligase, which was originally identified as a tumor suppressor associated with hereditary renal cell carcinoma.[89] In addition the enzyme has sterol-sensing capacity.[90] Recently it was shown that the US2 and US11 proteins of human cytomegalovirus trigger Trc8 to ubiquitylate the major histocompatibility complex class I (MHC I) receptor leading to its dislocation and degradation by the 26S proteasome.[91] With this mechanism cytomegalovirus misuses the ERAD system and Trc8 to reduce the overall abundance of MHC class I receptors on the cell surface to escape from immune response.[92]

A recent study revealed Rfp2 to be an additional ERAD ligase. The Rfp2 gene is reported to be frequently lost in various malignancies including subtypes of lymphoma, myeloma and several solid tumors making it a tumor suppressor gene candidate. Rfp2 is localized to the ER via a C-terminal transmembrane domain. It contains a RING domain and was shown to ubiquitylate the heterologously expressed proteolytic substrate CD3-δ and showed autoubiquitylation activity in vitro.[93] Native substrates of this ligase have not yet been described.

In addition, there are E3 ligases involved in ERAD of mammalian cells that are not ER membrane proteins but located in the cytosol. An example is the CHIP ligase which cooperates with membrane-bound RMA1 (RNF5) to target CFTRΔF508 for degradation via ERAD.[47] RMA1 was also shown to act upstream of gp78 in ubiquitylation of CFTRΔF508 suggesting that gp78 has an E4-like activity in this process.[94]

An additional example of such a cytosolic ligase is the two RING finger and cysteine-rich In-Between-RING fingers (IBR) region containing protein Parkin. A mutated version of Parkin is one of the main reasons for hereditary Parkinson's disease. The protein acts as an ubqiuitin ligase for polyubiquitylation of the Parkin-associated endothelin receptor- like receptor (Pael-Receptor).[95] This receptor is polyubiquitylated by HRD1 as well, suggesting that these two ligases, Parkin and HRD1, function in a common pathway.[96]

CONCLUSION

The different ubiquitylation systems used by the ER to remove misfolded proteins of the lumen and the membrane reflect the different tasks of recognizing the multitude of misfolded proteins with their many different misfolded domains on different sides of the ER to be able to finally send them to the proteasome. Here our understanding of the recognition processes is still very limited and requires intensive further research.

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CHAPTER 12

PUPYLATION

A Signal for Proteasomal Degradation in *Mycobacterium tuberculosis*

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Abstract: This chapter describes the identification of the first *p*rokaryotic *u*biquitin-like *p*rotein modifier, Pup, which covalently attaches to proteins to target them for destruction by a bacterial proteasome in a manner akin to ubiquitin in eukaryotes. Despite using a proteasome as the end point for proteolysis, Pup and ubiquitin differ in sequence, structure and method of activation and conjugation to protein substrates. Pup is so far the only known posttranslational protein modifier in prokaryotes and its discovery opens the door to the possibility that others are present not only for proteolysis, but also to regulate protein function or localization. Here, we discuss the putative mechanism of activation and conjugation of Pup (termed "pupylation") to target proteins. In addition, because it is unclear whether or not Pup, like ubiquitin, is recycled or degraded during substrate targeting to the proteasome, we propose methods that may identify Pup deconjugation enzymes ("depupylases"). Finally, we outline future directions for Pup research and anti-tuberculosis drug discovery.

INTRODUCTION

Unlike eukaryotes, prokaryotes lack well-defined sub-cellular compartments and therefore have additional requirements for the specificity and regulation of proteolysis. Bacterial ATP-dependent proteases, including ClpP, ClpQ (HslV), Lon and FtsH, provide "mini-compartments" or "barrel-shaped proteases" that tightly regulate the entrance of proteins into chambers enclosing proteolytic active sites (reviewed in refs. 1,2). In some cases bacteria also encode proteasomes that have high structural and chemical similarity

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to eukaryotic proteasomes.[3] As with eukaryotic proteasomes, bacterial proteasomes are likely to form a complex with AAA or AAA+ ATPases (*A*TPases *a*ssociated with a variety of cellular *a*ctivities), which serve as regulatory subunits that recognize, unfold and translocate protein substrates into the proteasome core.

Proteasomes are encoded in all sequenced Archaea but limited to bacteria of the order Actinomycetales, which includes the genera *Streptomyces*, *Rhodococcus*, *Frankia* and *Mycobacterium*.[4] Numerous studies of prokaryotic proteasomes were undertaken with the hope that these proteases would provide a simplified model system for understanding proteolysis by complex eukaryotic proteasomes. Bacterial core particles (CPs) share sequence, structural and functional similarity with eukaryotic CPs. Like eukaryotic 20S CPs, bacterial proteasome CPs are barrel-shaped proteases with 14 alpha (α, PrcA) and 14 beta (β, PrcB) subunits with amino (N)-terminal threonines residing in the β-subunits that provide the catalytic active site nucleophiles (reviewed in ref. 5). Unlike eukaryotic proteasomes, bacterial CPs are usually composed of homo-heptameric rings of α subunits and β subunits. With at least one exception,[6] the presence of one type of β-subunit appears to limit prokaryotic proteasomes to having only chymotryptic activity. Proteasome protease activity has been reconstituted in several Actinomycetales in vitro, however, these studies were carried out with model peptide substrates and not with native proteins, suggesting the need for additional factors for full proteasome function.[6-9]

Putative proteasome-associated genes colocalize with proteasome CP genes in Actinobacteria (Fig. 1A). These genes were initially identified based on comparisons with the genomic region of other proteasome-containing bacteria. Whereas prokaryotic proteasome core subunits were identified based on sequence homology to their eukaryotic counterparts,[10] most of the putative proteasome-associated genes in the vicinity of the proteasome genes do not share any similarity with those found in eukaryotes. One exception is *arc* (*A*AA ATPase forming *r*ing-shaped *c*omplexes), a gene encoding an AAA ATPase with homology to those found in the 19S regulatory particle in eukaryotes (Fig. 1A).[11] Several biochemical studies demonstrated that this protein from *Rhodococcus erythropolis* formed hexameric or dodecameric rings with ATPase activity.[11,12] ARC could not form stable or robust interactions with bacterial 20S CPs in vitro, nor could they stimulate protein degradation by 20S CPs. This suggested that the interactions between ARC and CPs were either transient or required additional cofactors. Despite advances in its characterization, the function of proteasomes in *Rhodococcus* is not well understood, as neither *arc* nor proteasome mutants have been characterized in this bacterium.

mpa (*Mycobacterium p*roteasomal *A*TPase) is an orthologue of *Rhodococcus arc* in *M. tuberculosis* (Mtb). Mutants of this gene were identified in a screen for transposon disruption mutants sensitive to nitric oxide, an anti-microbial molecule made by activated macrophages.[13] Mutations in *pafA* (*p*roteasome *a*ccessory *f*actor A), another open reading frame near the *prcBA* genes, resulted in a similar phenotype to the *mpa* mutants. PafA was thought to participate in proteasome function because it is usually encoded near proteasome CP genes.[14] Two-dimensional protein gel analysis revealed that two proteins, FabD (malonyl coA-acyl carrier protein transacylase) and PanB (ketopantoate hydroxymethyltransferase), displayed altered steady state levels in an *mpa* mutant compared to wild type. This phenotype was also produced by treating wild type Mtb with eukaryotic proteasome inhibitors.[15] Ectopic expression of *fabD* and *panB* using a strong, nonnative promoter demonstrated that the over-produced protein accumulated in the *mpa* and *pafA* Mtb mutants as well as in proteasome inhibitor treated wild type Mtb, but not in

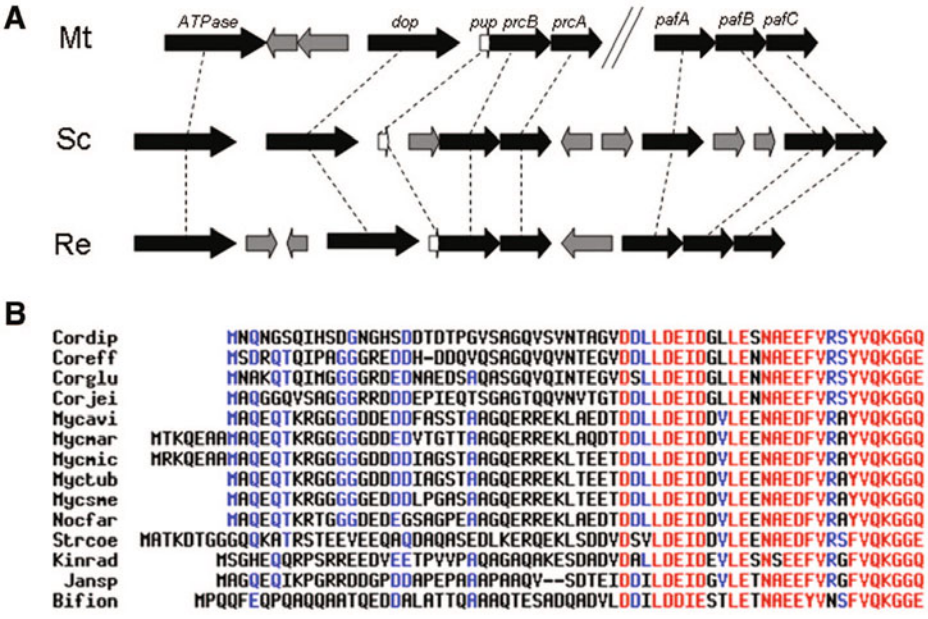

Figure 1. Proteasome-associated genes are present in bacteria of the order *Actinomycetales*. A) Genomic organization of putative proteasome genes in *M. tuberculosis* (Mt), *Streptomyces coelicolor* (Sc) and *Rhodococcus erythropolis* (Re). Proteasome associated genes are in black; homologues are connected by dashed lines. Figure is adapted from Figure 4 in reference 9. B) Alignment of Pup from various Actinomycetales reveals a striking conservation in amino acid sequence at the C-terminus. Identical and similar amino acids are in red and blue, respectively.

the untreated wild type strain. Although these data strengthened the association of Mpa and PafA with proteasome function, neither had been shown to directly interact with the proteasome CPs or the degradation substrates. Thus it remained to be determined how Mpa and PafA targeted proteins like FabD and PanB for proteasomal degradation.

DISCOVERY OF A BACTERIAL "UBIQUITIN-LIKE" MODIFIER

Although the proteasome, putative proteasome-associated proteins and endogenous substrates had been identified in bacteria, it was unclear how proteins were targeted for degradation by this machinery since ubiquitin-like modifiers had not been identified in prokaryotes. The combination of bacterial 20S CPs and Mpa in vitro did not facilitate the degradation of FabD or PanB (M. Pearce, K.H. Darwin, unpublished). In an effort to understand how proteins were targeted to the proteasome, Pearce and coworkers used an *E. coli* bacterial two-hybrid system screen to identify Mtb proteins that interact with Mpa. Rv2111c, a 64 amino acid protein of unknown function encoded directly upstream the proteasome CP genes, was identified in this screen.[16] The addition of purified Rv2111c to the in vitro system, however, failed to stimulate degradation. Furthermore, expression of recombinant Mtb *PrcBA*, *Mpa* and Rv2111c in *E. coli* failed to degrade FabD (K.H. Darwin, unpublished).

It was possible that additional proteins specific to proteasome-bearing bacteria were required for proteolysis. The development of a mycobacterial two-hybrid system allowed this hypothesis to be tested by looking for interactions between proteasome components and degradation substrates in the proteasome-bearing bacterium *M. smegmatis*, a nonpathogenic relative of Mtb.[17] A positive interaction was detected between the substrate FabD and Rv2111c, a result that was confirmed in a pull down experiment from mycobacterial lysates. Surprisingly, FabD and Rv2111c were isolated as a covalently linked complex, where Rv2111c formed an isopeptide bond between its carboxyl (C) terminus and the ε-amino group of a specific lysine (Lys173) in FabD. Mutagenesis of FabD's modified Lys dramatically stabilized this substrate in wild type mycobacteria.[16] In addition, pulse-chase analysis also showed that proteins modified with Rv2111c had longer half-lives in an *mpa* mutant of *M. smegmatis*. The modification of FabD with Rv2111c was reminiscent of ubiquitylation of proteasome substrates in eukaryotes, thus Rv2111c was named *p*rokaryotic *u*biquitin-like *p*rotein (Pup).

In a separate study, Burns and colleagues independently noticed that *pup* encoded a small protein with a di-glycine motif at the penultimate position of the C-terminus, followed by either glutamine (Gln) or glutamate (Glu) (depending on the organism) (Fig. 1B).[18] They speculated that Pup could covalently attach to bacterial proteasome substrates, despite the lack of overall sequence homology to ubiquitin. Using epitope-tagged Pup from *M. smegmatis*, two covalently linked proteins, super oxide dismutase (SodA) and myo-inositol-1-phosphate synthase (Ino1), were identified. Burns and coworkers also showed that several pupylated proteins were more stable in a proteasome CP mutant when compared to wild type *M. smegmatis*,[18] consistent with the Pearce et al study.[16]

By analogy with ubiquitin processing and activation,[19] it was hypothesized that the C-terminal residue of Pup is removed to expose the di-glycine (Gly-Gly) motif for activation by an E1-like enzyme. Mass spectrometry analysis revealed that this is not the case. Not only had Pup retained its C-terminal amino acid upon conjugation to its substrates, it was shown that the C-terminal Gln was deamidated, converting it to a Glu.[16,18] Deletion of the C-terminal Glu or penultimate Gly abrogated pupylation.[18] When unconjugated Pup was purified from mycobacteria and then analyzed by mass spectrometry, nearly all molecules were deamidated ("PupGGE"); in sharp contrast, the majority of Pup purified from *E. coli* ended in Gln ("PupGGQ").[16] This result suggested that a specific activity is present in mycobacteria that deamidates Pup prior to covalent attachment to substrate proteins. Alternatively, this result may indicate that Pup-target complexes are hydrolyzed, releasing PupGGE for recycling. These studies showed that proteasome substrates are posttranslationally modified with Pup, which is first processed at the C-terminus in a manner different than the proteolytic processing of ubiquitin and therefore likely requires a different activation pathway for conjugation onto substrates.

PUP CONJUGATION ("PUPYLATION")

Pup appears to be deamidated in *Mycobacteria* prior to conjugation to target proteins. Striebel and coworkers confirmed this observation and showed that the reaction was catalyzed by Dop (*d*eamidase *o*f *P*up), which is encoded upstream of *pup* in several bacterial genomes and is highly similar to PafA (Figs. 1A and 2).[20] Dop shares no homology to ubiquitin-activating enzymes (E1) or ligases (E2, E3). Bioinformatics analysis suggests structural homology to the glutamine synthetase fold family, with Dop and PafA most

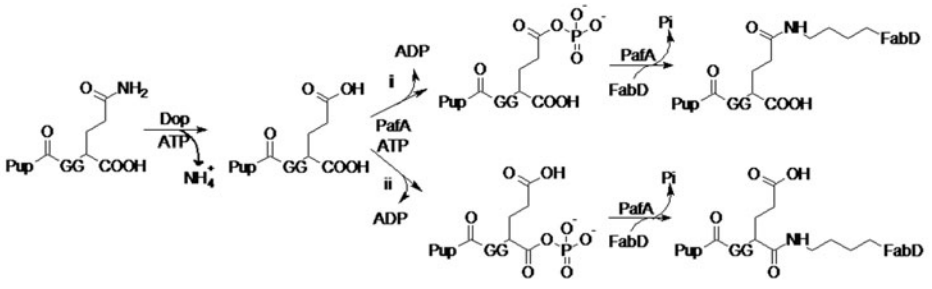

Figure 2. Pup conjugation. Following a deamidation reaction catalyzed by Dop, Pup is conjugated to a substrate (e.g., FabD) via an isopeptide linkage between a Lys of the substrate and either the C-terminal side chain carboxylate of Glu (i) or the C-terminal backbone carboxylate (ii). The ligation reaction is catalyzed by PafA and is ATP-dependent. Ligation is shown through the γ-carboxylate of Glu for simplicity.

likely belonging to the carboxylate-amine/ammonia ligase super family, similar to γ-glutamyl-cysteine synthetases.[21] This family of enzymes catalyzes ligation reactions involving phosphorylation of a carboxylate group followed by ligation of an amino group, resulting in an amide linkage. The deamidation reaction generates ammonia and ATP is not hydrolyzed during the reaction but serves as a cofactor.[20] The deamidation step may serve as a regulatory mechanism in organisms where Pup terminates in Gln. It is furthermore of note that bacteria encoding PupGGE have retained the *dop* gene, possibly suggesting roles in addition to deamidation for Dop.

The Mtb *pafA* mutant, which had previously been shown to have a defect in proteasome function,[13] was unable to pupylate target proteins and was thereby implicated in the activation and/or conjugation of Pup to substrates.[16] PafA was shown to catalyze the conjugation of Pup to a known proteasome substrate, FabD, in the presence of ATP and Dop (Fig. 2).[20] It is unclear which C-terminal carboxylate (the backbone carboxylate or the γ-carboxylate on the Glu) is conjugated to substrates (Fig. 2). PupGGE is a substrate for PafA-catalyzed conjugation in the absence of Dop, suggesting deamidation precedes conjugation and that Dop and PafA are not necessarily coupled. ATP is hydrolyzed during the course of the reaction and one molecule of ADP is generated per molecule of conjugated Pup.[20] These data suggest that the PafA-catalyzed ligation reaction proceeds through a phosphorylated intermediate (Fig. 2) as hypothesized by bioinformatics analysis,[21] although this intermediate has yet to be detected.

Both in vitro as well as in vivo experiments indicate that a single Pup moiety is conjugated onto a particular Lys residue on a target; chains of Pup have not been observed.[16,18,20] Because Pup has three Lys, it is quite possible that "polypupylation" occurs. In addition, a single substrate may have multiple pupylated Lys.

More than 600 distinct mammalian proteins are thought to be involved in the ligation of ubiquitin to substrates.[22] It is the multitude, diversity and combination of these ubiquitin ligases that allows a variety of substrates to be ubiquitylated in a specific and regulated manner. In Mtb, a *pafA* mutation abrogates pupylation,[16] raising the obvious question as to how pupylation is regulated. Preliminary data suggest that there are potentially up to 155 pupylation targets in *M. smegmatis* (J. Watrous, P. Dorrestein, unpublished) and over 600 in Mtb (F. McAllister, J. Mintseris, S. Gygi, unpublished). The number and diversity of putative pupylation targets suggest the requirement for additional factors to

accommodate selective protein targeting through pupylation. Furthermore, the ligation of Pup catalyzed by PafA on FabD in vitro was slow (17 h),[20] perhaps suggesting the requirement of additional cofactors required for optimal pupylation.

PUP DECONJUGATION ("DEPUPYLATION")

Unlike ubiquitin and other ubiquitin-related modifiers, the processing of de novo synthesized Pup prior to substrate ligation may not require a Pup protease ("depupylase") activity. In addition, although there is no evidence for poly-Pup chains that would necessitate a depupylating activity, the recycling of Pup from substrates would provide an energy efficient means of protein degradation and regulation. There are no homologues of eukaryotic DUBs (*de**ub*iquitinating protease) or ULPs (*u*biquitin-*l*ike specific *p*rotease) present in the vicinity of proteasome genes in bacteria and it is unknown if Pup is deconjugated prior to target degradation, or if it is simply degraded in the process.

Pup attaches to a target via an isopeptide bond, most likely at the γ-carboxylate position of the C-terminal glutamate. One would anticipate putative depupylases to be proteases that can recognize and hydrolyze the amide bond between Pup and Lys residues of target proteins. Hydrolysis by a depupylase then results in a PupGGE sequence, primed for additional substrate conjugation reactions (Fig. 3A). Alternatively, a transamidation reaction with ammonia would regenerate PupGGQ (Fig. 3A), which would require deamidation by Dop prior to conjugation to substrates. Both the peptidase and transamidase reactions could in theory proceed through a protein-substrate

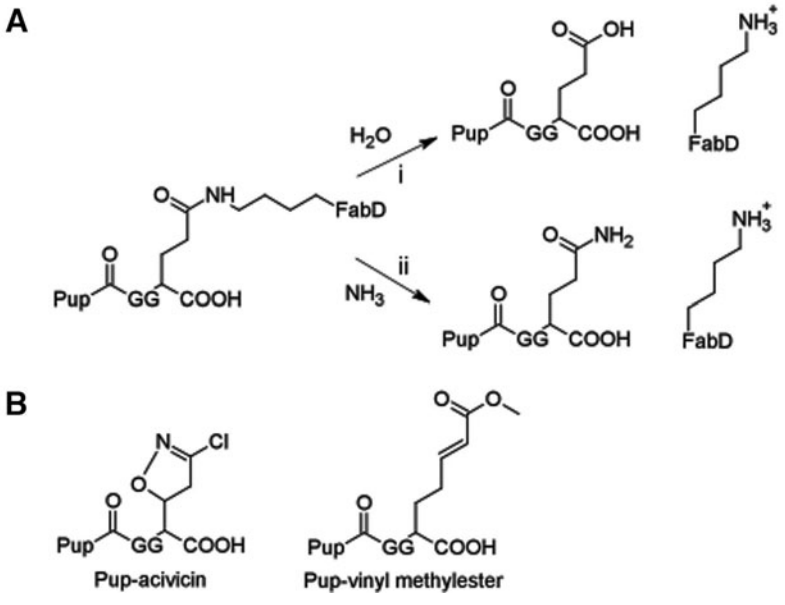

Figure 3. A depupylase? A) Proposed depupylase reaction by either hydrolysis (i) or transamidation (ii). B) Possible probes to test and trap putative depupylase activity.

intermediate complex that can be exploited for developing activity-based probes to trap depupylases, if present. The probes would be designed to crosslink the depupylases with Pup, similar to those used for the general identification of DUBs and ULPs that belong to the cysteine family of proteases.[23,24] An example of such a probe would be Pup vinyl methylesters (Fig. 3B), analogous to the ubiquitin vinyl methylester probe generated to trap DUBs, which has been instrumental to the identification of numerous novel DUBs, including novel DUB families.[24] Alternatively, Pup could be modified at the C-terminus with acivicin or DON (6-diazo-5-oxo-L-norleucine) (Fig. 3B), known traps for deamidases.[25,26]

Due to the similarity in chemistry proposed for depupylation and deamidation (Fig. 2 and Fig. 3A), it is possible that these probes will react with Dop. As mentioned previously, bioinformatics analysis suggests that organisms that naturally encode PupGGE instead of PupGGQ also have *dop*, suggesting a possible depupylase role for Dop in addition to deamidation. Whether or not Dop can serve as a depupylase, its observed deamidation activity should enable it to react with one or more of the proposed probes.

Over 100 DUBs have been identified in mammalian cells,[27] with functions ranging from recycling ubiquitin prior to target degradation by the proteasome to rescuing proteins from degradation.[28] Some DUBs simply bind ubiquitin with high affinity. Certain DUBs, however, do not function in concert with the proteasome, as extensively discussed in this book. Although it is currently unknown whether depupylases exist in bacteria, it is imperative to investigate whether such proteins, if identified, play a more sophisticated role in protein homeostasis, similar to their eukaryotic counterparts. Thus, in addition to trapping proteasome-associated depupylases, the probes highlighted in Fig. 3B may trap proteasome-independent depupylases.

CONCLUSION AND FUTURE PERSPECTIVES

Pup posttranslationally tags proteins for degradation by the proteasome and it is the only currently known prokaryotic protein that is functionally similar to ubiquitin. Despite the functional homology, Pup differs from ubiquitin in many other aspects. With little sequence and no structural homology to ubiquitin, Pup is first deamidated by Dop and subsequently conjugated to a variety of substrates by PafA, two proteins highly similar to each other but bearing no resemblance to eukaryotic ubiquitin/proteasome-associated proteins (Fig. 4). Similar to ubiquitin, pupylation is through a C-terminal carboxylate to substrate lysines. Pupylation dooms proteins to the proteasome for destruction, however, additional roles for pupylation cannot be ruled out.

Many aspects of the Pup-proteasome system remain to be resolved. How is Pup recognized by the proteasome system in bacteria? Pup is an intrinsically disordered protein,[29,30] very different from the highly structured ubiquitin.[31] Pup binds Mpa,[16,20,29,30] most likely to target proteins for proteasomal degradation, but detecting interactions between Mpa and the 20S proteasome have been elusive in prokaryotes. It is unclear if this interaction is transient or whether additional factors are involved in directing substrate specificity at the proteasome. Pup also stably binds to Dop and PafA[20] (F. Cerda, K.H. Darwin, unpublished), however, similar to Mpa, it is unclear what these associations mean in the context of the proteasome. It is intriguing to hypothesize the presence a Pup interaction motif, analogous to the ubiquitin interacting motifs, whereby

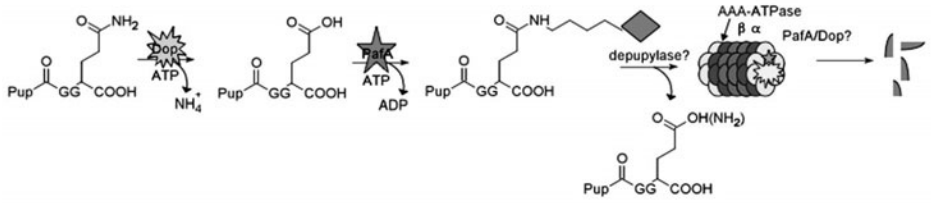

Figure 4. Model of the Pup-proteasome system. Following deamidation by Dop, Pup is ligated to substrate Lys via an isopeptide linkage to the C-terminal carboxylate of Glu. Ligation is shown on the γ-carboxylate for simplicity. Pup interacts with the AAA-ATPase Mpa, which presumably unfolds substrates for delivery into the catalytic chamber of the proteasome core for degradation. It is currently unknown whether additional factors are required for optimal conjugation and delivery to the proteasome, if PafA and Dop interact with the proteasome, or if Pup is recycled or degraded.

Pup becomes ordered upon binding distinct motifs on proteasomal components. Due to the lack of structural information for Dop and PafA, we are unsure whether this motif exists. The presence of a Pup interaction motif may help guide the identification of additional proteasome components, including potential depupylases, regulators and specificity factors.

It should be mentioned that various organisms have only parts of the Pup-proteasome system. For example in Archaea, proteasome-dependent proteolysis has been demonstrated in vitro and proteolysis was stimulated by the presence of the AAA+ ATPase PAN (*p*roteasome *a*ctivating *n*ucleotidase).[32] Archaea, however, do not have homologues of Pup or bacterial proteasome-associated proteins such as PafA. The PAN-proteasome complex may serve as a general protease in Archaea, similar to Clp proteases in bacteria. In addition, *Corynebacteria* encode homologues of proteasome-associated proteins, including PafA, Dop, AAA ATPase and Pup, however, proteasomes are absent from these organisms. It is unknown whether *Corynebacteria* Pup is able to conjugate proteins and if so, what purpose it serves. It is intriguing to hypothesize a signaling or regulatory role for pupylation in the absence of a proteasome.

Although the chemistries of the ubiquitylation and pupylation systems differ, many of the principles and techniques used to study the ubiquitin-proteasome system can be applied to unravel the Pup-proteasome system. Similar to the ubiquitin system, we will not only begin to understand key players involved in target recognition, but also the significance of the Pup signal in bacterial physiology and disease, opening novel options for therapeutic intervention of Mtb. The Pup-proteasome system is essential for the pathogenesis of Mtb, one of the most deadly bacterial pathogens in the world (WHO; http://www.who.int/en). Thus the identification of players in this pathway may also provide ideal drug targets for the development of novel tuberculosis chemotherapies.

ACKNOWLEDGEMENTS

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CHAPTER 13

SUMO CONTROL

Katharina Maderböck and Andrea Pichler*

Abstract: Sumoylation, the covalent attachment of SUMO peptide to cellular proteins, is an essential regulator of protein function involved in a wide range of cellular events. Deregulation of the SUMO pathway is implicated in the pathogenesis of several diseases, so it is important to understand how this system is controlled. Sumoylation is a highly dynamic regulatory mechanism, involving an energy dependent enzyme cascade for conjugation and another set of enzymes for deconjugation. In this chapter we will highlight the different mechanisms controlling the SUMO system.

INTRODUCTION

Posttranslational modification by SUMO (small ubiquitin related modifier) is an effective means to reversibly regulate protein function. Like other protein modifications, sumoylation is essential for the dynamic control of cellular processes and the rapid reaction to environmental changes without de novo protein synthesis. Sumoylation plays a role in most cellular events including transcriptional regulation, chromatin structure, DNA repair, nuclear transport, signal transduction and protein degradation, reviewed in refs. 1-4.

SUMO is a small protein, about 11 kDa in size and is ubiquitously expressed in all eukaryotic organisms, but absent from eubacteria and archea. Less complex eukaryotes have a single SUMO protein whereas plants and vertebrates express several SUMO variants (e.g., ref. 5). All SUMO members are expressed as precursor proteins and require maturation prior to conjugation. Mammals have four SUMO paralogs with SUMO2 and 3 being nearly identical. In contrast to the ubiquitously expressed SUMO1-3 members, SUMO4 expression is restricted to the kidney, spleen and lymph nodes.[6,7] Surprisingly, loss of SUMO1 in mice is not lethal and can be compensated by SUMO2/3 variants,[8] which is consistent with partially overlapping substrates for SUMO1 and SUMO2.[9]

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The covalent substrate linkage is an isopeptide bond between the SUMO C-terminal glycine and the ε-amino group of the target substrate lysine often embedded in a SUMO consensus motif (ΨKxE/D, Ψ is a bulky aliphatic amino acid and x can be any residue). The structural context of a SUMO consensus motif is important since it can only be recognized by the sumoylation machinery when present in an extended loop or unstructured area[10] but not in compact helical structures.[11] Two extended variants of this motif have been described, which both have negatively charged residues downstream of the SUMO consensus motif: the phosphorylation dependent SUMO motif (PDSM) consists of a phosphorylated serine and proline (ΨKxE/DxxSP)[12] whereas the negatively charged SUMO motif (NDSM) contains additional acidic residues.[13]

Besides covalent modification, SUMO can also regulate protein function by noncovalent interaction via a so-called SUMO interaction motif (SIM) or SUMO binding motif (SBM) in the target protein.[14-18] Such a motif is a short hydrophobic stretch which is often flanked by an acidic region that adds a β-strand to SUMOs β-sheet. There is increasing evidence that some SIMs discriminate between SUMO variants, emphasizing their specific functions.[5,19,20] Other SIMs depend on adjacent phosphorylation for noncovalent SUMO interaction.[21]

SUMO is mostly conjugated as a single moiety although SUMO2/3 chain formations have been observed. The consequences of sumoylation are alterations in protein/DNA/RNA interactions caused by SUMO physically interfering with existing binding sites or by providing a new binding site via SIM binding (e.g., refs. 11,22-26). Sumoylation has also been shown to change the conformation of its target via intramolecular SIM binding.[27] The subsequent biological consequences of sumoylation are highly diverse, ranging from changes in cellular localisation to altering the stability and activity of the respective target.

Sumoylation is a highly dynamic event and depends on the equilibrium between conjugation and deconjugation. Conjugation occurs via a specific ATP-dependent enzymatic cascade which includes E1 activating-, E2 conjugating- and E3 ligating-enzymes, whereas deconjugation is performed by specific SUMO proteases. Modifying hundreds of targets requires a highly specific and tightly controlled system, which cannot easily be explained by the relatively low number of sumoylation enzymes that have been identified. In this chapter we aim to concentrate on different mechanisms controlling sumoylation and will discuss examples for fine-tuning substrate selection and also for regulating the global SUMO machinery.

THE SUMO MODIFICATION CYCLE ALLOWS DIFFERENT LEVELS OF REGULATION

Newly translated SUMO precursor proteins have C-terminal extensions of variable length and depend on proteolytic processing to free the C-terminal Gly-Gly motif required for conjugation (Fig. 1, PROCESSING). In an ATP-dependent reaction, SUMO is activated and forms a thioester linkage with the E1 activating enzyme; a heterodimer formed between Aos1 and Uba2 (also referred to as SAE1/SAE2) (Fig. 1, ACTIVATION).[28,29] SUMO is then transferred to the E2 conjugating enzyme Ubc9, again resulting in a thioester linkage (Fig. 1, CONJUGATION).[30,31] The catalytic cleft of Ubc9 directly binds to the SUMO consensus motif within the SUMO substrate, but this interaction is not in itself enough for efficient modification.[10] In the past, different mechanisms were identified which stabilize

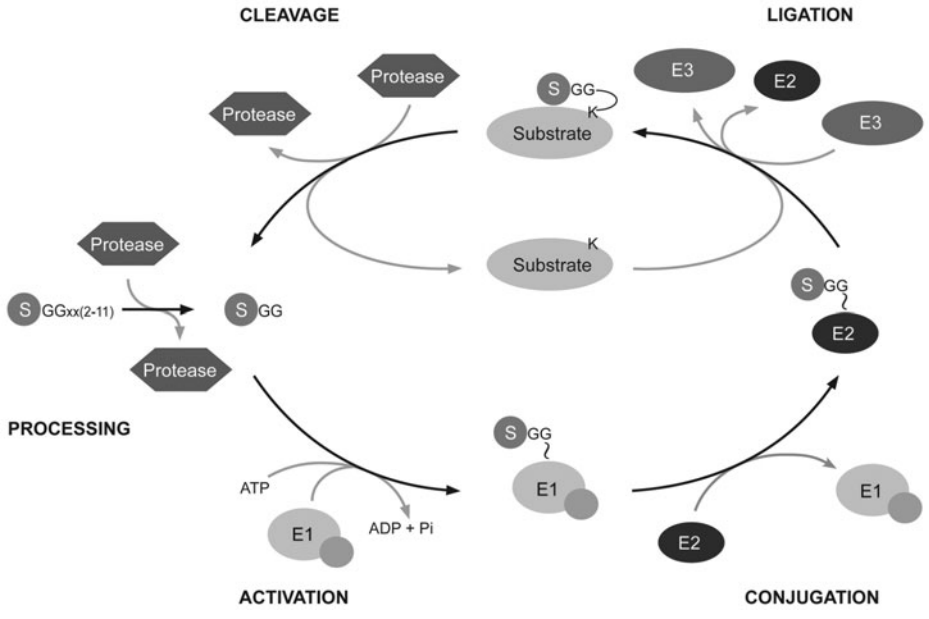

Figure 1. The SUMO conjugation cycle. All SUMO members need to be processed prior conjugation to free their Gly-Gly motif. SUMO is then activated in an ATP-dependent manner forming a thioester with the E1 enzyme. Subsequently SUMO is transferred to the E2 again resulting in a thioester bond. In the final step SUMO is transferred directly or with the help of an E3 ligating enzyme to a lysine in the substrate. SUMO can be cleaved from the substrate by SUMO specific proteases.

this interaction and consequently accelerate the SUMO transfer from Ubc9 to the target lysine (Fig. 1, LIGATION). The classical mechanism is via a third class of enzymes, the E3 ligases, which enhance SUMO conjugation by increasing the affinity between the target and the SUMO-loaded E2.[32] This is best understood for E3 ligases of the Siz/Pias family. Alternatively, substrate modification is enhanced by optimal positioning of the SUMO-loaded E2, as shown for the E3 ligase RanBP2.[33] Efficient modification does not always require E3 ligases. The prime example is RanGAP1, which stably interacts with Ubc9 involving a second binding surface.[10]

Also noncovalent interactions via a SIM in the substrate can enhance the affinity between the SUMO-loaded E2 and the substrate. Since selected SIMs discriminate between SUMO variants, the SIM in the substrate can determine the choice of SUMO paralog selection.[5,19] Whether the SIM in the substrate accelerates modification simply by increasing the affinity for the SUMO-loaded E2 or if additionally SUMO positioning is involved awaits further analysis. An additional mechanism is provided by posttranslational modifications on either the substrate or the E2 enzyme, which increases E2/substrate affinity in an E3 independent manner and consequently leads to enhanced modification (see below). SUMO modification can be reversed by isopeptide cleavage performed by SUMO specific proteases (Fig. 1, CLEAVAGE) (e.g., refs. 34-37).

To accomplish spatially and temporally controlled SUMO substrate modification, there are different levels of modulating the system. Regulation of sumoylation takes place largely at the level of the individual target proteins but the enzymes for conjugation and

deconjugation can also be regulated. As well as transcriptional and posttranscriptional regulation, both localization and posttranslational modifications can be regulated to control sumoylation.

REGULATING THE SUBSTRATE

One key mechanism for controlling substrate-specific sumoylation is by modulating the target itself. The most understood way of increasing the magnitude of regulation is by using the variety of posttranslational modifications such as phosphorylation, ubiquitination, acetylation and methylation. Such modifications can regulate substrate sumoylation in every conceivable manner, so here we will highlight a few examples.

Large range of SUMO substrates depend on prior phosphorylation either in close proximity to the sumoylation motif (e.g., Hsf1[38] and Mef2)[39] or at other positions in the protein, before they can be sumoylated. Phosphorylation enhances substrate sumoylation by increasing its affinity for either the E2[40] or the E3 enzyme. Phosphorylation-dependent changes in localization or structural changes can also be envisioned. Importantly, phosphorylation can also negatively regulate sumoylation, most likely by interfering with the SUMO conjugation enzymes (e.g., p53,[41] IKBα,[42] c-jun,[43] c-fos[44] and Elk1).[45]

Another mechanism directly or indirectly impairing sumoylation is modification of the same acceptor lysine by ubiquitination (e.g., PCNA[46] and IKBα),[42] acetylation (e.g., Sp3[47] and H2B)[48] and most likely also by methylation. Whether these modifications are directly competing with sumoylation or working independently is not clear, but both scenarios have been discussed. Certainly, modifying different lysines can also affect sumoylation. An example for the complexity of regulatory modification crosstalk is provided by the yeast histone H2B. C-terminal ubiquitination at K123 of H2B opposes its sumoylation at different N-terminal lysines. In turn, sumoylation inhibits acetylation by involving the same acceptor lysine residues.[48] However, modification-dependent conformational changes can also mask or unmask the site for sumoylation.

Alternatively, changes in the intracellular localization can determine the sumoylation status of a protein, both dependently and independently of other modifications. For example, the nuclear speckle component Sp100 depends on nuclear import for efficient sumoylation.[49]

REGULATING SUMO

SUMO1 appears to be limiting in the cell and is mainly found conjugated to its substrates, while SUMO2/3 is primarily found in its free form and only conjugated upon specific stress conditions.[50] Our understanding of how the SUMO family is regulated is poor, but different studies have indicated that they are controlled at different levels. SUMO1-3 are ubiquitously expressed but show tissue specific variations.[51] SUMO1 expression is elevated in response to hypoxia[52,53] and SUMO4 levels are restricted to specific tissues.[6,7]

Posttranslational modifications also appear to play a role in controlling SUMO members, since SUMO1 was found modified by both acetylation and phosphorylation.[54,55] Functional consequences of these modifications are currently unknown. In certain cases sumoylated substrates are recognized by the ubiquitination machinery[4] raising the idea that

SUMO family members themselves may also be regulated via proteasomal degradation. Such a mechanism is assumed for SUMO4, which is rapidly degraded under physiological conditions and only gets stabilized, processed and conjugated by stress induction.[56]

REGULATING THE E1 ACTIVATING ENZYME

Sumoylation involves one E1 enzyme, a heterodimeric protein between Aos1 and Uba2 (also known as SAE1 and SAE2).[28,29] Targeting this essential enzyme for SUMO conjugation suggests impairment of global sumoylation. This was indeed demonstrated for the CELO virus protein Gam1 which hijacks the SUMO system by inducing proteasomal degradation of E1.[57] A very different mechanism of E1 regulation occurs during oxidative stress. Low H_2O_2 concentrations induce reversible disulfide bond formation between the catalytic cysteines of the Uba2 E1 subunit and the E2 enzyme Ubc9 resulting in a loss of SUMO conjugation and consequently desumoylation of most cellular SUMO targets.[58]

REGULATING THE E2 CONJUGATING ENZYME

Ubc9 is the sole E2 enzyme for sumoylation.[30,31] It has two functions in SUMO conjugation: accepting SUMO from the E1 by forming a thioester linkage and subsequently transferring SUMO to the substrate with or without the help of an E3 ligase. To execute these functions it requires binding interfaces for the E1, the substrate and the E3 ligases.

Different mechanisms regulating Ubc9 have been described, such as the above mentioned disulfide bond formation between Ubc9 and the E1 subunit Uba2 under oxidative stress.[58] In addition, posttranslational modifications were found to regulate the E2 enzyme. Ubc9 sumoylation is identified in every screen for novel SUMO substrates and we could recently show that this modification regulates Ubc9 function in target discrimination. Sumoylated Ubc9 can enhance modification of selected SIM-containing SUMO substrates via an increase in substrate affinity whereas other substrates are not affected or even impaired in modification.[23] Currently the global biological role for Ubc9 sumoylation is unclear. Ubc9 was additionally shown to be modified by S-nitrosylation upon treatment with the nitric oxide donor GSNO but so far no specific function has been identified.[59]

Ubc9 levels vary between different organs and tissues[60] and are frequently upregulated in human malignancies.[61] Less is known about the molecular mechanisms controlling Ubc9 levels but increasing evidence indicates a role for transcriptional and posttranscriptional mechanisms along with differential intracellular localization.[62-64]

REGULATING THE E3 LIGASES

E3 ligases are the enzymes assumed to ensure substrate specificity. Most E3 ligases interact with both the SUMO~Ubc9 thioester and the substrate and bring them in close proximity for efficient SUMO transfer (e.g., refs. 32,65). Alternatively, catalysis can be accelerated by the optimal positioning of the SUMO-loaded E2 for SUMO transfer to the target lysine.[33]

Sumoylation targets hundreds of proteins but currently only a handful of E3 ligases have been described and it is still not clear how substrate specificity occurs. On the one hand, it is likely that many E3 ligases await their identification but on the other hand tight regulation may determine substrate specificity. Therefore, E3 ligase control is as influential as regulation of the specific substrate. At present, we are just starting to gain insights into the complexity of E3 ligase regulation and here we will describe a few examples.

One important regulatory mechanism is the spatial and temporal co-occurrence of target and E3 ligases, as shown for septins in yeast. Septins only get sumoylated during mitosis when the E3 ligase Siz1 translocates from the nucleus to the bud neck where it meets its targets. Exactly at this time Siz1 gets phosphorylated, although the functional consequence of this modification is not understood.[32]

To date, the mechanism leading to substrate/E3 colocalization remains unclear but recently one attractive hypothesis was put forward, which proposes that substrate binding contributes to locate the E3 ligase in the cell.[66] This idea is supported by the finding that multiple domains of Siz E3 ligases contribute to target selection[66] and that mutations in many different domains of the mammalian relative Pias3 all impaired nuclear retention.[67] For such a mechanism one would expect that substrate and/or E3 ligase availability is limited or tightly regulated probably via posttranslational modification. Substrate sumoylation would then most likely be determined by the presence of the SUMO loaded Ubc9.

Posttranslational modifications for several E3 ligases are frequently identified. The meiosis-associated E3 ligase Zip3 regulates global SUMO modification along meiotic chromosomes at synaptonemal complex assembly in budding yeast. Zip3 is regulated oppositional to Siz1 and Siz2 at and after mid-prophase. At this time Zip3 is posttranslationally modified by phosphorylation and sumoylation suggesting an important role for these modifications in executing meiosis-specific functions.[68] Several E3 ligases of the Siz1/PIAS family are targets of phosphorylation but so far none have been directly linked to the regulation of E3 function.[69,70] Nevertheless, phosphorylation-inhibited E3 activity has been described for KAP1 and Topors (topoisomerase I-binding protein). The multifunctional KAP1 protein, which has internal E3 activity, is phosphorylated in response to DNA damage leading to inhibition of sumoylation.[71] Topors has both SUMO and ubiquitin E3 ligase activity. Plk1 (Polo-like kinase 1) mediated phosphorylation of Topors switches its activity by inhibiting sumoylation and promoting ubiquitination on its substrate p53.[72]

Phosphorylation can also enhance E3 activity as has been described for the polycomb group member Pc2. It interacts with HIPK2 (homeodomain-interacting protein kinase 2), which is activated upon DNA damage and phosphorylates Pc2 at multiple sites. In turn, phosphorylation activates Pc2´s E3 ligase activity and accelerates HIPK2 sumoylation.[73] In addition, HIPK2 binding controls Pc2´s intracellular localization but this is independent of its sumoylation activity on HIPK2.[73]

Another way regulating E3 activity was found for Piasy. Here, sumoylation alters its intracellular localization and leads to enhanced E3 activity for the transcription factor Tcf4 via an unknown but localization-independent mechanism.[74] Another substrate of Piasy is NEMO and it was demonstrated that genotoxic stress increases E3/substrate interaction in the nucleus but is mutually exclusive with IKK (IKB kinase) interaction.[69] An indirect mechanism for impairing E3 function was found for Pias3. Nitric oxide stimulates its S-nitrosylation, which allows recruitment of the ubiquitin machinery and accelerates its degradation.[59]

Localization-specific target recruitment may also play a role in regulating the vertebrate E3 ligase RanBP2, which forms filaments of the nuclear pore complex and is therefore restricted to the nuclear envelope in interphase.[11,75,76] During mitosis when nuclear pores disassemble, RanBP2 gets redistributed throughout the cell and is enriched at kinetochores and at the mitotic spindle,[77,78] making it accessible for a different set of substrates. How RanBP2 substrate selection is performed is currently unclear since the small RanBP2ΔFG fragment which bears the E3 activity does not directly interact with its substrate.[79] Two alternative scenarios can be envisioned: In vivo, cofactors like transport receptors deliver the substrate to RanBP2 which has docking sites for such receptors. Alternatively, a posttranslational modification such as RanBP2 sumoylation could be involved in substrate recruitment since Sp100 requires a functional SIM for efficient RanBP2ΔFG dependent modification.[23]

Together these findings point to an important role for posttranslational modifications and regulated intracellular localization for SUMO E3 ligase activity. To date less is known about transcriptional and posttranscriptional mechanisms regulating E3 activity but the existence of such mechanisms is obvious.

REGULATING THE SUMO SPECIFIC PROTEASES

A major means for controlling sumoylation is at the level of deconjugation. This step is performed by SUMO-specific proteases of which all belong to the ubiquitin-like protease (Ulp) family, named after the founding member Ulp1 in yeast.[80] To date, two members have been described in yeast (Ulp1 and Ulp2) and six in humans (Senp1-3 and Senp5-7), with Senp1, Senp2, Senp3 and Senp5 related to Ulp1 and Senp6 and Senp7 to Ulp2. All known SUMO proteases share a C-terminal 200 amino-acid-core domain with the catalytic triad (Cys-His-Asn) but they significantly differ in their N-terminal regions.[34-37]

SUMO proteases carry out three distinct functions: SUMO processing, SUMO isopeptide cleavage from the substrate and SUMO chain editing. Indeed, different proteases are dedicated to these different functions. Senp1 and Senp2 have both C-terminal hydrolase and isopeptidase activity for all SUMO variants[81] whereas Senp3 and Senp5 have isopeptidase activity preferentially for SUMO2/3.[82,83] Senp6 and Senp7 function in SUMO2/3 chain editing.[84] Division of work is also found for the yeast counterparts, where Ulp1 displays hydrolase and isopeptidase activity and Ulp2 is thought to function in SUMO chain editing additional to isopeptidase activity.[85]

One striking feature of the mammalian Senps is that they display functional preference for selected SUMO variants. Senp1 and Senp2 do not discriminate for SUMO cleavage but show selectivity in SUMO processing. Senp1 has higher efficiency for SUMO1 than SUMO2 and Senp2 displays controversial specificity, while both enzymes show poor activity for SUMO3. More detailed investigations identified the C-terminal residues of the cleavage site which determine the differences in processing.[86] For the very poor SUMO3 maturation efficiency, residue Pro94 was found to be responsible.[51] Enzymatic analysis indicates similar Km values for SUMO1 and SUMO3 but significantly (50-fold) lower Kcat for values for SUMO3 compared to SUMO1.[87]

As for the E3 ligases, it is also important to understand how a small number of SUMO proteases serve hundreds of substrates. Again, the best understood control system is based on differences in cellular localization, determined by the diverse N-termini of the proteases. Senp1, Senp6 and Senp7 are enriched in the nucleoplasm[35,81,84,88] whereas Senp2 is located

at the nuclear pore and in nuclear speckles,[82,83,88] and Senp3 and Senp5 have a nucleolar distribution.[89-91] The yeast enzymes also display different cellular distributions. Ulp1 is restricted via its N-terminus to the nuclear site of nuclear pore complexes, whereas Ulp2 is found in the nucleoplasm.[85,92] That localization determines substrate specificity is supported by the finding that deletion of Ulp2, which results in cellular defects, can only be repressed by the Ulp1 catalytic domain but not by the full length protein.[92] Senp5 provides another example in which regulated subcellular localization determines target selection. In interphase cells, Senp5 mainly resides in the nucleoli but relocates to the mitochondrial surface at G2/M where it desumoylates a number of mitochondrial SUMO substrates.[93]

An interesting novel mechanism for how isopeptidase activity indirectly discriminates between SUMO paralogs is by involving sumoylation-dependent binding partners. As already mentioned above, some SUMO binding proteins discriminate between SUMO variants via their SIM. Higher binding affinity to the substrate modified with the preferred SUMO member protects from isopeptidase cleavage.[94]

SUMO proteases are suggested to be controlled by transcriptional regulation since their expression levels differ in various tissues.[51] Alternative splicing generates at least three different Senp2 isoforms with strikingly different cellular localization.[34] Transcription can further be stimulated upon UV irradiation as has been described for Susp4, a mouse isoform related to Senp2.[95]

To date, less is known about how posttranslational modifications regulate SUMO protease function. One example comes from Senp3, which is sequentially phosphorylated, polyubiquitinated and rapidly degraded by the proteasome system in a p19Arf-dependent manner.[96] Similar to the SUMO E1, the activity of Senp1 but not Senp2 can also be inhibited upon oxidative stress by reversible disulfide bond formation.[97]

CONCLUSION AND FUTURE PERSPECTIVES

Sumoylation has important functions in DNA damage response, cell-cycle regulation, proliferation, apoptosis and transcriptional regulation. Hence, alterations in the SUMO-cycle have major impact on cell growth, neuronal inclusions, cancer development, alternative lengthening of telomeres and drug responsiveness.[3,61,98] Consequently it is fundamental to understand the different mechanisms controlling this system. We are just in the early stages of gaining insights how the system is regulated but it is already clear at this point that both enzymes and substrates participate in modulating the system. The underlying mechanisms are diverse and often involve posttranslational modifications but in many cases the exact consequences are unclear. Also the cellular localization of substrates and enzymes is a key regulatory event but again the causes and consequences are often nebulous. Less is known about differences in expression levels, transcriptional and posttranscriptional regulation like alternative splicing. Certainly additional enzymes will be indentified in the future but we also expect many more clues regarding the mechanisms controlling SUMO.

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CHAPTER 14

THE IN VIVO FUNCTIONS OF DESUMOYLATING ENZYMES

Tasneem Bawa-Khalfe and Edward T.H. Yeh*

Abstract: This chapter reviews the current literature to highlight the biological mechanisms mediated via the enzymatic actions of the SUMO-specific protease family. All members of this cysteine protease family express isopeptidase activity to deSUMOylate conjugated cellular protein targets. Here, we discuss how SUMO proteases discriminate amongst the SUMOylated targets based on subcellular localization and conjugated SUMO isoform. Several signal transduction pathways modulate endogenous levels of the deSUMOylating enzymes to regulate cell growth, cell cycle progression and gene transcription. The ability of specific proteases to mediate these cellular events is presented. In addition, we examine cases in which aberrant SUMO protease expression affects normal embryonic development, carcinogenesis and the onset of additional pathophysiological conditions.

INTRODUCTION TO THE SUMO-SPECIFIC PROTEASE FAMILY

Ubiquitin-like specific protease 1 (Ulp1) is the first yeast SUMO-specific protease reported.[1] Li and Hochstrasser distinguished Ulp1 from other deubiquitylating cysteine proteases based on its selectivity for regulating the yeast SUMO ortholog Smt3 but not ubiquitin. These investigators also reported Ulp2 that also exhibited Smt3-specific protease activity.[2] Ulp2 is much larger than Ulp1 but both Ulps express a conserved C-terminal catalytic domain which requires two conserved cysteine and histidine residues for activity against Smt3.[1,2] Hence, a SUMO specific protease is defined by its (1) highly conserved catalytic domain and (2) specificity for regulating SUMO conjugation. The protease family regulates SUMO/Smt3 conjugation to cellular substrates (a process called SUMOylation) via its (1) C-terminal hydrolase and/or (2) isopeptidase activity (Fig. 1).

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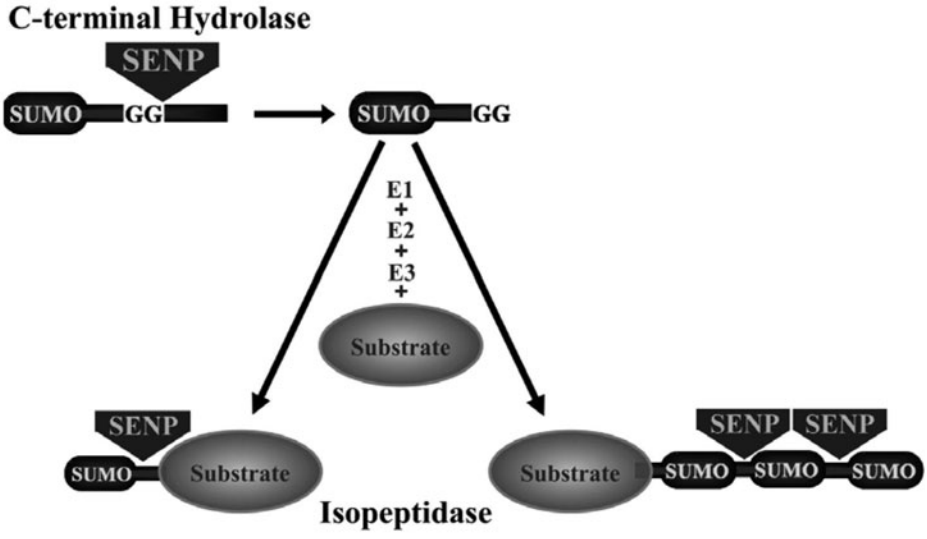

Figure 1. Enzymatic Activity of SUMO-specific Proteases. The illustration demonstrates how the mammalian SUMO proteases, SENP regulates the level of SUMO conjugated cellular substrates. The C-terminal hydrolase activity of SENPs initiates the first step towards SUMOylation which requires processing of the inactive SUMO precursor. Once in its active form, SUMO interacts with activating (E1), conjugating (E2) and ligating (E3) enzymes to conjugate to a cellular substrate. The SENPs utilize their isopeptidase activity to either cleave SUMO from the substrate or disrupt the poly SUMO side chain.

In 2000, our laboratory isolated the first mammalian SUMO protease.[3] The mammalian proteases have adopted the SENP nomenclature; previously, we referred to SUMO as Sentrin and hence SENP is an acronym for Sentrin-specific Protease. Initially, 8 SENPs were identified and named chronologically. However, it was later determined that SENP4 was a variant of SENP3 and SENP8 exhibits deconjugating activity for the ubiquitin-like molecule NEDD8 but not SUMO.[4] Currently 6 SENPs constitute the mammalian SUMO protease family.[3,5,6] Evolutionary conservation of the cysteine proteases is evident as the sequence of SENP1, SENP2, SENP3 and SENP5 is closely related to the yeast Ulp1 while SENP6 and SENP7 express sequences that are similar to Ulp2.[7] Like the yeast Ulps, the 6 enzymes all require the 200 amino acid C-terminal catalytic region for selectively deconjugating SUMOylated, but not ubiquitylated proteins.[3] Unlike the Ulps, the SENPs regulate three isoforms of SUMO in higher order organisms, specifically SUMO1, SUMO2 and SUMO3. A fourth SUMO isoforms was identified[8] however, in vivo, SUMO4 remains in the inactive form and its biological function is unclear.[9] The C-terminal catalytic activity of SENPs for select SUMO isoform is highlighted in Table 1.

Although the catalytic domain is conserved amongst all family members, the N-terminus of the SUMO proteases differs significantly in amino acid sequence and size. The N-terminal region of the protease dictates the enzyme's subcellular localization. The SENP's cellular localization is reviewed in Table 1 and its contributions to their function will be discussed in the subsequent sessions.

Based on their (1) N-terminus sequence homology, (2) subcellular localization and (3) specificity for deconjugation of specific SUMO isoforms, the mammalian proteases

Table 1. SUMO-specific proteast family

	Alternative Names	Enzymatic Activity		Subcellular
		Hydrolase	Isopeptidase	Localization
Yeast Proteases				
Ulpl	-	Smt3	Smt3	Nuclear Pore Complex
Ulp2	Smt4	No	Smt3	Nucleoplasm
Mammalian Proteases				
SENP1	SuPr-2	SUMO1 > 2 = 3	SUMO 1,2,3	Nucleoplasm, Translocates to/from Cytoplasm
SENP2	SuPr-1, Axam, SMT3IP2	SUMO1 = 2 > 3	SUMOl.2,3	Nuclear Pore Complex, Translocates to/from Cytoplasm
SENP3	SuPr-3,SMT3IPl, SUSP3, SSP3	SUMO2,3	SUMO2,3	Nucleolus, Translocates to Nucleoplasm
SENP5	SMT3IP3	SUMO2,3	SUMO2,3	Nucleolus
SENP6	SUSP1,SSP1	No	SUMO2,3	Nucleoplasm
SENP7	SUSP2, SSP2	No	SUMO2,3	Nucleoplasm

can be divided into independent subfamilies. The first family, which includes SENP1 and SENP2, exhibit 95% sequence homology with each other. SENP3 and SENP5 constitute the second family due to their localization in the nucleolus. The expression of a loop within the catalytic domain distinguishes the third family, SENP6 and SENP7 from the other 2 SENP families.

Recently, SUMO specific proteases were discovered in plants.[10-12] The function of the *Arabidopsis* deSUMOylating enzymes is not as well defined as that of their counterparts in yeast and mammals. Hence this work will discuss primarily studies on yeast and mammalian SUMO proteases.

C-TERMINAL HYDROLASE ACTIVITY

The C-terminal hydrolase activity of the SUMO protease family is required to cleave amino acids on the C-terminus adjacent to two glycine residues (Gly-Gly motif, Fig. 1). This processing of the precursor SUMO to expose the Gly-Gly residues and generate a mature SUMO molecule is the initial step for SUMOylation. For the SENPs, kinetics studies reveal that the SUMO protease family members exhibit less efficient hydrolase than isopeptidase activity; although the SENPs bind the precursor with high affinity, the rate of C-terminal hydrolase activity is relatively slow.[13-15]

Initial studies characterizing the yeast SUMO protease Ulp1 demonstrated that Ulp1 can actively process the Smt3 precursor molecule (Smt3-aty) to generate the mature

Smt3.[1] This generation of mature Smt3 is dependent primarily on Ulp1 as Ulp2 does not process Smt3-aty efficiently.[2]

SENP1 can process all three SUMO isoforms. However, data from the SENP1–/– embryos indicates a greater activity against SUMO1 precursors in vivo as mature SUMO1 levels are significantly diminished with ablation of SENP1.[16] Similar results were also reported in a kinetic study in which SENP1 cleaves the SUMO1 precursor more efficiently than the SUMO2 precursor.[15] Like SENP1, SENP2 can also process all three SUMO isoforms. SENP2 processes SUMO1 and SUMO2 precursors at approximately the same rate (K_{cat} value) but is significantly slower at cleavage of inactive SUMO3.[13] Of the nucleolar SENPs, we and others report greater SENP5 C-terminal hydrolase activity for SUMO2/3 but not SUMO1.[5,17] We observe a similar specificity for processing precursor SUMO2/3 by SENP3 (unpublished observation). Finally, neither SENP6 nor SENP7 exhibit efficient C-terminal hydrolase activity.[14,18]

ISOPEPTIDASE ACTIVITY

The SUMO proteases cleave the isopeptide bond between the carboxy terminus of SUMO and the lysine side chain of a substrate. The isopeptidase activity functions to deSUMOylate a specific protein target or disassemble polymeric SUMO side chains (Fig. 1).

The factors that regulate the isopeptidase activity of the SUMO proteases are not well defined. It is generally accepted that a change in the SENP's deSUMOylation activity is often due to changes in its subcellular localization or endogenous expression.

Subcellular Localization

SUMO protease isoforms differ in their localization in the cell (Table 1). This difference in subcellular local plays a critical role in defining which SUMO-conjugated substrates are targets for deconjugation by a specific SUMO protease isoforms. For example, a SUMO protease may exhibit isopeptidase activity against a particular SUMOylated protein in vitro but in vivo, the conjugated protein may not be deSUMOylated by the SUMO protease due to differences in subcellular locale.

For the two yeast SUMO proteases, Ulp1 is localized in the nuclear pore complex[1] while Ulp2 is distributed throughout the nucleoplasm. Li and Hochstrasser demonstrated that the N-terminal region of Ulp1 is responsible for localization of the protease to the nuclear pore.[19] In the manuscript, the Ulp1 N-terminal domain was deleted to produce a functional active mutant expressing only the catalytic domain. The Ulp1 mutant did not concentrate at the nuclear envelope and caused an accumulation of Smt3-conjugated proteins. Similarly mislocation of Ulp2 to areas outside the nucleus causes accumulation of Smt3 conjugates and prompts poor growth of yeast; the authors concluded that improper distribution of the Ulp2 mutant induced these functional deficiencies.[19] Collectively, these studies demonstrate the importance of subcellular localization of the yeast SUMO protease in deconjugation of protein substrates.

The six mammalian SUMO proteases are expressed more diversely in the cell and several SENPs exhibit a preference for specific SUMO isoforms (Table 1). SENP1 is present throughout the nucleus with the exception of the nucleolus.[5] SENP2, like

Ulp1, is localized to the nucleoplasmic face of the nuclear pore complex and associates with nucleoporin that localize to the same region.[20,21] Interestingly, disruption of the interaction between SENP2 and the nucleoporin Nup153 prevented tethering of SENP2 to the nuclear envelop but increased its ability to deconjugate cellular substrates.[21] This induction of SENP2 activity is possibly due a loss of SENP2 specificity for a subset of conjugated proteins. Since both SENP1 and SENP2 can deconjugate the three SUMO isoforms with equal affinity, the difference in subnuclear localization would serve to differentiate between the SUMOylated proteins preferentially regulated by either one of the two SENPs. Along with the nuclear localization signal, both SENP1 and SENP2 also express a nuclear export sequence that allows the SENP to shuttle back and forth from the nucleus and cytoplasm.[22,23] SENP2 expression in the cytoplasm is responsible for its ubiquitin-mediated degradation;[23] it is currently unknown whether the same process regulates SENP1.

The subfamily of SENP3 and SENP5 constitute the nucleolar SENPs.[5,24-27] We identified that the first 168 amino acids of SENP5 are required for the nucleolar localization.[26] In the same study, we observed that both SENP3 and SENP5 exhibit greater isopeptidase activity for SUMO2 and SUMO3 conjugates than SUMO1.[26] The nucleolus is the site for ribosome synthesis and both SENP3 and SENP5 play a critical role in ribosome biogenesis.[25,27] Deletion of both SENPs in HeLa cells hinders each aspect of ribosome synthesis, specifically preribosomal RNA transcription, ribosomal RNA processing and ribosomal subunit assembly.[25] The two SENPs modulate ribosomal biogenesis via their isopeptidase activity on SUMO conjugated nucleolar proteins; one identified targets is B23/nucleophosmin.[25,27] Therefore, the nucleolar localization of SENP3 and SENP5 dictates their substrate specificity.

SENP6 and SENP7 are located in the nucleoplasm.[5] Initial studies suggested that SENP6 was expressed in the cytoplasm.[28] However the group later indicated that the cytosolic SENP6 expression was due to the N-terminal GFP-tag; GFP-tagged at the C-terminus of SENP6 and endogenous SENP6 exhibit nuclear staining patterns.[29] Collectively, these results, other additional reports and our data validate that SENP6 is exclusively in the nucleus.[5,30] Deletion of either SENP6 or SENP7 leads to accumulation of SUMO2/3 conjugates specifically in promyelocytic leukemia protein (PML) containing nuclear bodies.[30,31] Hence, both SENPs deconjugate protein targets that are modified by the poly-chain forming SUMO2/3 and can modulate the movement of target substrates into and out of subnuclear compartments.

Level of Expression

Sine SUMO proteases are not processed from an inactive precursor state like SUMO, it is accepted that the level of SUMO protease expressed in the cell is proportional to its enzymatic activity. In the literature, several signal transduction pathways and posttranslational modifications alter endogenous SENP levels in the cell. We and others observe an induction of SENP1 mRNA with introduction of the cytokine IL-6 or a hormone-activated androgen receptor (AR).[5,32,33] To elucidate what dictates expression of SUMO proteases endogenously, we recently cloned and characterized the *SENP1* promoter (Fig. 2).[32] The *SENP1* promoter expressed an androgen response element that is readily bound by the hormone-activated AR to enhance *SENP1* transcription. We observe a similar induction of *SENP1* promoter activity with two additional hormone-activated

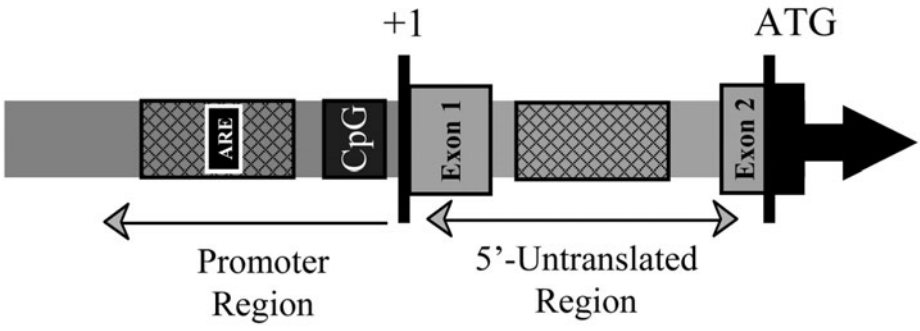

Figure 2. Features of the *SENP1* Promoter. The schematic illustrates the promoter and 5′-untranslated regions of the *SENP1* gene. The *SENP1* promoter includes a large CpG island ("CpG" labeled box) and lacks the conventional TATA box for recruitment of the general transcription machinery. We identified a functional androgen response element (black box) located within a highly conserved region (cross-hatched box in the promoter region).[32] A similar conserved region was previously identified in the 5′-untranslated region of the *SENP1*_gene (cross-hatched box in this region); disruption of this homologous domain in intron 1 lead to diminished expression of SENP1 in mice.[53]

nuclear receptors, specifically the glucocorticoid receptor (GR) and the estrogen receptor (ER). It is feasible that these receptors bind their respective response elements to illicit this response; a similar approach as described for the AR could be used to delineate the mechanism for SENP1 induction by ER and GR. Collectively, the results indicate that steroid receptor family can regulate SENP1 expression at the transcriptional level.

A previous report identified that SENP3 levels are regulated by the tumor suppressor protein p19(Arf).[34] p19(Arf) prompts SENP3 phosphorylation, ubiquitylation and proteasome-mediated degradation to reduce SENP3 levels. However, it remains unknown what kinase(s) mediate SENP3 phosphorylation and whether this modification also regulates SENP3's isopeptidase activity and/or substrate specificity. We also observe ubiquitylation and subsequent proteasome-mediated degradation of SENP3.[35] In our recent study, we report that mild oxidative stress prevents ubiquitylation and prompts accumulation of SENP3 in the nucleoplasm following translocation from the nucleolus. Enhanced expression of SENP3 in the nucleoplasm subsequently leads to deSUMOylation of p300 and induction of HIF1α-transcriptional activity. Hence in this system, SENP3's enhanced expression and change in subnuclear locale prompted isopeptidase activity against a nonnucleolar SUMOylated substrate, p300.

GENE TRANSCRIPTION AND mRNA PROCESSING

Numerous studies have validated the importance of the SUMO peptidases in the regulation of gene transcription. SENPs use their isopeptidase activity on SUMOylated transcriptional factors and nuclear receptors that bind promoter elements and directly mediate gene transcription. In addition, the isopeptidases also modulate the activity of SUMO-conjugated coregulatory proteins. These proteins can either facilitate (co-activator proteins) or inhibit (corepressor proteins) the transcriptional activity of specific transcription factors or nuclear receptors to which they are bound (Fig. 3).

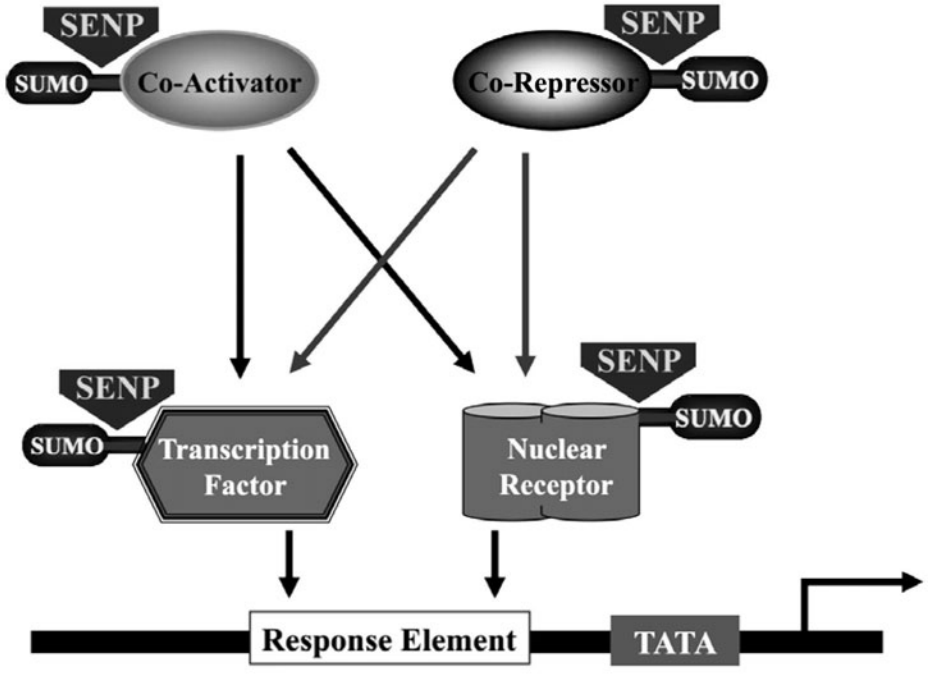

Figure 3. Regulation of Gene Transcription by SENPs. SUMO and SENPs modulate the function of several key regulators of gene transcription. The SUMOylation status of transcription factors or nuclear receptors can directly affect their ability to bind their respective response elements. In addition, the ability of co-activators and corepressors to modulate their downstream transcription factors and/or nuclear receptors can also be dictated via SUMOylation/deSUMOylation. It is likely that in vivo, the SUMOylation/deSUMOylation machinery work in concert at multiple levels to facilitate or repress a specific gene.

SENP2 regulates the SUMOylation and function of Sp3, a member of the GC box-binding transcription factors.[36] Conjugation of SUMO to Sp3 represses Sp3-mediated transcription while the deSUMOylating ability of SENP2 (referred to as SuPr-1 in the manuscript) prompts Sp3 transcriptional activation. This effect on Sp3-dependent transcription is selective for the SENP2 isopeptidase because neither SENP3 (referred to as SuPr-3) nor an additional peptidase homologous to SENP3 (recorded as SuPr-4) had any effect. In contrast, the activity of the transcription factor MEF2D is selectively regulated via the deconjugation activity of SENP3; SENP1 is less efficient at deSUMOylating modified MEF2D than SENP3. DeSUMOylation by SENP3 prevents the SUMO-mediated inhibition of the transcriptional activity of MEF2D and consequently MEF2D-mediated myogenesis is increased with SENP3 expression.[37]

A yeast two-hybrid demonstrated the interaction between SENP6 (referred to as SUSP1) and the nuclear receptor, retinoid X receptor α (RXRα).[29] SENP6 deSUMOylated RXRα but not two other SUMO conjugated nuclear receptors, peroxisome proliferator-activated receptor γ or AR. The authors went on to demonstrate that the isopeptidase activity of SENP6 directly increases transcriptional activity of the RXRα in a reporter assay while a catalytically inactive SENP6 mutant had no effect on RXRα-mediated transcription.

Another nuclear SUMO protease SENP1 also regulates the transcriptional activity of additional nuclear receptors. Overexpression of SENP1 enhances AR-dependent transcription.[38,39] In COS-1 cells, SENP1 and SENP2 (but not SENP3, SENP5, or SENP6) deSUMOylated modified AR and enhanced AR activity on a luciferase-reporter gene fused to 2 androgen response elements (ARE) and a TATA box.[39] In contrast, the same reporter system expressed in the prostate cancer cell line LNCaP exhibited increased AR-mediated luciferase activity with overexpression of SENP1 but not SENP2. Prior to this study, we had reported a similar induction of AR-dependent transcriptional activity with enhanced SENP1 levels in LNCaP cells using a slightly different reporter construct.[38] In our hands, although SENP1 could deconjugate SUMOylated AR in LNCaP cells, both wild-type AR and a SUMO-deficient AR mutant exhibited elevated transcriptional activity in the presence of SENP1. Instead the ability of SENP1 to induce AR-mediated transcription was reduced with targeted knockdown of the AR corepressor HDAC1; deSUMOylation of HDAC1 by SENP1 relieves its repression on AR-dependent transcription.

We and others have observed that SENP1 and SENP2 independently regulate the transcriptional activity of c-Jun.[40,41] Interestingly, the isopeptidase activity of SENP2 is not required for induction of c-Jun activity as a catalytically inactive SENP2 mutant is also able to potentiate c-Jun-dependent transcription.[40] In contrast, SENP1 requires its isopeptidase activity to mediate this elevation in c-Jun transcriptional activity. SENP1 deconjugates SUMO-modified c-Jun co-activator p300. SUMOylation of p300 is responsible for the cis-repression function of the CRD1 domain of p300 and prevents the transactivation capabilities of p300. SENP1 relieves this internal repression and enhances p300-mediated transactivation.

SENP2 has been shown to modulate the Wnt signaling pathway via regulation of the co-activator β-catenin and the transcription factor Tcf-4.[42–44] The highly conserved Wnt pathway mediates the transcription of genes that are critical for development and carcinogenesis. Initial studies identified the rat SENP2 ortholog Axam as an interacting protein of Axin, which is responsible for phosphorylation and subsequent degradation of β-catenin.[42,45] In fact, Axam expression directly facilitated degradation of β-catenin but required an intact isopeptidase domain to prompt this change in β-catenin levels.[42] The exact target that Axam deSUMOylated to mediate β-catenin degradation was not identified however in a subsequent report, the group identified that Axam regulates Tcf-4, another key component of the Wnt pathway.[43] The transcription factor Tcf-4 is SUMOylated. Overexpression of the E3 ligase PIASy enhances Tcf-4 SUMO conjugation and potentiates β-catenin-induced Tcf-4 transcriptional activity while enhanced expression Axam counters both PIASy induced events. Recently, similar results were obtained with SENP2 in two human colorectal cancer cell lines; expression of SENP2, but SENP1, deSUMOylated Tcf-4 and inhibited Tcf-4 transcriptional activity.[44] In addition, enhanced expression of SENP2 prevented the association of β-catenin and Tcf-4. Therefore, it appears that SENP2 regulates the propagation of the Wnt signaling pathway by dictating the interaction between the transcription factor Tcf-4 and its co-activation β-catenin.

In addition to its ability to regulate gene transcription, SENP2 also functions to mediate 3′ mRNA processing.[46] SENP2 interact with and deSUMOylates two components of the 3′ mRNA processing complex, specifically cleavage/polyadenylation specificity factor-73 (CPSF-73) and symplekin. This deSUMOylation activity of SENP2 is required to inhibit the two step 3′ end processing; SENP2 inhibits both (1) cleavage at the 3′ of the pre-mRNAs to generate a 3′-OH ends and (2) formation of the poly (A)-tail. In addition, increasing amounts of SENP2 prevented the association of polyadenylation complexes.

CELL CYCLE AND CELL GROWTH

In its initial discovery and characterization, the yeast SUMO protease ortholog Ulp1 was identified as a key regulator of growth and cell cycle progression.[1] Yeast strains deficient of Ulp1 exhibited cell growth arrest due to inability to transition past the G2/M stage of the cell cycle. Changes in cell growth induced with knockout of Ulp1 in *Schizosaccaromyces pombe* was not rescued with overexpression of mature Smt3, suggesting that it regulates cell growth via deconjugation of Smt3 substrates.[47] In contrast, Ulp2 plays a more specialized role in the regulation of the cell cycle. It dictates recovery of cells from transient checkpoint arrest due to DNA damage, defective DNA replication or incomplete spindle formation.[2,48]

Similarly, small interference RNA (siRNA) knockdown of SENP5 leads to decreased cell proliferation in HeLa cells.[17] SENP5 downregulation increases the number of binucleate cells that are due to defects in mitosis and/or cytokinesis; hence SENP5 mediate normal cell division. In addition to SENP5, SENP1 and SENP7 also appear to positively regulate cell proliferation. Our studies demonstrated that SENP1 overexpression potentiates AR transcriptional activity[38] and inhibition of SENP1 significantly reduces AR-mediated LNCaP cell growth.[32] In the same cell line, stable overexpression of SENP1 enhances the cell cycle regulator cyclin D1 whereas the expression of the catalytically inactive SENP1 mutant has no effect;[5] we are currently investigating how SENP1 alters cyclin D1 levels. In human foreskin fibroblast cells, retroviral SENP1 inhibits cell proliferation without inducing apoptosis or necrosis.[49] Instead, SENP1 deficient fibroblast cells undergo cellular senescence and hence are arrested in the G1 phase of the cell cycle. Absence of p53 activity with either shRNA treatment or stable overexpression of a p53 dominant negative mutant prevented cellular senescence induced with SENP1 knockdown suggesting that the senescence response is mediated via a p53-dependent pathway. In the same cell line, downregulation of SENP2 and SENP7, respectively, also inhibited cell proliferation and caused cellular senescence; however, changes in cell cycle progression and the role of p53 in cellular senescence were not defined. Interestingly, SENP2 ablation in a mouse model produces a defect in the G1-S transition with increased number of trophoblast stem cells in the G1-phase.[50] p53 also contributed to this cell cycle defect in the trophoblast stem cells of SENP2 knockout mice. SENP2 deficient trophoblasts exhibit enhanced SUMOylation of the p53-regulator Mdm2 that in turn prevents Mdm2 translocation to the cytosol where it facilitates ubiquitin-mediated p53 degradation. Hence, elevated p53 levels are observed in the SENP2 knockout trophoblasts which prompt G1 arrest and prevent trophoblast differentiation.

Min and colleagues demonstrated that SENP1 can also regulate the ASK/JNK apoptosis pathway. The Tumor Necrosis Factor (TNF) activates the apoptosis signal-regulating kinase-1 (ASK-1) via mediating the deSUMOylation of homeodomain-interacting protein kinase 1 (HIPK1).[51,52] In endothelial cells, TNF prompts the translocation of SENP1 to the cytoplasm, SENP1-mediated deSUMOylation of HIPK1 and subsequent ASK1-dependent apoptosis.[52] It is plausible that SENP1 initiation of either cell proliferation or apoptosis depends on the signaling pathway initiated, its ability to translocate to the cytosol and/or the cell types.

ROLE IN DEVELOPMENT

Studies in whole animals have provided great insight into the role of SUMO proteases in development. A random retroviral insertion in the promoter region of the mouse SENP1 ortholog SuPr-2 gene leads to diminshed expression of SuPr-2.[53] Reduced SuPr-2 leads to death midgestation between e12.5 and e14.5 due to placental abnormalities that prevented normal gas and nutrient exchange.

We generated SENP1 knockout mice using a gene trap vector that inserted into intron 8 of the SENP1 locus and prevented the expression of a SuPr-2 with a functional catalytical domain.[16] We also observe embryonic death during midgestion, specifically between e13 and e15. Severe fetal anemia was observed due to defective erythropoiesis in the SENP1–/– embryos because of insufficient erythropoietin (Epo) production which is required for red blood cell production. SENP1 dictates stability of hypoxia inducible factor-1 α (HIF1α) and thereby modulates transcription of the HIF1α-regulated *Epo* gene with onset of hypoxia. In the absence of SENP1, HIF1α is SUMOylated and subsequently degraded via a ubiquitin/proteosome-dependent pathway. Hence, in SENP1–/– embryos, inadequate transcription of *Epo* and consequently fetal anemia is observed due to enhanced degradation of HIF1α.

Loss of SENP2 in mice, using the lox-Cre system, also causes embryonic lethality due to a placental defect; SENP2–/– embryos die between e10.5 and e11.5.[50] Trophoblast stem cell differentiation plays a pivotal role in the formation of a normal placental. SENP2 ablation increases p53 levels and thereby impairs cell cycle progression (discussed above) and maturation of trophoblasts.

Collectively these studies indicate that deSUMOylation via the isopeptidase activity of SENP1 and SENP2 are critical for normal mammalian development. However, the contribution of the other four SUMO proteases to development has not been investigated.

CONTRIBUTION TO PATHOPHYSIOLOGICAL CONDITIONS

As discussed above, the isopeptidase activity of the SUMO proteases is heavily dependent upon the level expressed in the cell. Under normal physiological conditions, a balance must exist between the level of SUMO conjugated and deconjugated target proteins. Changes in the expression level of one SENP would greatly effect this equilibrium; where induction of a specific SENP would facilitate greater deSUMOylation of cellular substrates and inversely reduction of a SENP would enhances SUMO conjugation of target proteins. Hence, it is feasible that shifts in this SUMOylation/deSUMOylation equilibrium could contribute to the onset of various pathophysiological conditions. In fact, several carcinomas do exhibit altered levels of the SUMO machinery including changes in SENP expression (Table 2).[5,54-61]

In situ hybridization studies indicate that SENP1 mRNA levels are elevated in precancer prostatic intra-epithelial neoplasia (PIN) lesions and remain elevated with the onset of prostate carcinoma.[5] Our previous studies in prostate cancer cells suggest that induction of SENP1 prompts several oncogenic pathways.[5,32,38,40] Therefore, we generated

Table 2. SUMO machinery and cancer

	Altered Expression	Cancer	Reference
SUMO Conjugating Proteins			
Ubc9	Single Nucleotide Polymorphisms	Breast	(60)
	Upregulated	Melanoma Ovarian Lung Adenocarcinoma	(54, 57-58)
PIAS3	Upregulated	Breast Lung Adeno-carcinoma Prostate Colorectal Brain	(61)
SUMO Deconjugating Proteins			
SENP1	Fused Gene	Infantile Sacrococcy-geal Teratoma	(55)
	Upregulated	Prostate Thyroid Oncocytic Adenocarcinoma	(5, 59)
SENP6	Fused Gene	T-cell lymphoma	(56)

transgenic mice with the mouse SENP1 transgene fused to androgen-driven promoter, which prompted enhanced expression of SENP1 in mouse prostate epithelial cells. Initial studies reveal aberrant epithelial hyperplasia and signs of low-grade PIN in 2 founder transgenic mice.[5] Currently, we are further characterizing and delineating the mechanism for the prostate dysplasia in 2 transgenic mice lines. These studies provide initial insight into how deregulation of SENP1 could contribute to carcinogenesis. SENP1 induction has also been reported in thyroid oncocytic adenocarcinoma[59] but how this induction participates in the onset of the adenocarcinoma is currently undefined.

In contrast, SENP1 mRNA and proteins levels are significantly decreased in the synovial fibroblasts from rheumatoid arthritis patients as compared to osteoarthritis patients. Meinecke and colleagues suggest that the reduction of SENP1 allows the fibroblasts to remain resistant to Fas-induced apoptosis and contribute to the development of a more aggressive disorder. Reduced SENP1 causes enhanced SUMO1 conjugation of PML, which in turn recruits and sequesters the proapoptotic protein DAXX in the PML nuclear bodies. Therefore, increasing SENP1 levels would be advantageous for regulating the pathogenesis of this disorder.

Chromosomal translocation and generation of a fusion gene is a common event in cancer and two *SENP* genes are subject to this genetic phenomenon. In a patient with germ cell tumor specifically infantile sacrococcygeal teratoma, there is a translocation of a portion of chromosome 12 that leads to the fusion of the first 2 exons of SENP1 with a portion of the embryonic polarity-related mesoderm development gene MESDC2 to generate 2 fusion genes: SENP1-MESDC2 (SEME) and MESDC2-SENP1 (MESE).[55] SEME prevents the translocation of MESDC2 to the endoplasmic reticulum. The MESE fusion gene maintains normal SENP1 isopeptidase activity as observed on SUMO-conjugated PML however it remains unknown whether SENP1 substrate specificity is lost. In addition, the role of SENP1 in additional cellular funtions could be affected since the SENP1 expression is under the control of the MESDC2 promoter with the MESE gene.

Similarly, SENP6/SUSP1 fuses to the novel gene T-cell lymphoma breakpoint associated target 1 (TCBA1) in the HT-1 cell line which is derived from a T-cell lymphoblastic lymphoma.[56] The SENP6 is truncated after nucleotide 550 and fused to TCBA1 only in HT-1 cells. The authors suggest that the fusion gene may contribute to tumorigenesis but the mechanism remains undefined.

CONCLUSION AND CURRENT PERSPECTIVE

Initial studies established the role of the cysteine proteases in cell cycle progression and regulation of gene transcription. Recent studies in whole animals have expanded our knowledge of the importance of mammalian SUMO-specific proteases in embryonic development and carcinogenesis. However, many questions remain unanswered especially with respect to the mammalian SENPs. The biological function of several SENPs is currently undefined. It is unclear what allows the SUMO proteases to discriminate amongst the SUMO-conjugated proteins. It is known from the current literature that the protease and SUMOylated protein must be in the same subnuclear locale and the SENP exhibit specificity for deconjugating a particular SUMO isoform. What other factors are involved? Could specific signals be responsible for the recruitment of a specific SENP to mediate a specific cellular or physiological event? Could facilitating or hindering SENP recruitment be used as a therpeutic target to prevent the onset of a disease and/or disorder?

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CHAPTER 15

REGULATORY FUNCTIONS OF UBIQUITIN AND SUMO IN DNA REPAIR PATHWAYS

Stefan Jentsch* and Stefan Müller*

Abstract: Ubiquitin and SUMO are structurally related protein modifiers that are covalently attached to lysine residues of target proteins. While ubiquitin is traditionally known as a signal for proteasomal degradation, its nondegradative actions are equally important in the control of cellular key processes. Similarly, the SUMO system primarily acts in a nondegradative manner. Accumulating evidence indicates that these nonproteolytic functions of ubiquitin and SUMO are particularly important in the control of the DNA damage response network, which coordinates a set of DNA repair pathways and allows cells to cope with different types of genotoxic stress. In this chapter we will illustrate some key functions of ubiquitin and SUMO in the control of selected DNA repair pathways.

MECHANISMS OF UBIQUITIN AND SUMO CONJUGATION

The reversible posttranslational modification of proteins provides a versatile mode to control cellular functions. The modification of specific amino acids by small chemical groups or macromolecules typically regulates the dynamics and specificity of protein-protein interactions by attracting or repelling a binding partner. In many cases this directs the assembly or disassembly of protein complexes. A paradigm is the phospho-regulated modulation of protein networks, where a binding partner is recruited to a phosphorylated amino acid residue through docking of a defined phospho-binding module.[1] Ubiquitin and its relative SUMO are structurally related small protein modifiers that are covalently attached to lysine residues of target proteins (Fig. 1).[2] A single, highly conserved ubiquitin form is found in all eukaryotes. SUMO is evolutionary less conserved

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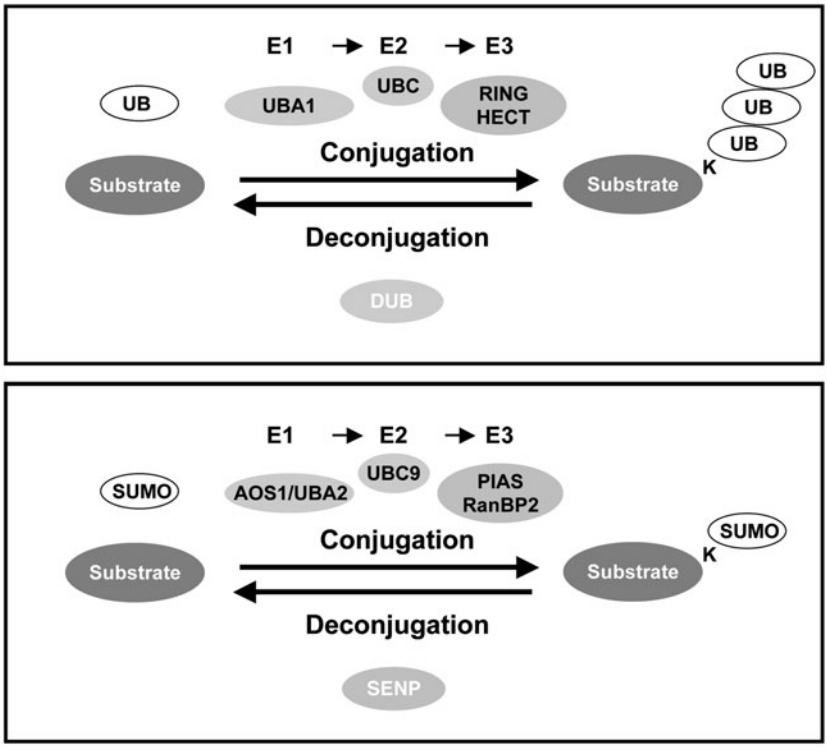

Figure 1. The pathways of ubiquitin and SUMO conjugation and deconjugation. The modifiers ubiquitin (UB) (top) and SUMO (bottom) are attached to lysine residues of target proteins. Conjugation generates an isopeptide bond between the C-terminal glycine residues of UB/SUMO and an ε-amino group of a lysine residue. Conjugation to internal lysine residues of UB and SUMO can lead to the formation of poly-chains. The respective conjugation processes involve E1 activating enzymes, E2 conjugating enzymes (UBCs) and E3 enzymes. In the ubiquitin system RING-type and HECT ligases comprise the largest ligase families, while in the SUMO system RanBP2 and members of the PIAS family are best characterized. Demodification of ubiquitin is catalyzed by deubiquitinating enzymes (DUBs), while SUMO is deconjugated by members of the SENP family.

and more diversified. Lower eukaryotes have a single SUMO protein, while vertebrates express three SUMO paralogs (SUMO1, 2 and 3). Ubiquitin and all SUMO forms are synthesized as precursor proteins that are processed prior to conjugation. The respective conjugation processes, termed SUMOylation or ubiquitylation, further require an E1 activating enzyme, an E2 conjugating enzyme and in most cases involve E3 ligases as specificity factors. SUMOylation uses Ubc9 as the sole E2 enzyme, while ubiquitylation can be catalyzed by a family of E2 ubiquitin conjugating (Ubc) enzymes. Only few E3 ligases have been identified in the SUMO pathway so far, whereas the two major classes of E3 ubiquitin ligases, HECT-domain and RING-type ligases, comprise large protein families. Importantly, SUMOylation and ubiquitylation are reversible processes. In human cells about 100 deubiquitylating enzymes, which can be subdivided in five families, have been identified. Deconjugation of SUMO from target proteins is catalyzed by SUMO-specific cysteine proteases of the SENP/Ulp family, which consists of Ulp1 and Ulp2 in yeast and has six members in humans.

Ubiquitin and SUMO are attached to proteins either as monomers or as polymeric chains that are linked via internal lysine residues. Distinct functions of mono- or polymodification with SUMO are just beginning to be uncovered, but at least in some contexts polymeric chains of SUMO2 appear to destabilize proteins by recruiting a SUMO-binding ubiquitin ligase.[3,4] The functional divergence of mono- and polymodification in the ubiquitin pathway is well established. Further functional diversity is generated by the use of distinct residues for polyubiquitin chain formation. For example, K48-linked polyubiquitin chains typically mark proteins for proteolytic degradation by serving as a targeting signal to the proteasome. Monoubiquitylation or K63-linked chains in turn act in a nondegradative way in cellular signaling pathways, mostly by modulating distinct protein-protein interactions. Both, the proteolytic and nonproteolytic functions depend on the recognition of an ubiquitylated substrate by specialized interaction modules. Currently over 15 different types of ubiquitin-binding modules have been defined and at least some exhibit monoubiquitin-specific or linkage-selective polyubiquitin binding.[5] Signaling by SUMO appears to follow the same general principle and accordingly involves the noncovalent interactions of SUMO conjugates to specialized SUMO interaction motifs (SIMs).[6]

UBIQUITIN AND SUMO AS KEY REGULATORS OF DNA REPAIR PATHWAYS

While proteasomal degradation has long been considered to be the central function of ubiquitin, it is now evident that the nondegradative ubiquitin actions govern many critical cellular processes. This concept has been expanded by the discovery of ubiquitin-like modifiers, which mostly do also not serve as address tags to the proteasome. Genetic and biochemical studies have revealed a particularly important role of nonproteolytic actions of ubiquitin and SUMO in DNA repair pathways and many components of these pathways were identified as substrates for ubiquitylation or SUMOylation.[7]

DNA damage results from the continuous exposure of cellular DNA to endogenous or exogenous agents, such as reactive oxygen species, γ-irradiation or UV light. The repair of the resultant DNA lesions or the elimination of cells that have accumulated severe lesions is of crucial importance for genome integrity and tumor prevention in mammals.[8] To correct the different types of DNA lesions highly complex repair systems have evolved. All rely on the coordinated action of protein networks and require the timely and spatially controlled assembly and disassembly of protein complexes. Recent work demonstrated that many of these processes are orchestrated by posttranslational modifications. In this chapter we will illustrate some of the basic regulatory principles of SUMO- and ubiquitin-dependent control of selected DNA repair pathways. We will particularly focus on the intersections of SUMO and ubiquitin in these pathways and the crosstalk of both modifiers to other posttranslational modification systems.

A SUMO-UBIQUITIN SWITCH ON PCNA

The crosstalk of ubiquitin and SUMO is impressively illustrated on the proliferating cell nuclear antigen (PCNA) (Fig. 2). PCNA is essential for a variety of DNA transactions, including DNA replication and DNA repair.[9] It is loaded as trimeric ring on DNA strands and functions as a processivity factor for DNA polymerases and as a binding

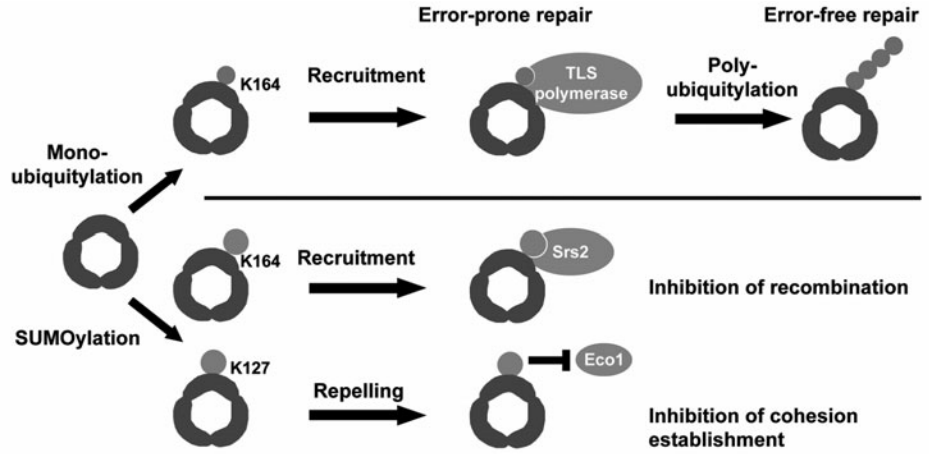

Figure 2. A SUMO-ubiquitin switch on PCNA. PCNA is loaded as a trimeric ring on DNA strands and acts as a processivity factor for DNA polymerases and as a binding platform for replication-associated factors. In S phase PCNA is alternatively modified by monoubiquitylation, K63-linked polyubiquitylation and SUMOylation at K164. SUMOylation also targets K127. Ubiquitylation is triggered by DNA damage and is essential for DNA repair by the error-free and error-prone branches of the *RAD6* pathway. Monoubiquitylation, which is catalyzed by the E2 enzyme Rad6 together with the E3 ligase Rad18, facilitates TLS synthesis by recruitment of TLS polymerases that contain specific ubiquitin binding motifs. K63-linked polyubiquitylation involves the dimeric E2 Mms2/Ubc13 and the ligase Rad5. How multiubiquitylation directs PCNA to the error free pathway is still unknown. SUMOylated PCNA inhibits recombination by triggering the recruitment of the anti-recombinogenic factor Srs2 via a SIM in Srs2. SUMOylation of K127 inhibits PCNA/PIP-box interactions, as demonstrated for the cohesion-linked protein Eco1.

platform for additional factors that regulate replication-associated processes, such as sister chromatid cohesion. PCNA can be alternatively modified by monoubiquitylation, K63-linked polyubiquitylation and SUMOylation at a specific lysine residue (K164), which is conserved from yeast to the mammalian PCNA.[10] Both, ubiquitylation and SUMOylation of PCNA occur in S phase and the ubiquitylation is induced upon DNA damage. In a series of biochemical and genetic experiments it was demonstrated that ubiquitylation of PCNA is essential for DNA repair by the *RAD6* pathway, which acts when replication forks have encountered a lesion in the DNA template. These lesions can either be bypassed by translesion synthesis (TLS), which uses error-prone TLS polymerases, or resolved by an error free mechanism, which relies on template switch and recombination.[8,10] Preferential activation of the error-free or the error-prone branch may depend on the specific type of lesion. Importantly, polyubiquitylation of PCNA is elementary for the error-free branch of DNA repair, while monoubiquitylation triggers error-prone repair. Monoubiquitylation is catalyzed by the E2 conjugating enzyme Rad6 in conjunction with the RING-type E3 ligase Rad18.[10] The monoubiquitylated PCNA facilitates TLS synthesis by recruitment of TLS polymerases that contain specific ubiquitin binding motifs.[11] In the error-free branch extension of monoubiquitin to the K63-linked ubiquitin chains involves the dimeric E2 enzyme Mms2/Ubc13 and the RING-type ligase Rad5.[10] How polyubiquitylation directs PCNA to the error free pathway is still unknown, but K63-linked chains may favor interaction with a specific binding partner.

Independent from DNA damage, lysine 164 of PCNA is also targeted by SUMO in S-phase.[10] SUMOylated PCNA facilitates the recruitment of the anti-recombinogenic factor Srs2 via a C-terminal SIM in Srs2.[12,13] The recruitment of Srs2 inhibits recombination by displacing the recombinase Rad51 from chromatin. SUMOylation of PCNA therefore helps to prevent unwanted recombination events during the replication process. In addition to K164, SUMO targets a second, nonconserved lysine residue in yeast PCNA (K127). Interestingly, K127 resides in the critical binding region for PCNA interacting proteins, which contact PCNA via a so-called PIP-box (PCNA-interacting protein) motif. SUMOylation of K127 seems to prevent PCNA/PIP-box interactions, as demonstrated for the cohesion-linked protein Eco1, whose PIP-box-dependent binding to PCNA competes with SUMOylation.[14] In summary, these data substantiate the concept that the ubiquitin/SUMO switch on PCNA is a crucial regulatory mechanism that allows the exchange of binding partners on PCNA.

AN INTRAMOLECULAR SUMO/SIM-MEDIATED INTERACTION CONTROLS A CRITICAL STEP OF BASE EXCISION REPAIR

SUMO/SIM-dependent recruitment of Srs2 to PCNA exemplifies the general principle of how SUMO-regulated changes in intermolecular interactions can affect protein functions. A prominent example of direct protein regulation by an intramolecular SUMO/SIM-dependent interaction has been elucidated in the base excision repair (BER) pathway (Fig. 3). BER is a system for the selective replacement of irregular bases in DNA duplexes.[8] BER is initiated by a DNA glycosylase that recognizes and removes the aberrant base. Thymine DNA glycosylase (TDG) releases thymine or uracil from G:T and G:U mismatches. After base excision the enzyme remains first bound to the resultant so-called abasic site (AP-site) most likely to avoid exposure of this potentially harmful repair intermediate. The subsequent release of TDG is coordinated with the transfer of the lesion to the AP-endonuclease (APE), which together with a cascade of downstream enzymes completes the repair process. TDG is modified by SUMO and initial biochemical data provided evidence that the unmodified TDG is the high affinity DNA binding form that is released from the AP-site by SUMO conjugation.[15] The recently solved crystal structure of the TDG-SUMO conjugate strongly supports this model.[16] The structure reveals a SUMO/SIM-dependent intramolecular interaction that induces a conformational change in TDG. This leads to the formation of an α helix, which protrudes from the surface of TDG and likely interferes with DNA binding. This work therefore illustrates that at least in some contexts SUMOylation can affect protein function by inducing structural changes.

A UBIQUITYLATION CASCADE FOR THE ASSEMBLY OF DNA REPAIR COMPLEXES ON CHROMATIN

DNA double-strand breaks (DSBs) are particularly hazardous lesions because a failure in repair will generate gross chromosomal rearrangements that can lead to tumor formation in mammalian cells. DSBs are repaired by either the homologous recombination (HR) or the nonhomologous end-joining pathways.[8] Among the key factors of DSB repair in mammals are the adaptor protein MDC1, the recombinase RAD51

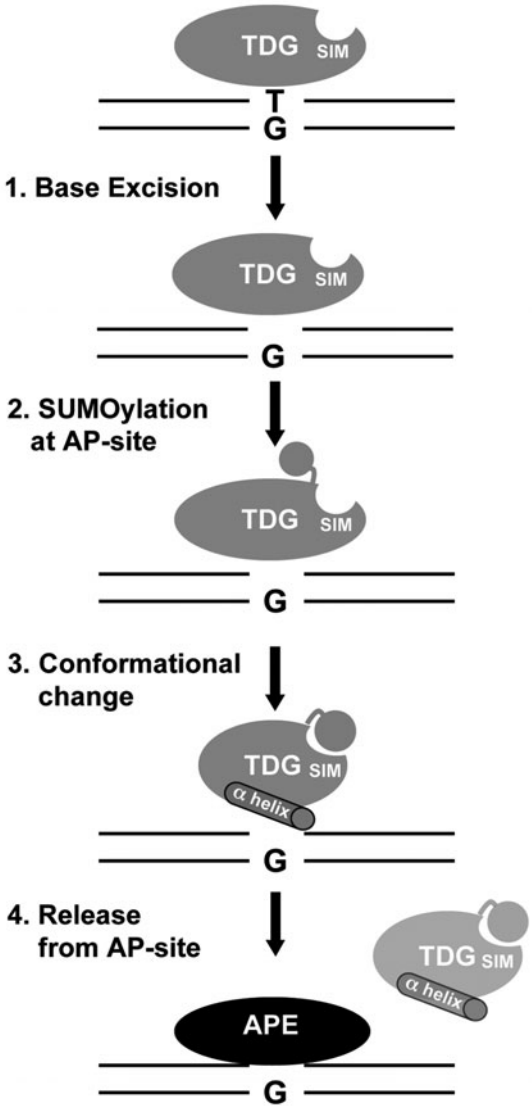

Figure 3. A model for the SUMO-mediated release of TDG from DNA. Initially TDG recognizes a G:T or a G:U mismatch and removes the aberrant base. The enzyme remains bound to the abasic site (AP-site) and undergoes SUMO modification. A SUMO/SIM-dependent intramolecular interaction leads to the formation of an α helix, which protrudes from the surface of TDG and likely interferes with DNA binding. The release of TDG is coordinated with the binding of the AP-endonuclease (APE) to the lesion.

and the ubiquitin-ligase BRCA1, which is lost or inactivated by mutations in familial breast cancer. A hallmark of the DNA damage response is the rapid accumulation of BRCA1 and other repair factors in subnuclear foci. These foci are formed around the DSB and are marked by the phosphorylated form of the histone variant H2AX, known as γH2AX. Recent work has delineated a pathway that integrates phosphorylation and

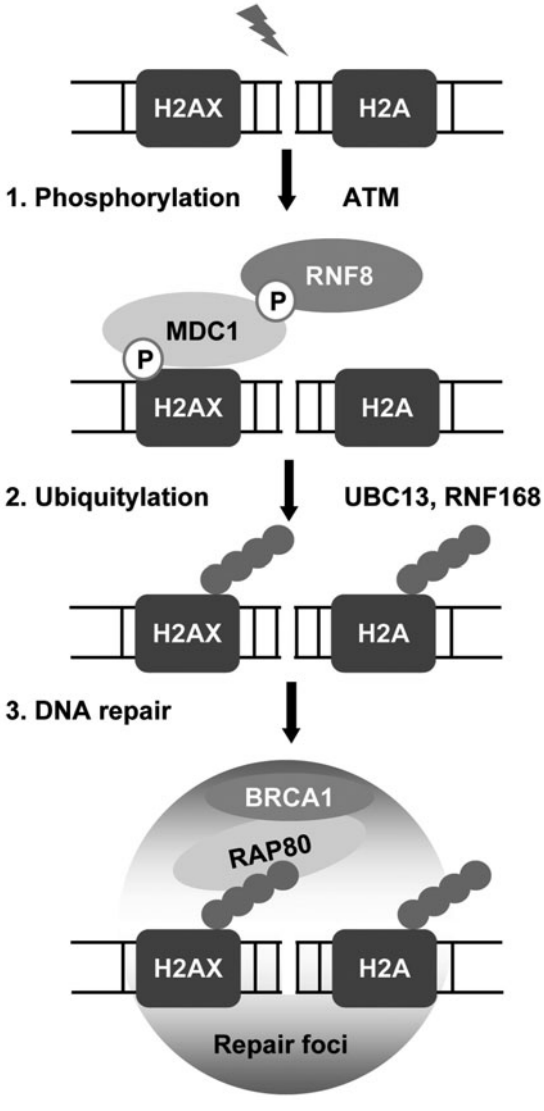

Figure 4. Ubiquitin-mediated assembly of repair foci at chromatin. A DSB in DNA activates the checkpoint kinase ATM, which phosphorylates H2AX thereby providing a docking site for MDC1, which is itself a substrate for ATM. Phosphorylated MDC1 is bound by the phospho-binding FHA domain of the ubiquitin-ligase RNF8, which cooperates with the E2 enzyme UBC13 to mediate K63-linked ubiquitylation of histones, including H2A and H2AX. The ubiquitylation is amplified by RNF168, another RING-type ubiquitin ligase, which contains a K63-specific ubiquitin-binding domain (UIM). The K63-linked chains also serve as an anchor for the UIM-domain of the RAP80 protein, which finally brings BRCA1 into repair foci.

ubiquitylation-dependent processes to trigger the assembly of these structures on the damaged chromatin[17] (Fig. 4). The signaling cascade is initiated by recognition of the break by the MRN (MRE11-RAD50-NBS1) complex, which acts as a break sensor

and recruits the checkpoint kinase ATM to phosphorylate H2AX. γH2AX provides a docking platform for the adaptor protein MDC1, which is itself a substrate for ATM. Phosphorylated MDC1 is bound by the phospho-binding FHA domain of the RING-type ubiquitin-ligase RNF8 thereby transmitting the phosphorylation signal to a component of the ubiquitin system. RNF8 cooperates with the E2 enzyme UBC13 to mediate K63-linked polyubiquitylation of histones, including H2A, γH2AX, H2B and H3. This ubiquitylation process is sustained and amplified by the ubiquitin-dependent recruitment of RNF168, another RING-type ubiquitin ligase, which contains a K63-specific ubiquitin-binding domain (UIM). The K63-linked chains further serve as an anchor for the UIM-domain of the RAP80 protein, a binding partner of BRCA1 that finally brings BRCA1 into repair foci. The role of BRCA1 at the sites of DNA damage has not yet been fully established, but it may function to amplify or remodel the existing ubiquitin chains. In summary this pathway provides an intriguing example for the crosstalk of phosphorylation and ubiquitylation in the DSB break response.

Ubiquitin-dependent chromatin recruitment of repair factors is also hallmark of the Fanconi anemia (FA) repair pathway. FA is a rare genetic disease that is characterized by developmental defects and susceptibility to cancer.[18] 13 distinct FA complementary groups have been identified and the corresponding proteins have been assigned to the FA pathway. Components of the FA pathway are thought to integrate NER, HR and TLS to allow repair of DNA interstrand crosslinks. Importantly, the eight proteins of the FA core complex form a large multi-subunit ubiquitin ligase with FANCL being the catalytic core of the complex. Substrates for ubiquitylation are the downstream FA proteins FANCD2 and FANCI, which are both monoubiquitylated in response to DNA damage. Importantly, the modification is required for localization of both proteins to chromatin-associated γH2AX-positive repair foci, which contain many of the above-mentioned factors, including RAD51 and BRCA1. The exact function of monoubiquitylated FANCD2 and FANCI in repair foci is still unknown, but ubiquitylation is clearly an essential step in FA activation and is indispensable for the DNA repair process, since cells expressing ubiquitin-deficient variants of FANCD2 or FANCI are hypersensitive to DNA cross-linking agents. Interestingly, the loss of the deubiquitylation enzyme USP1, which catalyzes the deubiquitylation of FANCD2 and FANCI, leads to a similar hypersensitivity indicating that a tightly balanced level of FANCD2 and FANCI ubiquitylation is critical for the proper functioning of this pathway.[19]

SUMO-DEPENDENT SUBNUCLEAR COMPARTMENTALIZATION OF DOUBLE-STRAND BREAKS AND TELOMERES

While DNA repair by HR is important for the maintenance of genomic integrity, repetitive DNA elements, such as telomere ends or ribosomal DNA (rDNA) repeats, are prone to undesired homologous recombination events. One way to deal with this problem appears to be the spatial control of HR processes. To protect the nucleolar rDNA repeats from uncontrolled recombination HR components are largely excluded from the nucleolus.[20] For association with the recombination machinery and recombinatorial repair a double-strand break in rDNA must therefore be relocated from the nucleolus to an extranucleolar site. In the yeast *Saccharomyces cerevisiae* these nuclear sites are marked by the Rad52 protein, which mediates the exchange of the early recombination factor RPA by the recombinase Rad51. Rad52 is modified by SUMO upon induction

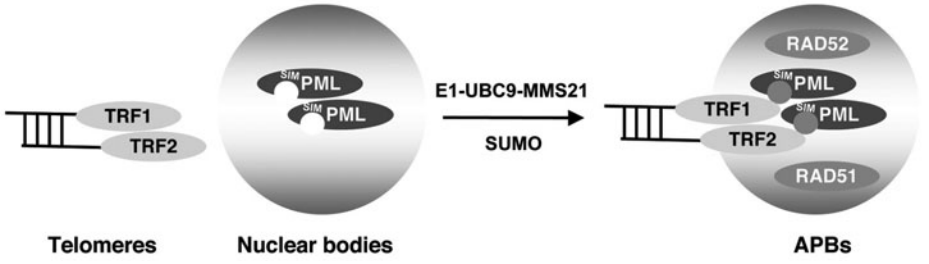

Figure 5. A model of SUMO-mediated recruitment of telomeres to ALT-associated PML-NBs. SUMOylation of the telomere binding proteins TRF1 and TRF2 by the E3 ligase MMS21 is required for the recruitment of telomeres to ALT-associated PML nuclear bodies (APBs) that contain PML and repair factors, including RAD51 and RAD52. Direct binding of SUMOylated TRF1/TRF2 to the SIM region of PML has not yet been demonstrated experimentally.

of double-strand breaks and the modification appears to protect the protein from ubiquitin-dependent degradation.[21] Moreover, SUMO modification of Rad52 is crucial for repair of an rDNA lesion by mediating the nucleolar exclusion of the break.[22] Mutations that abrogate SUMOylation result in the formation of Rad52 foci within the nucleolus and cause rDNA hyper-recombination thus highlighting the crucial importance of the nuclear relocation for the protection of rDNA repeats from unwanted recombination. The mechanistic details of the relocation are still unclear, but it seems that break sensing by the yeast MRX complex, the counterpart of the mammalian MRN complex and SUMOylation of Rad52 take place in the nucleolus. The subsequent SUMO-dependent compartmentalization of rDNA-associated Rad52 may involve enhanced binding of the SUMO-moiety to a nucleoplasmic SIM-containing interaction partner or be the result of decreased interaction with a nucleolar component.

Telomeric chromosome ends resemble double-strand breaks and therefore are also potential substrates for HR. Normally this is prevented by protection of telomere ends with telomere-binding proteins. However, telomerase-negative cancer cells explicitly make use of a HR pathway to maintain telomere length. This pathway of alternative lengthening of telomeres (ALT) is compartmentalized in specific subnuclear structures termed ALT-associated PML bodies (APBs)[23] (Fig. 5). PML nuclear bodies that are defined by the presence of the PML tumor suppressor are found in all mammalian cells and control a wide variety of cellular processes. ABPs are a specialized type of PML bodies that contain telomeres and many repair factors, such as the MRN complex, RAD51, RAD52 or BRCA1, supporting the idea that HR-mediated ALT occurs within these structures. Interestingly, depletion of the SUMO ligase MMS21 in an ALT-cell line causes progressive shortening of telomeres and telomere dysfunction indicating that SUMOylation is critically involved in the ALT pathway.[24] MMS21 has indeed shown to be essential for the recruitment of telomeres to these structures. Critical targets of MMS21-induced SUMOylation in this process are the telomere-binding proteins TRF1, TRF2 indicating that their modification tethers telomeres to ABPs. A receptor of SUMOylated TRF1/2 within APBs has not been identified, but a likely candidate is the PML protein itself, as a SIM in PML has already been demonstrated to be involved in the recruitment of SUMO-modified proteins to canonical PML NBs.

Interestingly, inhibition of Mms21 in budding yeast impairs the clustering of telomeres at the nuclear periphery suggesting that in yeast the SUMO system is also involved in the

subnuclear compartmentalization of potentially hazardous DNA elements.[25] In support of this idea persistent, irreparable double-strand breaks are also tethered to the nuclear periphery in a process that involves SUMOylation of the histone variant H2A.Z and the nuclear pore associated Slx5/Slx8 SUMO-dependent ubiquitin ligase.[26,27] Although the exact underlying mechanisms and the functional consequences of these processes are not fully understood, the ensemble of these observations substantiate the view that the SUMO system may exert a conserved function to sequester telomeres and DSB away from bulk DNA.

CONCLUSION AND FUTURE PERSPECTIVES

While accumulating data support the concept that the combinatorial use and the interconnections of posttranslational modification systems expand the regulatory repertoire in signaling pathways, we may currently only see the tip of the iceberg. For example, the recent work on the RNF4/Slx5-Slx8 pathway has not only unraveled the novel concept of SUMO-dependent ubiquitin-ligases, but has also provided evidence for the existence of mixed ubiquitin-SUMO chains.[3] To understand the function and the signaling mechanisms of these chains as well as the interdependencies of SUMO and ubiquitin with other modifications will therefore be a major challenge in the future.

ACKNOWLEDGEMENTS

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CHAPTER 16

SUMOYLATION AS A SIGNAL FOR POLYUBIQUITYLATION AND PROTEASOMAL DEGRADATION

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Gerrit J.K. Praefcke and R. Jürgen Dohmen*

Abstract: The small ubiquitin-related modifier (SUMO) is a versatile cellular tool to modulate a protein's function. SUMO modification is a reversible process analogous to ubiquitylation. The consecutive actions of E1, E2 and E3 enzymes catalyze the attachment of SUMO to target proteins, while deconjugation is promoted by SUMO specific proteases. Contrary to the long-standing assumption that SUMO has no role in proteolytic targeting and rather acts as an antagonist of ubiquitin in some cases, it has recently been discovered that sumoylation itself can function as a secondary signal mediating ubiquitin-dependent degradation by the proteasome. The discovery of a novel family of RING finger ubiquitin ligases bearing SUMO interaction motifs implicated the ubiquitin system in the control of SUMO modified proteins. SUMO modification as a signal for degradation is conserved in eukaryotes and ubiquitin ligases that specifically recognize SUMO-modified proteins have been discovered in species ranging from yeasts to humans. This chapter summarizes what is known about these ligases and their role in controlling sumoylated proteins.

INTRODUCTION

Posttranslational protein modification is one of the main cellular mechanisms to regulate the fate of a protein. Ubiquitin and SUMO (small ubiquitin-like modifier) are the most prominent members of a conserved family of ubiquitin-like (UBL) posttranslational modifiers (for a recent review, see ref. 1). UBL proteins share an analogous structure termed the ubiquitin fold. In addition, they have a similar conjugating machinery comprised of specific activating and conjugating enzymes, termed E1 and E2, respectively, that catalyse

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Conjugation and Deconjugation of Ubiquitin Family Modifiers, edited by Marcus Groettrup.

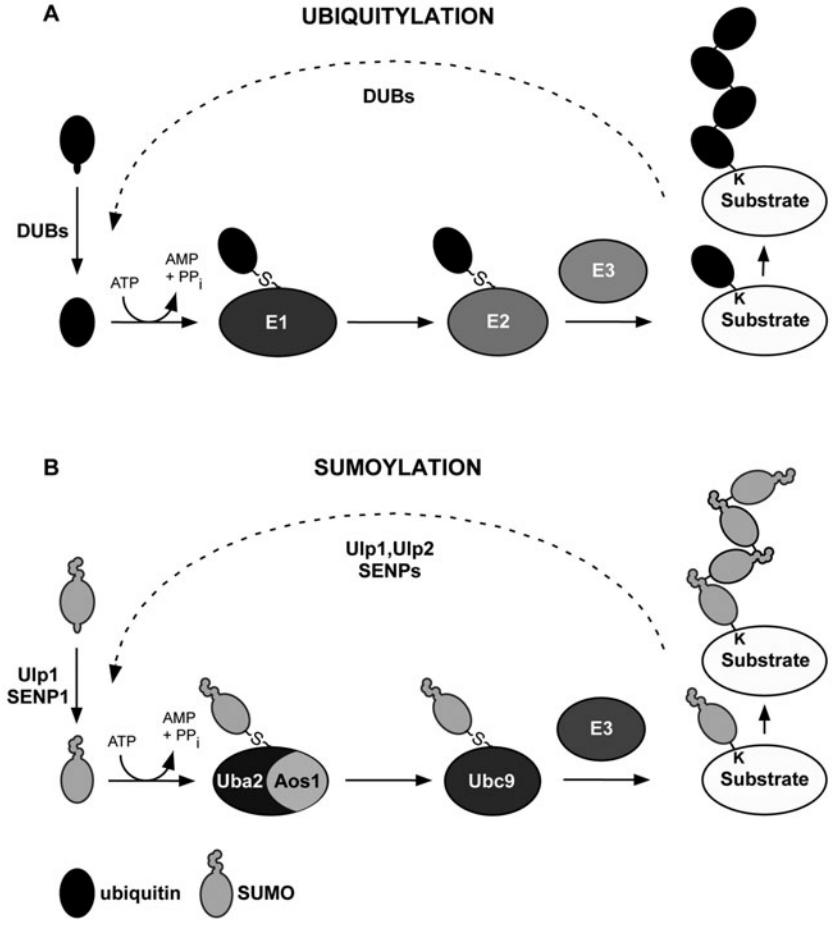

Figure 1. Comparison of ubiquitin and SUMO conjugation systems. Ubiquitin (Ub) and SUMO conjugation involves the activities of related enzymes. A) After processing of its precursor forms by deubiquitylating enzymes (Dub), Ub is activated by Ub-activating enzyme (E1) und subsequently conjugated to substrates by complexes of Ub-conjugating enzymes (E2) and Ub ligases (E3). Attachment of Ub to Ub leads to the formation of substrate attached chains. Ub isopeptidases (Dub) can regenerate free Ub from substrates. B) The analogous enzymes as shown in A) are shown for the SUMO system. Here the activating enzyme is composed of two subunits (Uba2 and Aos1) and only a single conjugating enzyme (Ubc9) is used. Desumoylating enzymes are Ulp1 and Ulp2 in budding yeast and several SENPs in mammals.

the attachment of an UBL via its C-terminal glycine residue to a lysine residue of the target protein via an amide bond. Substrate specificity is achieved by the activity of additional UBL ligases (E3), which form complexes with specific E2 proteins (Fig. 1).

Invertebrates and yeasts express only one variant of SUMO, while mammals express three conjugatable SUMO variants. In *Saccharomyces cerevisiae*, SUMO is encoded by a single gene (*SMT3, for Suppressor of Mif Two*), which was originally isolated as a high-copy suppressor of mutations affecting the centromeric protein Mif2.[2,3] While the budding yeast *SMT3* gene is essential for viability, its fission yeast orthologue *pmt3* is not.[4,5] Mutants lacking *pmt3,* however, display severe defects in genome maintenance and

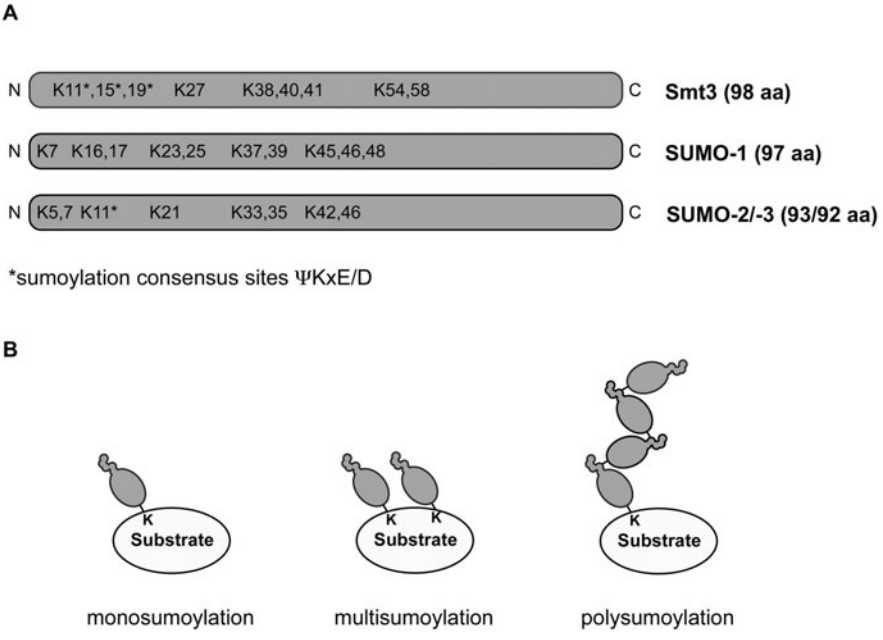

Figure 2. Yeast and mammalian SUMO orthologues and paralogues. A) Shown is a schematic representation of the single essential budding yeast SUMO (Smt3) and the three conjugatable SUMO isoforms found in mammals (SUMO-1, SUMO-2 and SUMO-3). The latter are nearly identical differing only in 3 residues. The positions of Lys (K) residues are indicated. Sumoylation consensus sites (Ψ KxD/E, in which Ψ is a hydrophobic residue and x a variable residue) that are involved in the formation of SUMO chains are marked with asterisks. B) Depicted are the different types of SUMO modification referred to in the main text.

are barely viable.[5] The three mammalian SUMO isoforms (SUMO-1,-2 and -3) differ in sequence and function. SUMO-1 is up to 44% identical to SUMO-2/-3, while SUMO-2 and SUMO-3 are 97% identical. The latter are assumed to carry out largely overlapping functions. While sumoylation in general appears to be essential in mammals, as can be concluded from the lethality of *UBC9* ablation,[6] conjugation of SUMO-2 and SUMO-3 appear to be sufficient to compensate for a loss of SUMO-1 because mice with an inactivated SUMO-1 gene were reported to be viable.[7] Unlike SUMO-1, SUMO-2/-3 supply the main reservoir of free SUMO for conjugation in response to certain stress stimuli.[8-10]

SUMO can be attached either to a single or to multiple lysine (Lys) residues within a target protein (mono- or multisumoylation), which are often part of a ΨKxD/E consensus motif that is directly recognized by the SUMO conjugation enzyme Ubc9 (Fig. 2).[11,12] Similar to ubiquitin, SUMO is also attached to Lys residues within SUMO itself, which leads to the formation of SUMO chains (polysumoylation).[13,14] SUMO chain formation mainly involves Lys residues within the above-mentioned consensus motifs, three of which (K11, K15 and K19) are present in an N-terminal domain of *S. cerevisiae* SUMO, while only one (K11) is found in SUMO-2 and SUMO-3 of mammals (Fig. 2).[11,13,15] SUMO-1 in contrast, lacks a Lys residue in such a consensus motif, which appears to be the reason why it does not form chains efficiently.[13] SUMO-1, however, may be attached to Lys residues within SUMO-2/3 chains thereby preventing the elongation of such chains.[16]

The SUMO pathway has important functions in the regulation of a large variety of cellular processes such as progression through the cell cycle, transcriptional regulation, DNA repair, stress responses, cancer and aging.[17-27] The molecular mechanisms underlying the role of SUMO modification in these processes is still incomplete. Generally, with the exception of RanGAP1, only a small fraction of a given protein pool is sumoylated at a certain point in time.[19] Supported by exemplary cases, it is assumed that SUMO modification alters protein function by affecting protein-protein interactions or subcellular localization. Until recently, SUMO and ubiquitin were thought to have opposing functions with sumoylation saving proteins from degradation by occupying the same lysine residue that is required for their ubiquitylation. The example leading to this concept was the modification of IκBα, an inhibitor of the transcriptional activator NF-κB.[28] Upon activation of the inflammatory response pathway, IκBα is ubiquitylated and degraded by the proteasome releasing active NF-κB.[297] IκBα, however, can be sumoylated on the same lysine residue preventing its ubiquitylation and leading to a stabilization of the protein.[28,30] Based on this finding it has been suggested that SUMO modification of IκBα serves to inhibit the induction of NF-κB-dependent transcription.[28]

Another example of SUMO-ubiquitin crosstalk was revealed by studies on postreplicative DNA repair and its key player PCNA (proliferating cell nuclear antigen). Also for PCNA, sumoylation and ubiquitylation target the same lysine residue, but in this case SUMO modification blocking ubiquitylation apparently does not only prevent ubiquitylation but in addition directs PCNA to a distinct interaction. PCNA is monoubiquitylated by Rad6 and Rad18 at the conserved lysine residue 164 in response to DNA damage, while the assembly of a Lys63-linked ubiquitin chain is catalyzed by a different subset of enzymes.[31,32] Monoubiquitylation triggers error-prone bypass replication, whereas polyubiquitylation results in an error-free bypass mode.[31] SUMO conjugation to Lys164 seems to inhibit DNA repair when the *RAD6* pathway is not functional.[33,34] Sumoylated yeast PCNA recruits the helicase Srs2, which prevents Rad51-dependent recombination by disrupting nucleoprotein filaments required for recombination and thereby prevents unwanted sister chromatid recombination during replication.[35] In this case, SUMO and ubiquitin can operate on the same lysine residue as a switch between different functional forms of a given protein.

The two examples described above gave only a glimpse to the range of functional interactions of these two Ubl modifier systems. Recent discoveries indicated that interconnections between SUMO and the ubiquitin/proteasome system (UPS) are far more widespread than anticipated. Contrary to previous assumptions, these studies have identified sumoylation as a targeting signal for ubiquitylation and ubiquitin-dependent degradation.[33,36-40] Our current understanding of these processes and their putative functions will be discussed in this chapter.

FUNCTIONAL INTERACTIONS OF SUMOYLATED PROTEINS AND THEIR REGULATION

Sumoylation of proteins alters their biochemical properties in a way that may either promote a specific function, for example by enabling or enhancing an interaction with another protein, or inhibit a function by preventing interactions. Examples, in which sumoylation was shown to promote specific protein interactions, are the binding of

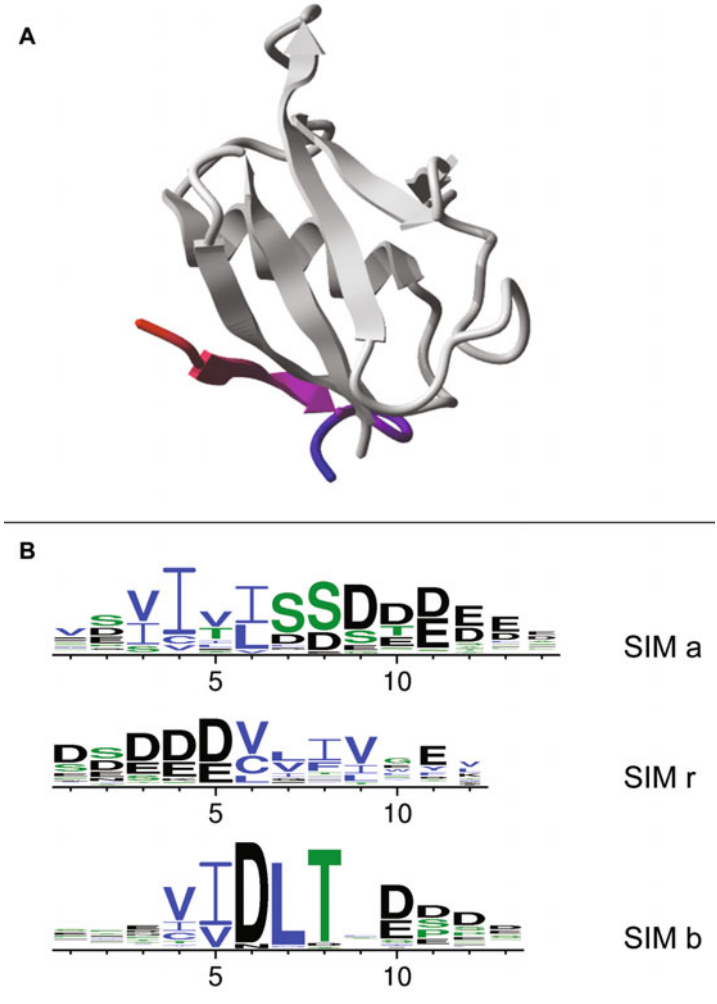

Figure 3. SUMO interaction motifs. A) Shown is the ribbon diagram of the SIMb from PIASx bound to human SUMO-1 (based on structure PDB: 2ASQ). B) Residue conservation of the three SIM types is shown in a sequence logo representation. Overall height of a position indicates its information content, height of individual residues indicates their frequency at that position. The residues are color coded (black: charged, green: polar, blue: hydrophobic).

Ran-GAP-SUMO to RanBP2,[41-44] the binding of PCNA-SUMO to Srs2[34,35] and the interactions of PML-SUMO proteins with each other as well as with other proteins, which are thought to promote the formation of PML nuclear bodies.[45,46] The interaction of sumoylated proteins with their partners involves specific SUMO interaction motifs (SIMs) in the latter proteins (Figs. 3 and 4, see next section).[47] The identification of ubiquitin ligases that bind sumoylated proteins via such motifs led to the discovery of a novel targeting pathway, to which this chapter is devoted. In other cases, such as thymine-DNA glycosylase, sumoylation terminates a function or interaction of the modified protein.[48,49]

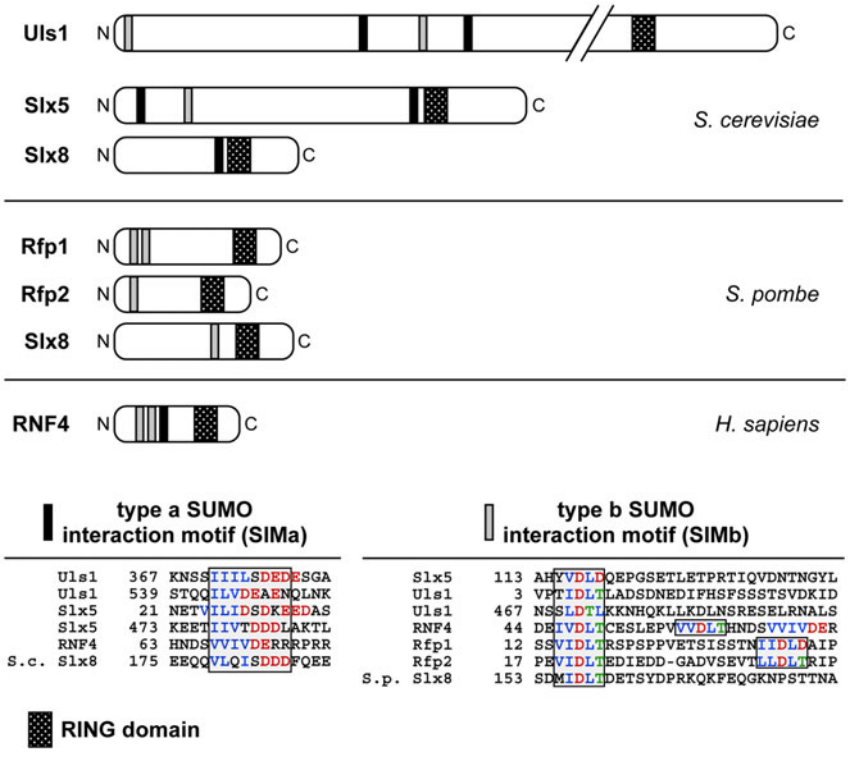

Figure 4. Ubiquitin ligases recognizing SUMO (ULS). Shown are schematic representations of recently discovered SUMO binding ubiquitin ligases. In budding yeast two such enzymes, Uls1 and the heterodimeric Slx5-Slx8, were identified. In fission yeast, complexes formed by Slx8 and either Rfp1 or Rfp2 carry out similar function as Slx5-Slx8 in budding yeast. The human RNF4 protein can functionally replace the Slx8-based ligases in fission and budding yeasts. The positions and sequences of two different types of SIMs in these ligases and the RING motifs are depicted.

Considering the fact that of a given protein only a small fraction is commonly found in the sumoylated state and that this modification is transient, it is an important question how the modified state of the protein is regulated in the cell.[19] The most direct way involves the activity of SUMO isopeptidases, which revert the substrate into its unmodified state (for recent reviews see refs. 50,51). While two SUMO isopeptidases, Ulp1 and Ulp2, were found in *S. cerevisiae*,[52-54] human cells express at least six such enzymes (SENP1,2,3,5,6 and 7).[50,51,55] Distinct specificities have been assigned to these enzymes. Ulp2 in yeast and SENP6 (also known as SUSP1) or SENP7 in mammals are efficient in the disassembly of SUMO chains (Fig. 1).[14,56] Aside from reversion of SUMO conjugation, another way to regulate the function of a sumoylated protein is to add further modifications. Conjugation of additional SUMO moieties to other Lys residues in a substrate (multisumoylation) or to an already conjugated SUMO (polysumoylation) may determine an altered fate of the so modified protein. As discussed in more detail below, recent evidence indicates that polysumoylation serves as a preferred recognition signal for specific ubiquitin ligases, which may ultimately target a SUMO substrate for ubiquitin-dependent degradation by the proteasome.

SUMO INTERACTION MOTIFS (SIM)

Although ubiquitin and SUMO share considerable similarity in both sequence and structure, the nature and binding mode of their interaction partners are fundamentally different. Ubiquitin can be bound by more than 10 different classes of autonomously folded recognition domains; the interaction typically involves a hydrophic surface region surrounding the critical Ile44 residue in ubiquitin.[57] By contrast, SUMO does not seem to employ folded interaction domains, but seems to be exclusively recognized by short conserved motifs called SIMs (for SUMO interaction motifs). The first motif to specifically recognize SUMO was described in 2000 by Minty et al.[58] A consensus motif, consisting of four hydrophobic residues followed by a serine-rich spacer and a group of acidic residues, was identified in human SAE2, PML and the PIAS family. Residues of this motif—particularly those within the hydrophobic part—were shown to be important for SUMO recognition. Since then, many more SUMO binding proteins in mammals and yeast have been identified and the definition of the SIM consensus has undergone several rounds of refinement.[36,44,59-61] At present, most experimentally proven SUMO-binding motifs can be classified by their residue conservation into three major SIM types (Fig. 3B).[36]

- **SIMa:** Motifs belonging to the SIMa type are characterized by four consecutive hydrophobic residues, immediately followed by a mixed cluster of Ser/Asp/Glu residues. When analyzing the sequence conservation of SIMa motifs, a certain variability at the third hydrophobic position becomes apparent. This position is not only less conserved than the other hydrophobic positions, it can even accommodate nonhydrophobic residues.
- **SIMr:** The second SIM type resembles the SIMa, but has a reversed orientation. In the SIMr motif, the four hydrophobic positions are preceded by an acidic cluster. In keeping with the inverse theme, the second hydrophobic position of SIMr—which corresponds to the third hydrophobic position of SIMa—is the most variable one and can occasionally accommodate nonhydrophobic residues.
- **SIMb:** The third SIM type is shorter than SIMa, but generally better conserved and easier to recognize. Most type b SIMs strictly adhere to the consensus sequence V-I-D-L-T, with some variability in the first two hydrophobic positions. The third position has a strong preference for Asp, unlike the usually hydrophobic residue found at the corresponding position of SIMa. Several SIMb motifs, including those of the PIAS family, are followed by a serine/acidic region resembling that of SIMa. However, this stretch is not crucial for SIMb function, as there are several documented instances of non-acidic SIMb motifs, including those of the RNF4 family.

While most established SIMs follow one of the three consensus motifs, there is also evidence that the three SIM classes form a continuum. The SIMb, when followed by an acidic stretch, is not much different from a SIMa, with the third (variable) hydrophobic position filled by an Asp residue. In addition, there are putative SIMs consisting of four hydrophobic residues flanked by acidic residues on both sides; thus precluding a classification as SIMa or SIMr. It is at present not clear if the SIMs class has a major influence on the recognition properties of the SIM. Mammals and several other taxa have multiple SUMO versions, often with differing properties and expression patterns. It has been proposed that in human SIMs the acidic stretch is important mainly for SUMO-1 binding, while SUMO-2 binds equally well to SIMs with and without acidic regions.[61]

However, this proposal is mainly based on the highly atypical and not conserved SIM of the human TTRAP protein; its generalization clearly requires further investigation. Interestingly, there are several examples where orthologous proteins from closely related species use different SIM types at a corresponding position, which supports the idea that different SIMs might be functionally interchangeable.

The recognition mode of SUMO by SIMs has been elucidated by a series of structural studies.[59,61-63] Three high-resolution structures of SIMs in contact with SUMO are currently available: the SIMb of PIASx bound to SUMO-1 (PDB: 2ASQ),[63] the SIMb of MCAF1 bound to SUMO-3 (PDB: 2RPQ)[63] and a (partial) SIMr of RANBP2 bound to SUMO-1 (PDB:1Z5S).[63] As shown in Figure 3A, the hydrophobic residues of the PIASx-derived SIMb form a short β strand that interacts with SUMO at a region formed by its β sheet 2 and the α helix, which is remote from the Ile44 patch that is used by ubiquitin for its interactions. The SIMb of MCAF1 is closely related to that of PIASx; both consist of the archetypical V-I-D-L-T motif, followed by a serine/acidic stretch. The binding mode of the two SIMb examples is also similar, with the highly conserved Asp3 of the SIMb core contacting a lysine residue conserved in all human SUMO paralogs. The orientation of the conserved Thr5 at the C-terminal boundary of the SIMb differs between the two structures; the same is true for the flanking serine/acidic stretch, which does not seem to participate in any crucial SUMO contact. The structure of the reversed SIM of RANBP2 demonstrates that the SIMr indeed recognizes SUMO in a reversed orientation as compared to the SIMb structures. As the acidic residues of the SIMr are not included in the structure, their contribution to SUMO recognition by the SIMr motif cannot be judged.

Recently, another layer of complexity has been added to the regulation of SUMO/SIM interaction. As mentioned above and shown in Figure 3, several SIMs of all types contain serine residues at positions that are occupied by aspartate or glutamate in other SIM instances. Already in 2006, it had been noticed that SIM-associated serines can be phosphorylated,[61] adding a negative charge and making them similar to an acidic amino acid. More recently, it was shown that casein kinase 2 (CK2) phosphorylates serine residues in the SIMs of PIAS1, PML and PMSCL1 and that this phosphorylation is required for efficient recognition of both human SUMO-1 and SUMO-2.[64] The enhanced SUMO binding was shown to depend on a conserved lysine residue (Lys39 in SUMO-1, Lys35 in SUMO-2). Most likely, the phospho-serine residues form a salt bridge with the SUMO lysine. In the NMR structure of the PIASx SIMb, which is closely related to that of PIAS1, these contacts are not visible—probably due to the fact that the PIASx SIMb has been studied in the nonphosphorylated form.[59] The full extent of the crosstalk between kinase signaling and SUMO/SIM recognition remains to be explored—serine residues at susceptible positions are rather common in different SIM subtypes.

PROTEASOMAL DEGRADATION OF SUMO CONJUGATES

The sumoylation state of a given protein is a dynamic equilibrium that is regulated mainly by the conjugating and deconjugating activities of the SUMO pathway. Desumoylation is also required to counteract the formation of SUMO chains. Genetic analysis revealed that Ulp2, one of the two desumoylating enzymes in *S. cerevisiae*, is mainly responsible for this activity in this organism.[14] Mutants lacking Ulp2 are viable but have severe growth defects and are sensitive to various stresses.[53,54] Accumulation of polysumoylated proteins appears to be responsible for these defects.[14] These high

molecular weight SUMO conjugates (HMW-SC) are not detectable in yeast strains expressing a mutant SUMO variant (Smt3-R11,15,19) lacking lysine residues critical for SUMO chain formation (Fig. 2A). Importantly, this variant of SUMO is able to suppress the defects of the *ulp2*Δ mutant.[14]

HMW-SC occur not only in the *ulp2*Δ mutant but also in mutants deficient in the UPS such as *ubc4*Δ *ubc5*Δ, or upon inhibition of the proteasome.[36] The UPS apparently contributes to the removal of certain types of sumoylated proteins. Experiments with the Smt3-R11,15,19 mutant indicated that chain formation promotes targeting of sumoylated proteins in the UPS. Consistent with this notion, conjugates of the chain forming SUMO-2/3 isoforms accumulated in human cells to much higher levels upon inhibition of the proteasome than those of SUMO-1, which does not form chains efficiently.[13,36] The preference for a proteolytic targeting of polysumoylated proteins can be related to the properties of SUMO-specific ubiquitin ligases (see below).

UBIQUITIN LIGASES RECOGNIZING SUMOYLATED PROTEINS IN *S. CEREVISIAE*

In the UPS, recognition and targeting of substrates for proteasomal degradation is mediated by a class of enzymes known as ubiquitin protein ligases, E3s or recognins, which form complexes with ubiquitin-conjugating enzymes (E2s).[65-67] The largest group of ubiquitin ligases is characterized by a structural domain termed the RING finger, in which two zinc atoms are complexed by Cys and His residues.[68]

In *S. cerevisiae*, a new subgroup of RING finger ubiquitin ligases was discovered recently. One of their characteristic properties is that they contain multiple SIMs for binding to SUMO (Fig. 4). Therefore they were called SUMO-targeted ubiquitin ligases (StUbL) or ubiquitin ligases for SUMO conjugates (ULS) (Fig. 3A).[36-38,69] We and others identified the yeast proteins Slx5 (alias Hex3) and Uls1 (alias Ris1) as SUMO interactors in yeast two-hybrid screens.[36,60,70,71] Both proteins contain multiple putative SIMs (Fig. 3B and 4).[36,60] Slx5 contains two SIMa and one SIMb sequences, whereas Uls1 contains two SIMa and two SIMb sequences. Deletion of the *SLX5* or *ULS1* gene in yeast leads to the accumulation of HMW-SC.[36,37,72] Deletion of both genes results in an even stronger effect on SUMO conjugates pattern.[36]

PROPERTIES AND FUNCTIONS OF Slx5-Slx8

SLX5 and *SLX8* were identified in a genetic screen that selected for genes required in the absence of the only RecQ type DNA helicase Sgs1 in budding yeast.[73] Mutations in WRN and BLM, two human homologues of Sgs1, cause premature aging (Werner's syndrome) or genome instability often leading to cancer (Bloom's syndrome). Sgs1 is involved in various processes including replication and the S-phase checkpoint, double-strand break repair, recombination and telomere maintenance (for a review see ref. 74). Mutations in *SLX5* and *SLX8* were also found to cause a synthetic growth defect with mutations in the *SRS2* gene, which encodes another DNA helicase.[75,76] As mentioned earlier, Srs2 is recruited to replication forks by sumoylated PCNA and prevents unscheduled recombination (for review see refs. 26,77,78). *SRS2* mutations were also found to be synthetically lethal in combination with mutations affecting the SUMO-deconjugating

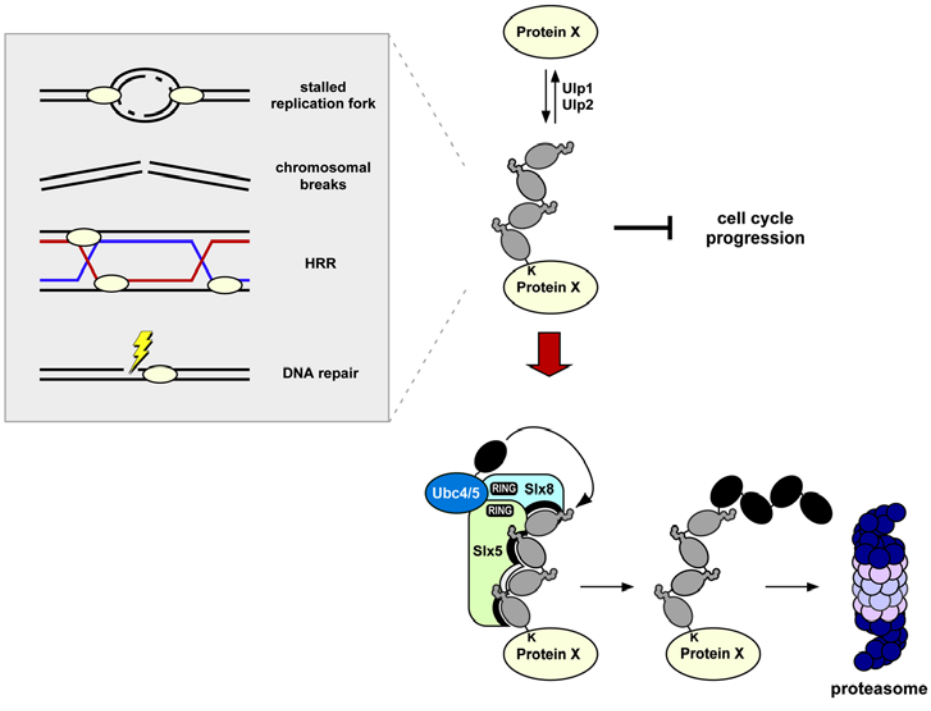

Figure 5. Role of SUMO-targeted ubiquitin ligases in genome stability. Processing of stalled replication forks and double strand breaks as well as homologous recombination repair (HRR) and DNA damage repair require the action of the ULS enzymes Slx5-Slx8 in budding yeast and Rfp1,2-Slx8 in fission yeast. These types of genomic instability coincide with the appearance of increased amounts of SUMO conjugates, which can lead to an arrest in the cell division cycle if they are not sufficiently controlled by Slx5-Slx8-mediated ubiquitylation and subsequent proteasomal degradation or by desumoylation.

enzyme Ulp1.[79] *Ulp1* mutations on the other hand were suppressed by overexpression of *SLX5*.[37] Interestingly, *sgs1*, *srs2* and *slx5* mutants all accumulate HMW-SC to a similar extent.[38] Together these observation are consistent with the idea that mutations affecting the two helicases Sgs1 and Srs2 accumulate sumoylated proteins and that Ulp1 and Slx5-Slx8 are required to limit the abundance of such conjugates either by desumoylation or by proteolytic targeting (Fig. 5).[37,38] If uncontrolled, such conjugates apparently lead to an arrest in cell division. Interestingly, the activity of the Ulp2 protein, which cleaves SUMO chains was shown to be required to recover from checkpoint arrests that were for example induced with hydroxyurea.[51] These observations together pointed to the fact that SUMO modification and its subsequent processing by Ulps, or its recognition by Slx5 and Slx8 are linked to processes of DNA repair, recombination and replication fork stability. In agreement with this notion, *slx5* and *slx8* mutants, similar to *ulp2*, are hypersensitive to DNA damage, or the replication inhibitor hydroxyurea, they accumulate gross chromosomal rearrangements (GCRs) as well as shortened telomeres and display rDNA hyperrecombination.[80-86] Consistent with an involvement in these processes, Slx5 or Slx8 have been localized to replication centers and damage-induced Rad52 nuclear foci.[82,87] Another study reported the recruitment of DNA double strand breaks to the nuclear pore complex in a Slx5-Slx8-dependent manner.[88]

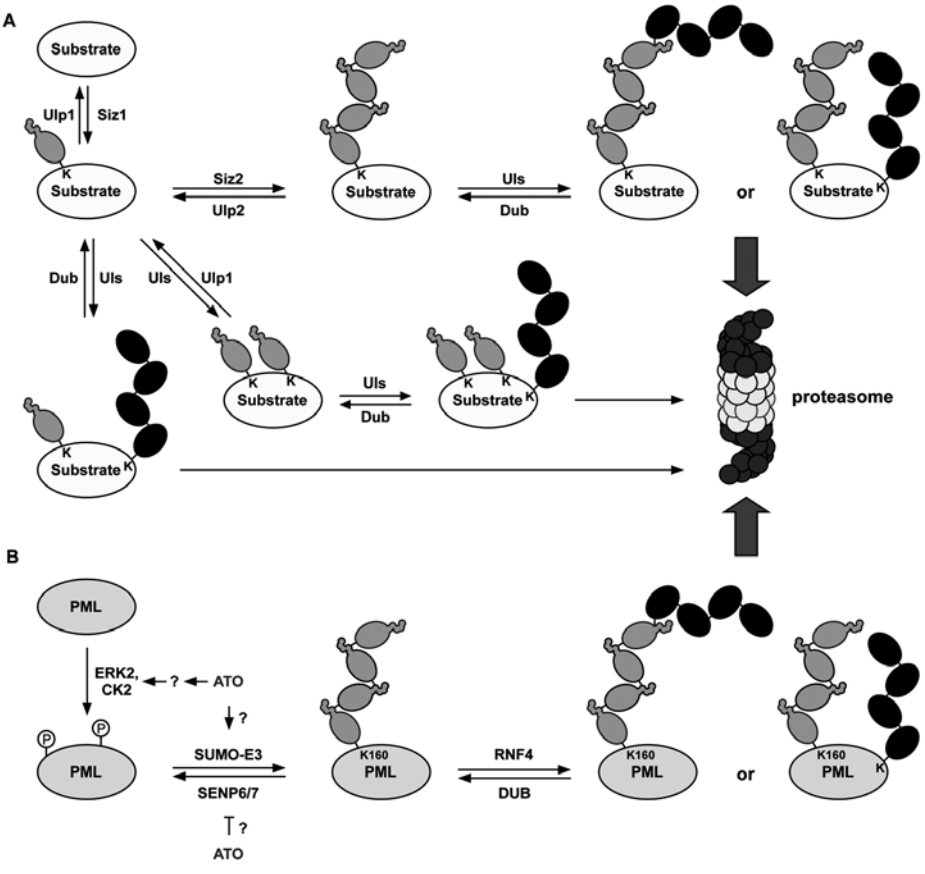

Figure 6. Ubiquitin-dependent proteolytic control of SUMO conjugates. A) Shown is a schematic model of the control of the sumoylated state of substrate proteins in budding yeast. Sumoylation on one side is controlled by the activity of SUMO ligases such as Siz1 and Siz2 and on the other side by the desumoylating enzymes Ulp1 and Ulp2, which have different activities towards SUMO chains. In addition, sumoylation in particular polysumoylation may trigger ubiquitylation by SUMO recognizing Ub ligases (ULS) and subsequent degradation by the proteasome. Ub may be attached either to Lys (K) residues of the substrate or in the SUMO chain. B) Model of ATO-induced SUMO-dependent proteolytic targeting of PML. ATO (arsenic trioxide) treatment of cells or APL patients results in an increased sumoylation of PML, which mediates its subsequent ubiquitylation by RNF4 and in turn its degradation by the proteosome. The molecular mechanism of ATO-induced PML sumoylation is still unclear (indicated by question marks). It may either involve stimulation of PML phosphorylation by ERK2 or CK2, or changes in the regulation of enzymes of the SUMO system.

Slx5 and Slx8 are both RING finger proteins that form a heterodimer.[72,89] Aside from interaction between Slx5 and SUMO,[36-38] Slx8 by itself was shown to bind to SUMO in vitro, indicating that the Slx5-Slx8 heterodimer has multiple SUMO binding sites distributed between the two polypeptides (Fig. 4).[38] Genetic and biochemical data support a view, in which Slx5-Slx8 has a role as a SUMO-dependent ubiquitin ligase that acts together with the redundant E2s Ubc4 or Ubc5 to target sumoylated proteins for degradation by the proteasome (Fig. 6A).[36-38] Consistent with this notion, in vitro experiments revealed that Slx5-Slx8 has ubiquitin ligase activity.[36-38,69] Slx8 and Slx5-Slx8 complexes, in contrast to

Slx5 alone, were shown to display autoubiquitylation activity in vitro.[36,37] Based on these and additional data, it was concluded that the core ubiquitylation activity is inherent to Slx8 and that Slx5 enhances this activity by a function relying on its RING domain and by binding to conjugated SUMO on a target protein.[37] The latter conclusion was derived from in vitro experiments that used Rad52 or a Rad52-SUMO fusion as substrates. In these assays, the Rad52-SUMO fusion protein was preferentially ubiquitylated.[37]

Binding experiments that used immobilized Slx5-Slx8 produced in *E. coli* revealed that this ligase preferentially binds to high molecular weight SUMO conjugates suggesting that poly- or multisumoylated proteins might be the preferred substrates of this ligase.[36] Supporting this notion, another study provided convincing in vitro evidence, using autosumoylated forms of the yeast SUMO ligase Siz2 as substrates, that poly-SUMO chains act as a preferred targeting signal for Slx5-Slx8-dependent ubiquitylation and suggested that ubiquitin modification occurs mainly on the terminal SUMO moiety.[38]

A recent study identified the transcriptional regulator Mot1 as a first in vivo substrate of Slx5-Slx8 in yeast.[69] In an earlier study by the same group, mutations in the *SLX5* and *SLX8* genes were found to suppress a mutation in the *MOT1* gene.[72] Interestingly, the same allele was also suppressed by mutations in the genes encoding components of the SUMO conjugation system. These authors propose a role for Slx5-Slx8-mediated SUMO-targeted ubiquitylation in the quality control of misfolded nuclear proteins. The increased turnover rates detected for the Mot1 mutant depended on SUMO modification and Slx5-Slx8. In additon, the turnover of wild-type Mot1 was stimulated by the same components when cells were treated with the amino acid analogue canavanine.[69]

What remains puzzling is the observation that combinations of mutations that interfere with SUMO conjugation (*uba2* or *ubc9*), or with the formation of SUMO chains (*smt3-R11,15,19*), with *slx5* or *slx8* mutations cause either synthetic lethality or sickness.[36,72] Intuitively, one would have expected the opposite, that the inability to turnover sumoylated substrates might be suppressed by a reduction in their formation. Indeed, a mutation of the *S. pombe* SUMO ligase Pli1 was found to suppress phenotypic effects of mutations in the Rfp1,2-Slx8 ligase, which is related to Slx5-Slx8 as discussed in a later section. A similar interrelation was also observed for mutations in the *ULP2* gene leading to a loss of a SUMO deconjugating enzyme, which were suppressed by mutations impairing the SUMO-activating enzyme or the SUMO precursor processing protease Ulp1.[53,54] The unsuspected synthetic effects of mutants affecting Slx5-Slx8 and SUMO conjugation could be explained by putative SUMO-independent functions of Slx5-Slx8, which might become essential when sumoylation is compromised. Another possibility is that low levels of sumoylation could be sufficient to direct certain essential substrates towards Slx5-Slx8, but at the same time might be insufficient to terminate the function of the same substrates more directly by generating multi- or polysumoylated forms of them. The latter explanation, however, is somewhat at odds with the observed binding preference of Slx5-Slx8 for HMW-SCs. Additional studies are required to resolve this issue.

PROPERTIES AND FUNCTIONS OF Uls1/Ris1

Compared to Slx5-Slx8, even less is known about the specific functions of the other SUMO-binding ubiquitin ligase Uls1/Ris1 in *S. cerevisae*. Uls1 is a member of the SWI/SNF family of DNA-dependent ATPases, for which a role in antagonizing silencing during mating-type switching was reported.[90] The presence of a RING domain as well

as of multiple putative SIMs (Fig. 4) and the fact that Uls1 interacts with SUMO and Ubc4 suggested a role for Uls1 in SUMO-dependent ubiquitylation.[36] In support of this hypothesis, the deletion of both *SLX5* and *ULS1* yielded a synthetic effect both on yeast growth and on the accumulation of HMW-SC.[36] The recently identified interaction between Uls1 and sumoylated Ebp2, a yeast protein related to Epstein-Barr virus nuclear antigen 1-binding protein 2, is also consistent with a significance of the SUMO binding properties of Uls1.[91] Apparent changes in the overall Ebp2 levels as a consequence of *ULS1* deletion, however, were not observed in this study suggesting that Uls1 may not control the stability of Ebp2. Polysumoylated forms of Ebp2, which are likely to be the preferred substrates of Uls1, however, may be too low in abundance and too heterogeneous to detect stabilizing effects of a *uls1* mutation. While genetic evidence strongly suggests that Uls1 acts as a SUMO-dependent ubiquitin ligase with functions partially overlapping with those of Slx5-Slx8, biochemical prove of this activity is still missing.

SUMO TARGETED UBIQUITIN LIGASE Rfp1,2-Slx8 IN *SCHIZOSACCHAROMYCES POMBE*

Ubiquitin ligases for SUMO modified proteins were also discovered in fission yeast.[33,39] The functionally redundant proteins Rfp1 and Rfp2 are RING finger proteins with multiple SUMO interacting motifs. By themselves, these polypeptides lack ligase activity, but upon interaction with Slx8 they form an enzyme that ubiquitylates Rad60, a protein that resembles a fusion of two SUMO domains,[92,93] or GST-SUMO in vitro.[33,39] Similar to the *slx5*Δ *uls1*Δ mutant in *S. cerevisae*, the fission yeast double mutant *rfp1*Δ *rfp2*Δ accumulates high molecular weight SUMO conjugates. Cells lacking Rfp1,2-Slx8 display genomic instability and hypersensitivity to genotoxic stress. Much in contrast to what was discussed above for Slx5-Slx8 in budding yeast, these defects can be suppressed by the deletion of the major fisson yeast SUMO ligase Pli1.[39] These data indicate that Rfp1,2-Slx8 is required to remove sumoylated targets that are generated as a result of DNA damage or replication fork arrests (for a recent review, see ref. 86). Together these studies showed that Rfp1,2-Slx8 complexes are SUMO-targeted ubiquitin ligases that are functionally related to the Slx5-Slx8 complex of *S. cerevisiae.*

MAMMALIAN SUMO TARGETED UBIQUITIN LIGASE RNF4

No apparent human homolog of the *S. cerevisiae* ULS proteins Slx5-Slx8 or Uls1 could be found in the human genome, but the *S. pombe* proteins Rfp1 or Rfp2, which form redundant complexes with Slx8 in fission yeast, show weak homology to the human protein RNF4 (alias SNURF).[39] RNF4 is a nuclear RING finger protein with ubiquitin ligase activity.[94] RNF4 can functionally complement the *rfp1*Δ *rfp2*Δ or *slx8*Δ mutations in *S. pombe* and *slx5*Δ or *slx8*Δ in *S. cerevisiae* suggesting that RNF4 alone provides a similar activity as the Rfp1,2-Slx8 or Slx5-Slx8 heterodimeric enzymes in these yeasts.[33,36,39,95]

RNF4 contains four putative SIMs, which are located closely spaced near its N terminus (3 of which are shown in Fig. 4).[86] In vitro studies showed that binding of RNF4 to polySUMO chains is mainly dependent on SIM2 and 3, which both fit into the 'Type b' class, with some contribution from SIM4, which is of 'Type a'. Complete loss of SUMO

binding was observed when all four SIMs were mutated.[96] Even though a contribution of SIM1 remained uncertain, these data demonstrated that multiple SIMs are required for efficient binding of RNF4 to SUMO-2 chains. This study also revealed, using a mixture of SUMO-2 conjugates, that for chains comprising 3 or more SUMO-2 moieties increasing chain length correlated with efficiency of RNF4 binding, while mono-SUMO or di-SUMO binding was undetectable under these conditions.[96] SUMO-2 chains were efficiently polyubiquitylated by RNF4 in vitro, while mono-SUMO was a poor substrate. Together these data characterized RNF4 as a mammalian poly-SUMO-dependent ubiquitin ligase that is functionally related to yeast SUMO-targeted ubiquitin ligases.[33,36,39,96]

REGULATION OF PML BY RNF4-MEDIATED SUMO-DEPENDENT PROTEOLYSIS

A hint to a physiological substrate of RNF4 was provided by the observation that it interacted with the promyelocytic leukemia protein (PML) in a SUMO-dependent manner.[97] PML and its sumoylation are essential for the formation of PML nuclear bodies (PML-NBs),[98-100] large protein complexes detected in mammalian cell nuclei.[101] Many other proteins such as Daxx, Sp100 and RNF4 are recruited to these dynamic PML-NBs.[97,101] Complex formation between these proteins is provided by SUMO/SIM interactions, which contribute to the interactions of sumoylated and unmodified PML-NBs proteins and thereby to the formation of these subnuclear structures.[45,46] PML-NBs have been implicated in the regulation of many important cellular functions including, transcription, apoptosis, DNA repair, antiviral defence, tumour suppression, senescence, proteolysis and storage of nuclear proteins.[101]

PML is also known as TRIM19 because it belongs to the tripartite motif family of proteins, which are characterized by a RING domain followed by two B-boxes and a coiled-coil region (for review see ref. 102). Eleven PML isoforms could be isolated from human cells that all differ at their C termini due to alternative splicing. Most isoforms are nuclear proteins; only two of them lack the NLS and are therefore cytoplasmic.[101,102] PML has three sumoylation sites K65, K160 and K490 (or K442 depending on the splice variant) and a single SIM of the 'a type' downstream of K490/442.[102] These properties are critical for the role of PML as a scaffold protein of PML nuclear bodies. PML was first identified in patients suffering from acute promyelocytic leukemia (APL). This leukemia is caused by a chromosomal translocation (t(15;17)) in myelocytic progenitor cells. This translocation results in a fusion of the N-terminal part of PML to the C terminus of retinoic receptor alpha (PML-RARα).[103,104] As a consequence, these progenitor cells proliferate without proper differentiation. This leukemia is treated with all-trans retinoic acid (ATRA) or arsenic trioxide (ATO), both of which induce terminal differentiation of these cells. High doses of ATO, in addition, induce apoptosis of APL cells (for a recent review see ref. 105).

A milestone finding towards an understanding of the therapeutic effect of ATO was that it induces sumoylation of PML-RARα and its subsequent degradation by the proteasome.[106] The discovery of SUMO-dependent ubiquitin ligases described above together with the observation that PML and RNF4 interact in a SUMO-dependent manner suggested that the sumoylation of PML may target it for ubiquitylation by RNF4 (Fig. 6B).[33,36,39,97] This conjecture has been proven recently identifying PML as a first bona fide substrate of SUMO-dependent ubiquitylation.[10,96,107] Knockdown of RNF4, mutation of K160 of PML to arginine, or simultaneous knockdown of SUMO-1,-2 and -3 isoforms all impaired

ATO-induced degradation of PML indicating that attachment of SUMO to K160 is essential for this proteolytic targeting mechanism.[96,107] Importantly, SUMO-ubiquitin hybrid conjugates of PML could be detected both in vivo, upon ATO treatment, and in vitro.[10,96] Mass spectrometric analyses of such conjugates indicated that ubiquitin is either conjugated to lysine residues of SUMO, including K11, or to various lysine residues in the C-terminal part of PML. Importantly, K160 was not found to be ubiquitylated in these experiments.[96,107] Consistent with an earlier observation that SUMO-2/3 conjugates are accumulating dramatically upon inhibition of the proteasome,[36] RNF4-mediated ubiquitylation and subsequent degradation of PML was promoted most effectively by SUMO-2/3 modification.[10,96,107] The situation, however, may not be quite that simple because SUMO-1 modified forms of PML were also ubiquitylated by RNF4 in a SUMO-dependent manner in vitro and a knockdown of SUMO-2/3 was insufficient to prevent ATO-induced degradation of PML.[10,107] In addition, it was suggested that modification of K65, either by SUMO-1 or SUMO-2/3, influences sumoylation at K160 indicating that there may be a crosstalk between modification of individual attachment sites by the different SUMO isoforms.[107]

POSSIBLE TARGETS OF ATO LEADING TO DEGRADATION OF PML

Stress induction appears to be a key trigger for increased SUMO-2/3 modification. SUMO-2/3 modification as well as subsequent ubiquitylation can be observed after many stresses including heat, osmotic or oxidative stress (H_2O_2), or treatment with ethanol or ATO.[8,10] The molecular targets relevant for these effects are still largely unknown. Among the possibilities is that compounds such as H_2O_2 or ATO cause an inhibition of certain cystein-containing enzymes mediating conjugation or deconjugation of SUMO isoforms or ubiquitin.[55,108] Inhibition of SUMO chain depolymerising isopeptidase activity could for example explain the observed increased levels of high molecular weight SUMO-2/3 conjugates following ATO or H_2O_2 treatment. Additional signals that specifically induce SUMO-dependent ubiquitylation, however, must be involved in the effect of ATO on PML, because up-regulation of SUMO-2/3 modification alone was not sufficient to induce PML degradation.[10] In line with this conclusion is the observation that down-regulation of the SUMO-chain specific protease SENP6 does not lead to smaller PML-NBs but instead to larger ones.[56]

Another possibility is that ATO-induced phosphorylation of the PML protein through a mitogen-activated protein (MAP) kinase pathway activates the RNF4-dependent pathway (Fig. 6B), as it was shown to cause increased PML sumoylation and PML-mediated apoptosis.[109] These phosphorylations occur near the N terminus and also between the third sumoylation site and the SIM in PML. Other serine residues of PML, which are part of its SIM, were found to be phosphorylated by CK2 after osmotic stress.[110] This study suggested that phosphorylation of the SIM in PML is necessary for stress induced degradation. The same phosphorylation was recently shown to enhance its interaction of PML with SUMO.[64] Another study, however, showed that, while this SIM is important for ATO-induced degradation of PML, its phosphorylation is not.[111] A possible way to explain the need for the SIM in RNF4-mediated degradation could be that it promotes autosumoylation of PML by recruiting SUMO-2/3-loaded Ubc9. A similar role of SIMs in promoting autosumoylation was observed for other proteins including human Daxx (death domain associated) protein.[46,112-114] This mechanism is reminiscent of an analogous mechanism for noncovalent ubiquitin/substrate interactions leading to monoubiquitylation.[115]

In conclusion, while the identification of SUMO-dependent ubiquitin ligases has significantly increased our understanding of ATO-induced degradation of PML, more studies are required to fully understand its mechanism in detail. ATO targeting of PML is of considerable interest also for the treatment of cancer types other than APL. Recent results have indicated that ATO treatment prior to conventional chemotherapy contributes to the activation and subsequent eradication of quiescent cancer initiating cells in chronic myeloid leukaemia.[116] Other important questions concern the physiological functions of the RNF4 pathway and its other substrates and how their targeting is regulated for example by cellular stress response pathways. RNF4 interacts with a variety of transcription factors that are known to be both sumoylated and ubiquitylated suggesting that it is involved in their regulation (for a recent review see ref. 86).

SUMO-DEPENDENT REGULATION OF HIF1α

SUMO-dependent proteolytic targeting was shown to contribute also to the regulation of hypoxia-inducible factor 1α (HIF1α) under hypoxic conditions.[40] Inactivation of the desumoylating enzyme SENP1 led to a stabilization of HIF1α under these conditions. According to the model proposed by these authors, SUMO-1 modification of HIF1α promotes its recognition by the von Hippel-Lindau (VHL) protein, a subunit of a ubiquitin ligase and subsequent degradation by the proteasome.[40] These results are still under discussion as other studies reported stabilizing effects of sumoylation on HIF1α under hypoxic conditions.[117-119]

CONCLUSION

The studies summarized above indicated that the UPS, via specific ubiquitin ligases, participates in the regulation of SUMO target proteins. Studies in budding and fission yeast have implicated ubiquitylation of SUMO targets in the control of genomic stability, while studies in mammalian cells on the functions of RNF4 have linked this mechanism to the regulation of PML, nuclear bodies and of transcription factors. For an individual substrate multiple scenarios can be envisioned. The sole function of sumoylation of a certain substrate may be to target it for ubiquitin-dependent degradation by the proteasome. In other cases, SUMO-mediated ubiquitylation may also have nonproteolytic targeting functions. For the bulk of targets, sumoylation is likely to serve to transiently regulate protein interactions. The transient nature of this modification on one side is provided by de-sumoylating activities and on the other side by formation of SUMO chains and proteolytic targeting via SUMO-dependent ubiquitylation. The latter scenario resembles the suicide model for activation of certain transcription factors that require monoubiquitylation for their activation, which however is also the beginning of their end since subsequent formation of a ubiquitin chain leads to their degradation.[120,121] This mechanism is thought to ascertain a transient nature of gene activation by so regulated transcription factors. This scenario also resembles that of other protein modifications such as phosphorylation, which can be reversed by dephosphorylation, but which can also trigger ubiquitin-dependent degradation.[122] Future studies on individual targets will reveal which of the described scenarios is relevant to the function of ubiquitin ligases recognizing SUMO modified proteins.

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CHAPTER 17

IN VIVO FUNCTIONS OF ISGYLATION

Klaus-Peter Knobeloch*

Abstract: This chapter recapitulates our current knowledge about the functions of the interferon stimulated gene 15 (ISG15) system in vivo with a specific focus on physiological aspects and the biological relevance of ISG15 conjugation and deconjugation. ISG15 contains two domains with structural similarity to ubiquitin and was the first ubiquitin like modifier (UBL) described. It can be conjugated to protein substrates in a process similar to ubiquitin modification termed ISGylation. Of all ubiquitin like modifications ISGylation exhibits the highest degree of interlace with the ubiquitin system and distinct ubiquitin ligases and isopeptidases can also mediate ISG15 linkage and deconjugation, respectively. The system is strongly induced by Type I interferons or microbial infections and studies based on gene targeted mice have shown that it plays an important role in antiviral defence.

INTRODUCTION

It is well established that posttranslational modification by ubiquitin and ubiquitin like proteins (UBLs) serves as a basic mechanism to control a wide range of cellular functions. Interferon-stimulated gene 15 (ISG15), initially named ubiquitin cross reactive protein (UCRP), was the first UBL described[1] and represents one of the genes most strongly up regulated by Type I Interferons (IFN), which serve critical roles in innate and adaptive immunity, antiviral defence and the control of malignant cell proliferation. Expression of ISG15 is induced by viral and bacterial infections or genotoxic stress and was also reported to be secreted from the cell raising speculations about a cytokine like function.[2-4,4] In analogy to ubiquitin or other UBLs, ISG15 is conjugated to a wide variety of target proteins mediated by the activity of E1, E2 and E3 ligases (Fig. 1).[5,6] The E1 responsible for the activation of ISG15 in the conjugation process is ubiquitin-activating enzyme E1-like (UBE1L) which is specific for ISG15.[7] Ubiquitin-conjugating enzyme

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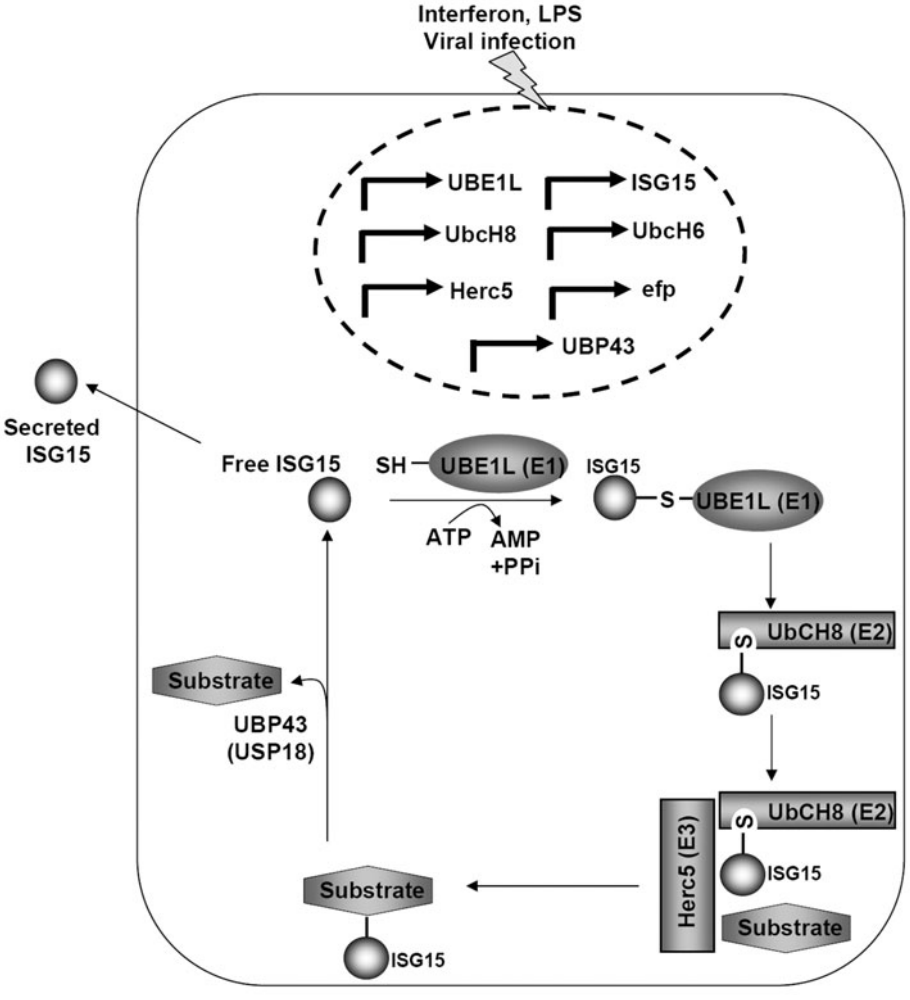

Figure 1. ISGylation system. Different stimuli like interferon, LPS or viral infections stimulate genes encoding for components of the ISG15 conjugation and deconjugation system. ISG15, depicted as a circle can either be secreted from the cell, remain intracellular in an unbound form or forms a thioester bond with the E1 activating enzyme UBE1L in an energy consuming reaction. From this activation complex ISG15 is transferred to the E2 enzyme (UbcH8 or UbcH6). Finally, an E3 ligase (HERC5, EFP, HHARI) mediates isopeptide-linkage to a lysine residue within the target substrate. The conjugation process is reversible by the action of isopeptidases (prototypically UBP43) which specifically cleaves the isopeptide linkage thereby removing ISG15 from the target substrate.

in humans (UbcH)6 and UbcH8 are E2s for ISG15[8,9] but also serve as conjugating enzymes for ubiquitin, demonstrating an overlap between ISGylation and the ubiquitin modification machinery. Reported E3 ligases for the ISGylation process are the HECT E3 ligase HERC5,[10,11] estrogene-responsive finger protein (EFP), which is regulated by auto-ISGylation and targets 14-3-3σ[12,13] and the UbcH8-interacting ubiquitin ligase *human homolog of Drosophila ariadne* (HHARI).[14] The observation that knockdown of

HERC5 almost completely abrogates ISGylation in human cells strongly suggests that this is the major E3 Ligase in the human system.[15,16] ISG15 conjugation represents a reversible process and ubiquitin specific protease 18 (USP18/UBP43), which was originally described as an ubiquitin deconjugating enzyme,[17,18] was later on reported to be an ISG15 specific isopeptidase.[19] Also other "ubiquitin specific proteases (USPs)" like USP2, USP5, USP13 and USP14 were shown to be ISG15-reactive proteases, further supporting the concept of overlapping functions between ISGylation and ubiquitin modification.[20] The main components of the ISGylation system (ISG15, UBE1L, UBCH8, HERC5, EFP and UBP43) are inducible by interferon, implicating a functional role of ISG15 modification in innate immunity and other interferon effector mechanisms. A strong hint for a role of ISG15 modification in antiviral activity came from the observation that different strains of influenza viruses have developed variable strategies to interfere with ISGylation.[21] Furthermore a critical role for ISG15 in the IFN mediated inhibition of late stages of HIV assembly and release was reported.[22] Using over expression of E1, E2 and a tagged version of ISG15 in HEK293T cells more than 200 target substrates were identified.[23,24] However, under these over expression conditions identified substrates did not exhibit an apparent specificity related to distinct pathways, protein families or sub cellular localization. Using different knockout models considerable progress has been made to define the biological relevance of ISGylation in the context of the whole organism. However, insights into the molecular function of ISG15 in vivo are just beginning to emerge.

IN VIVO ANALYSIS OF THE ISGYLATION SYSTEM

Figure 2 and Table 1 provide an overview about the consequences and phenotypes of different mouse models within the ISGylation system. The first animal model developed for a component of the ISG15 system was a gene targeted mouse lacking the ISG15 isopeptidase UBP43. UBP43 deficient mice develop severe phenotypic alterations characterized by premature death and brain abnormalities clearly demonstrating the extraordinary biological relevance of this protein.[25] In concordance with its role as a specific deISGylating enzyme, UBP43–/– mice exhibit enhanced levels of ISG15 conjugated substrates.[25] As proteolytic processing of the ISG15 precursor protein is a prerequisite for ISG15 conjugation, UBP43 is not the ISG15 precursor processing protease in vivo. This is consistant with results showing that the ISG15 processing enzyme is a 100 kD constitutively expressed protease.[26] Interestingly UBP43–/– mice die upon injection with the interferon-inducer Poly (I:C) and cells lacking UBP43 have highly elevated RNA expression levels of IFN target genes, demonstrating an essential role of UBP43 as a negative regulator of IFN responses.[27] Consistent with an overactivated IFN system UBP43 deficient animals were reported to exhibit enhanced resistance upon Vesicular-Stomatitis-(VSV) or Lymphocytic-Choriomeningitis-virus (LCMV) infections.[28] In line with these results knockdown of UBP43 in human cells enhanced the antiviral activity of interferon upon hepatitis C infection.[29] Due to the reported ISG 15 deconjugating activity it seemed to be clear that the severe phenotypic effects are caused by enhanced ISGylation of target substrates. In particular ISG15 modification of STAT1 was implicated to play a dominant role in the termination of IFN responses.[30]

Despite the fact that UBP43–/– mice represented only an indirect model system to study the role of ISG15 in vivo, there were no doubts that the phenotypic alterations are connected to the loss of deISGylating activity. However, when ISG15 knockout mice

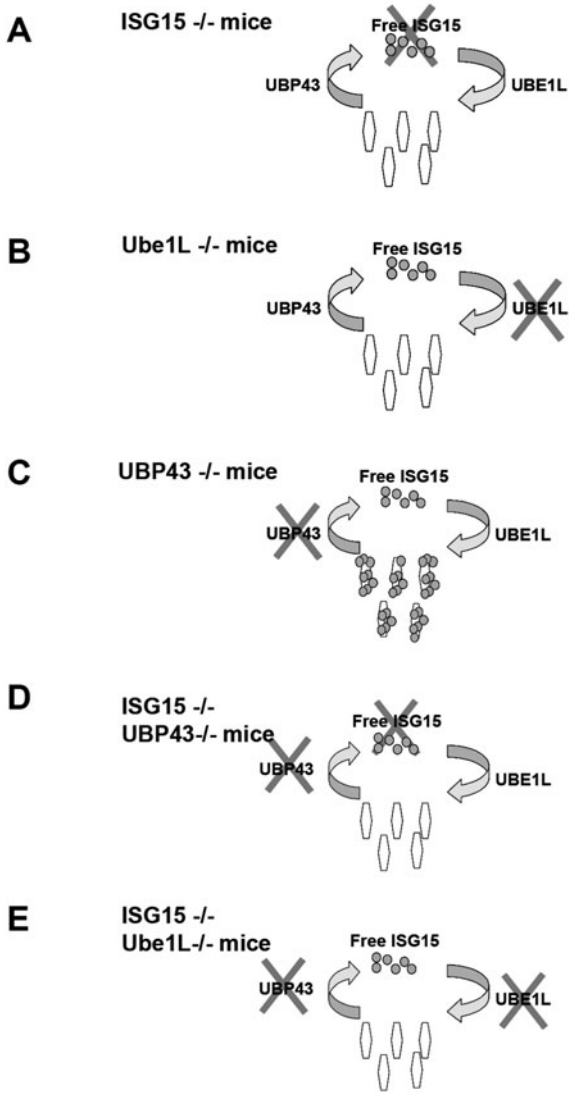

Figure 2. Gene knockout of different proteins involved in ISGylation. A) ISG15 knockout mice lack free ISG15 B) Ube1L knockout mice express free ISG15 but are deficient in conjugating ISG15 to target substrates. C) UBP43 knockout mice lack an ISG15 deconjugating enzyme resulting in enhanced of ISG15 substrate modification. D) ISG15–/–UBP43–/– doubleknockout mice. Lack of free ISG15 should neutralize phenotypic alterations in UBP43–/– mice caused by enhanced ISGylation E) Ube1L–/–UBP43–/– doubleknockout mice. Due to the lack of the ISG15 activating E1 enzyme phenotypic alterations in UBP43–/– mice caused by enhanced ISGylation should be abolished.

had been generated, these animals surprisingly did not show any abnormalities in terms of viability, lifespan, interferon signaling or antiviral defence against VSV or LCMV.[31] This lack of apparent phenotypic alterations in a steady state situation raised the question whether enhanced ISG15 modification is really causative for the phenotype observed in

Table 1. Phenotype of mice lacking distinct components of the ISG15 modification system

Mouse Model	Phenotype	Remarks
UBP43–/–	Enhanced level of ISGylation, premature death, brain injuries, development of a hydrocephalus[25]	Shows the highly relevant function of UBP43 in brain homeostasis and as a negative regulator of IFN signalling (not related to ISG15 deconjugating activity-see below).
	Lethal hypersensitivity upon PolyI:C injection, up regulation of IFN target genes and STAT signalling[56]	
	Enhanced resistance to vesicular stomatitis (VSV) and lymphocytic choriomeningitis virus (LCMV) infections[57]	Provides evidence for UBP43 as an attractive drug target
	Enhanced antibacterial potential against Salmonella infections[58]	
	Increased resistance to oncogenic transformation by BCR-ABL[59]	
ISG15–/–	Normal IFN signalling and response to VSV and LCMV[60]	Shows that ISG15 exerts antiviral activity in vivo
	Enhanced susceptibility upon Influenza, Herpes and Sendai virus infections[37]	
	Significant mortality and disease development upon infection with an attenuated vaccinia virus[61]	
Ube1L–/–	Normal IFN signalling and response to VSV and LCMV[33]	Shows that ISG15 rather than free ISG15 mediates antiviral activity in vivo
	Enhanced susceptibility upon Influenza and Sendai virus infections[40,41]	
UBP43–/– ISG15–/–	Premature death, brain injuries, development of a hydrocephalus, Lethal hypersensitivity upon PolyI:C injection[32]	Shows that severe phenotype of UBP43–/– mice is ISG15 independent
UBP43–/– Ube1L	Premature death, brain injuries, development of a hydrocephalus, Lethal hypersensitivity upon PolyI:C injection, enhanced IFN signalling[33]	Shows that severe phenotype of UBP43–/– mice is ISGylation independent

UBP43 deficient animals. To unequivocally clarify the contribution of enhanced ISG15 conjugation in the phenotype of UBP43 mice, animals lacking ISG15 and UBP43 or UBE1L and UBP43 were generated. As in both types of double k.o. mice ISG15 can no longer be conjugated to substrates, phenotypic effects caused by enhanced ISGylation should be rescued (Fig. 2). However, in both experimental settings, lack of ISG15 or ISG15 conjugation did not alter lifespan, diminish brain injuries or reverse the hypersensitivity

to poly (I:C) of UBP43–/– animals, clearly demonstrating that the biological function related to the severe phenotype is not connected to the ISG15 deconjugating activity of UBP43.[32,33] Thus, UBP43 must exert additional functions besides being an ISG15 specific isopeptidase by either possessing non-enzymatic properties and/or being a not strictly ISG15 restricted isopeptidase. Indeed, evidence for a non-enzymatic function of UBP43 was provided in cell culture based experiments, where UBP43 can bind to the interferon alpha receptor subunit 2 (IFNAR2) in an isopeptidase independent manner and compete with janus kinase 1 (JAK1) for binding.[34] In support of a molecular function tightly connected to IFNAR signalling it was published that UBP43 deficiency increased resistance to transformation by the BCR-ABL oncogene, which was not observed in UBP43–/– IFNAR–/– double knockout mice.[35]

As mentioned above, so far no apparent phenotype was detected in ISG15 deficient mice in a steady state situation and animals showed unaltered responses upon VSV and LCMV infection.[36] However, the observation that ISG15–/– animals responded normally to VSV and LCMV does not provide evidence for a generally obsolete role of ISG15 in antiviral defence. The immune system usually employs different redundant systems to impede viral immune evasion and lack of the ISG15 system might thus be compensated by other pathways. And indeed when ISG15–/– animals were challenged with other viruses it became clear that the ISGylation system exerts a fundamental role in innate immunity. ISG15 deficient animals exhibited enhanced susceptibility to Influenza A and B infections as demonstrated by enhanced lethality, increased weight loss and higher virus titers in the lung.[37] Interestingly, in cell culture no difference in virus load after influenza infection between ISG15–/– and wild-type murine embryonic fibroblasts (MEFs) could be detected underlining the importance of mouse models in infection studies. In the human system knock-down of ISG15 or UbCH8 already drastically enhanced influenza virus titers in cell culture, suggesting that in humans antiviral defence by ISGylation might be essential and non-redundant in a wider variety of cell types than in mice.[38]

Lack of ISG15 also increased lethality upon infections with Herpes virus demonstrating that the effector function of ISG15 is not limited to influenza infections but rather applies to a broader range of viruses. In line with this also challenge with recombinant Sendai virus caused accelerated and higher lethality in ISG15 deficient mice than in wild-type controls. Increased lethality here could be partially rescued with wild-type ISG15 but not by expression of ISG15 mutated in the LRLRGG motif which is essential for conjugation to substrates.[37,39] Using the same kind of rescue experiments it was shown that an arginine at position 151 of murine ISG15, which is required for efficient Ube1l-ISG15 binding and transthiolation to UbcH8, is essential for proper antiviral function in vivo. Both types of rescue experiments show that ISG15 modification rather than the function of free ISG15 is responsible for the antiviral activity.[40]

ISG15–/– mice and cells derived thereof were also challenged with a vaccinia infection model, as it was shown that wild-type vaccinia virus does not induce ISG15 expression while an attenuated vaccinia virus (VVΔE3) causes high levels of ISG15. VVΔE3 lacks the viral early protein 3 which represses different antiviral pathways. Thus this strain can only replicate in cells with a compromised interferon system and is nonpathogenic in mice. When cells were infected with a wild-type vaccinia strain, cytopathic effects and virus replication were only marginally affected by the lack of ISG15. In contrast, upon infection with the attenuated VVΔE3strain ISG15–/– embryonic fibroblasts showed 25 fold higher virus titers which could be reverted by ectopic expression of ISG15. Furthermore, ISG15–/– animals exhibited significant

mortality and higher virus loads upon infection with the VVΔE3 vaccinia virus strain than wildtype controls. These results strongly indicate that ISG15 mediates antiviral effects which are counteracted by the vaccinia E3 protein.

Mice lacking the ISG15 activating E1 enzyme Ube1L are deficient in ISG15 conjugation thus lacking ISG15 modified substrates. However, expression of unconjugated ISG15 protein is not impaired in these animals. This clearly shows that Ube1L is the E1 enzyme for ISG15 and that its function can not be compensated by other E1 enzymes in vivo.[33] Animals lacking Ube1L are a valuable tool to dissect physiological functions of free ISG15 from those mediated by ISG15 conjugated with substrates. This is particularly interesting as unconjugated ISG15 not only accumulates within the cell but was also reported to be secreted like a cytokine and can be detected in sera of interferon treated humans.[2,3]

Like ISG15−/− animals Ube1L−/− animals did not exhibit altered Interferon responses and were not impaired in their responses upon VSV and LCMV infections.[33] When infected with Influenza virus, Ube1L deficient animals exhibited the same increased lethality like ISG15−/− mice and also had increased viral titers, clearly indicating that ISG15 conjugation is essential for the antiviral activity of the ISGylation system in vivo.[41]

Collectively, all these infection experiments clearly show that ISG15 modification in vivo plays an important role in immune defence and adds ISGylation to the group of other Interferon induced antiviral effector system like the Mx-GTPase pathway, the 2′,5′-oligoadenylate-synthetase-directed ribonuclease L pathway or the protein-kinase R pathway.[33,39]

ISGYLATION AND CANCER

Several reports also suggest that ISGylation plays an important role in oncogenic transformation starting with studies showing that several cancer cell lines lack Ube1L.[42] Acute promyelocytic leukemia (APL) is characterized by the expression of a promyelocytic leukaemia (PML)/Retinoic acid receptor RAR protein; treatment with all-trans-Retinoic acid (RA) lead to PML/RAR degradation. RA induces Ube1L and ISGylation in APL cells and over expression studies suggested that Ube1L also mediated ISG15 modification of PML/RAR connected to RA mediated PML/RAR repression. Consistent with such an anti-oncogenic function would be the observation that APL cells resistant to RA treatment are also deficient for RA induced ISGylation.[43,44] In addition, Ube1L was described to promote complex formation of ISG15 with cyclin D1 and inhibition of cyclin D1.[45] Further support for a role of ISG15 as an antioncogenic molecule comes from reports showing that ISG15 is induced by the anticancer drug camptothecin (CPT) which inhibits topoisomerase I (TOP1).[46] In addition, highly elevated levels of ISG15 and ISG15 conjugates were found in bladder cancer and different tumors and tumor cell lines,[47,48] a recent report provides evidence for a role of ISGylation in prostate cancer[49] and melanoma cell conditioned medium contains high levels of ISG15. Furthermore, E-cadherin induction on dendritic cells by these melanoma cells could be abolished by ISG15 neutralizing antibodies raising speculations about a cytokine like function of ISG15 which could influence the migration of tumor infiltrating dendritic cells.[4] However, while the fundamental antiviral role of ISGylation in vivo is unambiguously reflected by the phenotype of the relevant k.o. mice, the proposed roles of ISG15 modification in tumor formation still awaits a final confirmation within the context of the whole organism. So far mice lacking distinct components of the ISGylation

machinery were not reported to exhibit enhanced susceptibility in tumor models and thus it is still unclear whether the connection of ISG15 modification to oncogenic transformation is simply correlative or causally determined.

MOLECULAR MECHANISMS

Although it is still enigmatic how ISG15 exerts its function within the context of the whole organism, cell culture based experiments have provided evidence for different modes of action, which might also apply in vivo. As shown in Figure 3 proposed

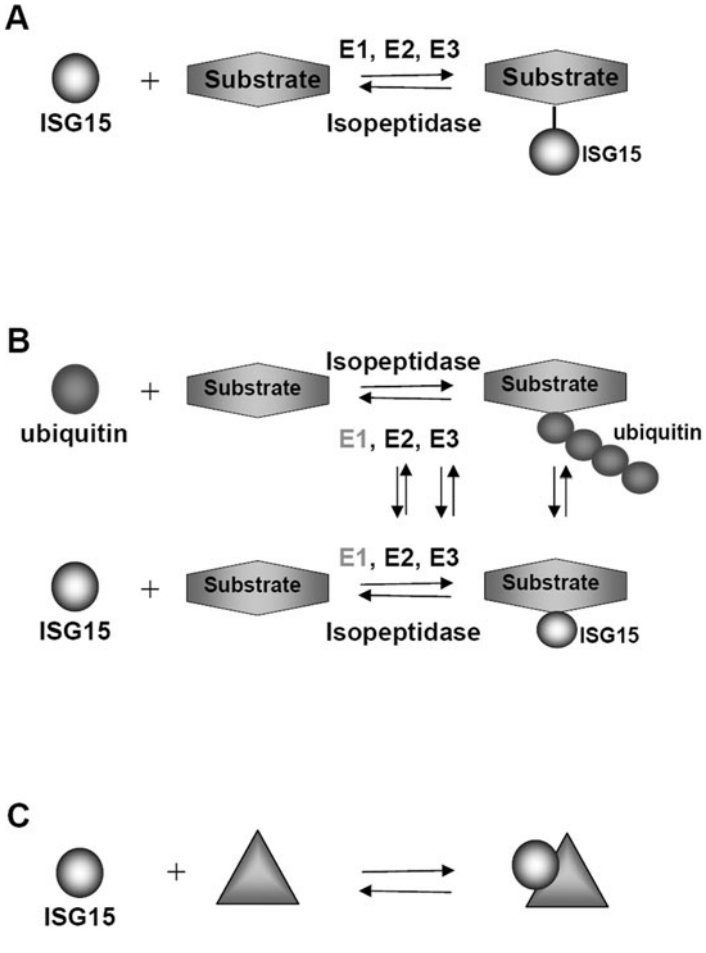

Figure 3. Potential ISG15 effectors functions. A) Covalent modification of ISG15 may alter functional properties of target substrates. B) Distinct E2 and E3 ligases as well as isopeptidases mediate ubiquitin- as well as ISG15-conjugation and deconjugation. ISGylation therefore might interfere with the ubiquitin modification machinery or compete with ubiquitin for lysine residues in target substrates. C) Unconjugated ISG15, which is present within the cell and can be secreted, might form non-covalent complexes with other proteins (depicted as triangle) to exert effectors functions.

molecular mechanisms of ISG15 in principle can be subdivided into the following groups: (i) ISGylation of proteins causing altered function of the target substrate. (ii) Competition with ubiquitin modification. (iii) Functions mediated by unconjugated ISG15.

ISGylation of Proteins Causing Altered Function or Stability of the Target Substrate

It is well established that the covalent modification of proteins by ubiquitin or UBLs like SUMO or NEDD8 can alter the function, binding properties, stability or localisation of target substrates. Although hundreds of ISG15 target substrates were identified, for most of these targets it is still unclear how ISG15 modifies their function. Generalized effects of ISG15 modification were not identified so far. Two reports describe the regulation of ubiquitin E2 enzymes by ISG15 modification, supporting the concept that ISGylation is tightly interlaced with ubiquitin modification. So it was shown that the E2 enzyme UbC13, which interacts with Mms2 and mediates formation of Lysine 63 linked (K63) ubiquitin chains, is ISG15 modified at a single lysine residue. As ISG15 modified UbC13 can no longer bind ubiquitin agarose, ISGylation of UbC13 might switch off the enzyme activity by interfering with the ability to form a thioester bond with ubiquitin. Thus, UbC13 mediated K63 linkage, which plays a fundamental role for example in NF-kappaB activation and DNA repair, might be negatively regulated by ISG15.[50]

It was also shown that UbcH6 is not only an E2 enzyme for ISGylation but can also be ISG15 modified itself at a lysine residue near the catalytic site. As described for Ubc13, this ISG15 modification disrupts the capability of UbcH6 to form a thioester bond with ubiquitin, providing evidence for a negative regulation of UbcH6 mediated ubiquitin transfer by ISG15 modification.[51] In contrast to the described negative regulation of enzyme activity also "gain of function" effects have been described for ISGylation. 4EHP, which is an mRNA 5′cap structure binding protein, was shown to be modified by ISG15, mediated by the E3 ligase HHARI. 4EHP acts as a translational repressor by competing with the translation initiating factor eIF4E for binding to the cap structure. ISGylation enhanced the cap structure binding activity of 4EHP, thereby negatively modulating translation, a feature which might be crucial in the inhibition of viral protein synthesis.[14] ISGylation can also alter binding properties of target substrates. Recent work showed that ISG15 modification of filamin B inhibits IFN-α induced JNK signalling. This specific pathway accelerates interferon induced apoptosis and filamin B facilitates signalling by acting as a scaffold protein.[52] ISGylation abrogates the scaffold function of filamin B, resulting in inhibition of the IFN induced JNK pathway. Thus promotion of IFN induced apoptosis might be blocked by ISGylation, which could be beneficial to prevent undesired apoptotic effects during viral infection.[53]

Competition with Ubiquitin Modification

As ISG15 and ubiquitin linkage are highly similar it is appealing to speculate about competitive binding of ubiquitin and ISG15 to the same lysine residues of a target protein. In addition, ubiquitin and ISG15 employ an overlapping set of E2 enzymes, E3 ligases and isopeptidases. Due to the large amounts of ISG15 expressed upon stimulation, it would be feasible to argue that enzymes usually employed for ubiquitin modification are titrated away by ISG15. Competition between ISGylation and ubiquitination would

be in line with reports of ISG15 conjugated substrates being inversely correlated with levels of ubiquitin modification.[47]

Functions Mediated by Unconjugated ISG15

In line with conjugation independent functions of ISG15 are results from cell culture experiments which provide evidence that free ISG15 counteracts virion release of Ebola virus like particles. Free ISG15 bound to the membrane-localized E3 Ligase NEDD4, blocked the interaction with E2 enzymes and thus prevented ubiquitin transfer. Resulting lack of Nedd4 ligase activity diminished ubiquitination of virus particles. As ubiquitination of viral matrix proteins by Nedd4 like proteins facilitates exocytosis of virions, ISG15 mediated suppression of ubiquitin transfer resulted in a reduction of virus like particles release, suggesting a critical role of ISG15 as a negative regulator of viral budding.[54,55]

Taken together, currently there is no common theme which can be ascribed to ISG15 modification and analyses of the molecular mechanisms exerted by ISG15 in vivo are still in their infancies.

CONCLUSION AND FUTURE PERSPECTIVES

Despite the fact that ISG15 was the first ubiquitin-like modifier discovered, remarkably less is still known about its function and molecular mode of action in vivo. For example till now nothing like an ISG15 interacting motif was discovered and it is unclear whether ISG15 modification can mediate distinct protein-protein interactions. It also needs to be clarified whether the reported secreted form of ISG15 binds to a distinct receptor or mediates signals in an autocrine or paracrine manner.

As it has been shown that the severe phenotype of UBP43–/– mice is not connected to the ISG15 deconjugating activity of this isopeptidase, the molecular function of UBP43 within the context of the whole organism remains enigmatic. It needs to be determined whether the negative regulation of interferon signalling based on the interaction of UBP43 with the IFNAR2 also accounts for the observed phenotypic alterations. Alternatively, as some ubiquitin isopeptidases were reported to also possess ISG15 deconjugation activity, one might speculate that UBP43 under certain circumstances is capable to cleave ubiquitin or other ubiquitin like modifiers from distinct substrates. Thus it will be interesting to evaluate whether and to what extend enzymatic and non-enzymatic functions of UBP43 account for the severe phenotype in UBP43–/– mice. This is particularly important as the observed enhanced viral resistance and resistance to BCR-ABL induced CML of UBP43–/– mice predestines the molecule as an attractive antioncogenic and antiviral drug target. Dissection of Isopeptidase dependent and independent functions will be detrimental to specifically target the relevant function with pharmacologic inhibitors.

It will be also interesting to uncover the degree of overlap between the ISG15 and ubiquitin modification system on the level of E3 ligases. In this context it is potentially interesting that there is no direct homologue of the major ISG15 E3 Ligase HERC5 in the mouse. Instead the closest homologue of human HERC5 encoded in the murine genome is misleadingly called mHERC5 but rather represents the gene homologous to human HERC6, which is also IFN inducible but no ISG15 E3 Ligase in humans. It

will be interesting to see whether the difference in the E3 usage might account for the differences observed between human and murine ISG15 modification. The major goal will be to further define molecular mechanisms of ISGylation and it will be exciting to evaluate whether a common mode of action underlies the effector functions mediated by ISG15.

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CHAPTER 18

IDENTIFICATION AND VALIDATION OF ISG15 TARGET PROTEINS

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Abstract: ISG15 is an interferon-induced ubiquitin-like protein (Ubl) that has antiviral properties. The core E1, E2 and E3 enzymes for conjugation of human ISG15 are Ube1L, UbcH8 and Herc5, all of which are induced at the transcriptional level by Type 1 interferon signaling. Several proteomics studies have, together, identified over 300 cellular proteins as ISG15 targets. These targets include a broad range of constitutively expressed proteins and approximately 15 interferon-induced proteins. This chapter provides an overview of the target identification process and the validation of these targets. We also discuss the limited number of examples where the biochemical effect of ISG15 conjugation on target proteins has been characterized.

INTRODUCTION

The innate immune response of mammalian cells is a first-line defense against viral and microbial infections.[1] The system is nonspecific with respect to antigen recognition and is instead geared to recognize classes of molecules that are commonly produced by microbes or viruses, such as lipopolysaccharides, flagellin and double-stranded RNA. These molecules are referred to as PAMPs, for Pattern Associated Molecular Patterns. Pathogen Recognition Receptors (PRRs), such as Toll-like receptors, RIG-I and double-stranded RNA-activated protein kinase (PKR), directly recognize PAMPs and trigger signaling pathways that lead to the production of Type I interferons (IFNs).[2] The two major forms of Type I IFNs are IFNα and β. IFNα is produced primarily by leukocytes, while IFNβ is produced by a wide range of cell types, including fibroblasts and epithelial cells. Type 1 IFNs are secreted by infected cells and bind to the interferon receptor (IFNAR) of surrounding cells, as well as

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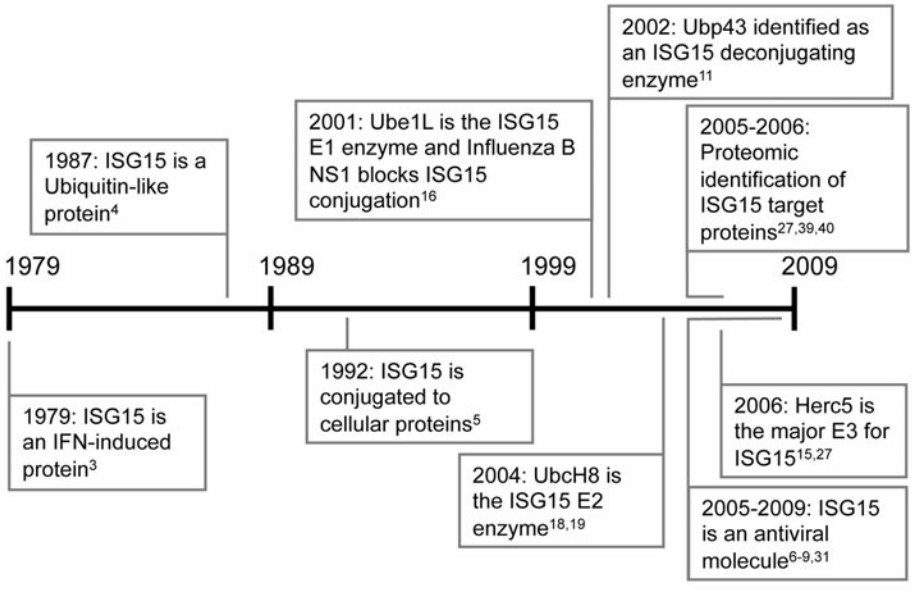

Figure 1. Important discoveries in the ISG15 field. The timeline highlights the progress made since the discovery of ISG15 in 1979.

the initially infected cell, resulting in the transcription of hundreds of interferon stimulated genes (ISGs). The expression of ISGs leads to the generation of a multi-faceted defense system that serves to limit viral or microbial infection.

ISG15 is an ISG denoted by the approximate molecular mass of the protein, although the actual molecular mass of the mature form of the protein is 17.1 kD. It was first identified based on its rapid induction in Ehrlich ascites tumor cells after IFN stimulation.[3] About a decade later, Art Haas and coworkers recognized ISG15 as what would turn out to be the first of many ubiquitin-like protein (Ubl) modifiers.[4,5] As discussed further below, it would be nearly another two decades before ISG15 was shown to actually have antiviral activity against a range of viruses, including Influenza, Sindbis, Herpes, HIV and Ebolavirus.[6-9] There have so far been no reports demonstrating an antimicrobial function for ISG15. A timeline of discovery for ISG15, spanning 30 years from 1979 to 2009, is shown in Figure 1.

Like ubiquitin (Ub) and other Ubls, ISG15 is synthesized as a precursor protein that must be processed to reveal a C-terminal glycine residue. The identity of the processing enzyme is unknown, but Ubp43, an ISG15 deconjugating enzyme, is clearly not essential for processing as the Ubp43 knockout mouse still generates processed and conjugation-competent ISG15.[10] There are also inconsistencies in the literature concerning the role of murine Ubp43 as a specific ISG15 deconjugating enzyme. It has been reported to have activity against both ubiquitin and ISG15[11,12] and neurologic phenotypes of the Ubp43 knockout mouse were also seen in the Ubp43/ISG15 double knockout mouse,[10,13] indicating that accumulation of ISG15 conjugates cannot account for the Ubp43$^{-/-}$ phenoytpes. The difficulty is identifying a specific deISGylating enzyme, if one indeed exists, is likely complicated by the fact that the last six residues of ISG15 are identical to

those of ubiquitin (LRLRGG) and these residues must be recognized by the active site of deconjugating enzymes. Consistent with this, other presumed deubiquitinating enzymes have also been shown to have activity against ISG15 conjugates.[14]

Conjugation of human ISG15 to target proteins occurs via an enzymatic cascade consisting of a single E1 enzyme (Ube1L), E2 enzyme (UbcH8) and a single major E3 enzyme (Herc5). The genes encoding these proteins are all transcriptionally induced by IFNα/β[15] although their induction is delayed relative to that of ISG15. Therefore, while free (unconjugated) ISG15 is rapidly induced by interferon stimulation,[3] conjugation of ISG15 does not occur to an appreciable degree until approximately 18-24 hours after stimulation.[5] It is not understood why this delay is built into the conjugation system, although it cannot be ruled out that that free ISG15 has a function independent of conjugation. Ube1L is the ISG15 E1 enzyme and this is a monomeric E1-like enzyme,[16] unlike the heterodimeric E1 enzymes for Nedd8 and Sumo. The E2 enzyme for ISG15 is UbcH8/Ube2L6.[17-19] While UbcH8 has been reported to be an E2 for ubiquitin,[20-26] determination of kinetic constants of Ube1L and Ube1 ($E1^{Ub}$) for UbcH8 and UbcH7 indicated that UbcH8 is unlikely to function as a ubiquitin E2 in vivo.[17] The major E3 for ISG15 in human cells is Herc5, a HECT domain ligase with N-terminal RCC1 repeats.[15,27] Herc5 depletion leads to a dramatic decrease in overall ISG15 conjugation activity, affecting conjugation to the vast majority of cellular target proteins.[15] While additional proteins may be required for ISG15 conjugation, Ube1L, UbcH8 and Herc5 form the core IFN-induced components of the system, as their co-expression with ISG15 reconstitutes robust ISG15 conjugation in non-IFN-stimulated cells.[15,27,28] ISGylation of target proteins has so far not been reconstituted in vitro. The difficulty appears to lay at the level of the E3 enzyme, as ISG15, Ube1L and UbcH8 are all biochemically active in vitro.[17]

Interestingly, human Herc5 does not have a direct equivalent in mice or rats.[29] However, the human Herc5 and Herc6 genes are adjacent to each other on chromosome 4 and are clearly related to each other through a gene duplication event.[15] Both genes are transcriptionally regulated by interferon signaling and the human Herc5 and Herc6 proteins are 50% identical with very similar domain organizations.[29] Mouse Herc6 is interferon-induced and located at the corresponding genomic position as human Herc6. While it was proposed that Herc5 was the result of a gene duplication of Herc6 in the primate lineage,[29] this is likely incorrect since other mammals (dogs, cow, sheep) have both the Herc5 and Herc6 genes in a similar arrangement as in the human genome, suggesting that Herc5 was lost in the evolution of the rodent lineage. Although there is no evidence that human Herc6 plays a significant role in ISG15 conjugation, it is conceivable that murine Herc6 plays the equivalent role to human Herc5 in conjugation of murine ISG15. To our knowledge, this possibility has not yet been experimentally tested.

Support for an antiviral function for ISG15 was first reported in 2001 when it was found that influenza B virus blocked the conjugation of ISG15 to cellular proteins.[16,30] Since then, ISG15 has been reported to have antiviral effects against Influenza, Sindbis, Vaccinia (VACV), Herpes Simplex I, murine γ-Herpesvirus, HIV-1 and Ebola virus.[6-9,31] Recent studies with mice lacking Ube1L highlight the importance of ISG15 conjugation, as Ube1L-null mice infected with either Sindbis or influenza B virus produced free ISG15, but no conjugates and were shown to have increased susceptibility to both viruses.[32,33] In addition to Influenza B, other viruses have also evolved mechanisms for interfering with ISG15 conjugation: SARS coronavirus encodes a protease that can deconjugate ISG15 from target proteins,[34] while vaccinia virus encodes a viral early protein, E3, which binds ISG15 and prevents ISGylation.[35]

Importantly, the precise biochemical function of ISG15 and the biochemical basis of its antiviral activities remain unknown. In order to understand the mechanism by which ISG15 conjugation contributes to antiviral responses, it is important to identify the proteins targeted by the ISG15 pathway. As described below, proteomic studies have identified over 300 cellular ISG15 targets. The functional consequences of ISGylation will also be discussed, although this has only been determined for a small number of target proteins.

DETECTION OF ISG15 CONJUGATION

ISGylation of target proteins can be detected by two cell-based methods: either by treatment of interferon-responsive cells (e.g., A549, HeLa) with purified IFN-β, or by cotransfection of non-interferon-stimulated cells with four plasmids expressing the core ISG15 conjugation components (ISG15, Ube1L, UbcH8 and Herc5). In HeLa cells, conjugation of ISG15 can be detected approximately 18 hours after treatment with IFNβ, with maximal accumulation of conjugates by 48 hours posttreatment.[5,16] For cotransfection experiments, HEK293T cells can be transfected with plasmids expressing Ube1L, UbcH8, Herc5 and tagged (3X-FLAG) or untagged ISG15. In both cases, extracts are typically prepared 24-48 hours after IFNβ treatment or transfection and total cellular proteins are separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane is then probed with anti-ISG15 antibody (Santa Cruz Antibodies) or with anti-FLAG antibody (Sigma) to detect conjugation of FLAG-ISG15. It is often useful to use a 12% acrylamide gel in order to observe the free (unconjugated) ISG15. Conjugates typically range in size from approximately 45 kD to very high molecular weight adducts that migrate near the top of the gel. Figure 2 shows a time course of ISG15 conjugation of

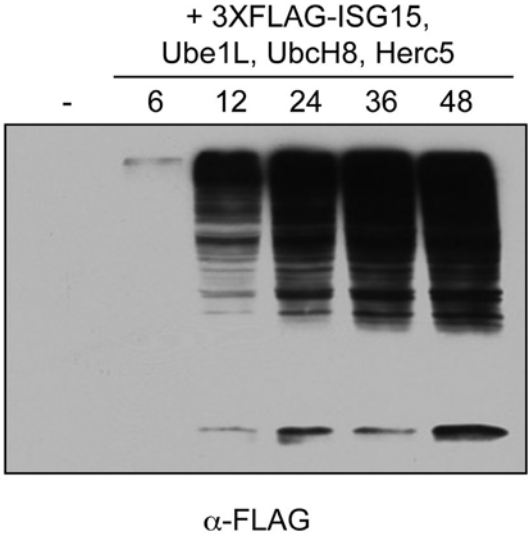

Figure 2. Timecourse of total ISG15 conjugation in non-interferon-treated cells. HEK293T cells were transfected with plasmids expressing Ube1L, UbcH8, Herc5 and 3X-FLAG-ISG15. Cell extracts were collected at the indicated time points and analyzed by immunoblotting with anti-FLAG antibody.

FLAG-ISG15 following cotransfection of plasmids expressing 3XFLAG-ISG15, Ube1L, UbcH8 and Herc5. It should be noted that, at least in our hands, HEK293T respond poorly to IFNβ stimulation and little if any endogenous ISG15 conjugation can be detected in these cells.

IDENTIFICATION OF ISG15 TARGETS

For ubiquitin, large-scale mass spectrometry-based identification of target in yeast proteins was first reported in 2003.[36] This approach involved expression of an N-terminally tagged (6xHis) ubiquitin molecule, affinity purification of conjugates under denaturing conditions and multidimensional liquid chromatography coupled with tandem mass spectrometry to identify conjugated proteins. The basis of mass spectrometry identification of ubiquitin and Ubl modifiers and the criteria for considering a protein as target have been discussed elsewhere.[37,38] Based on this general principle, three proteomics studies were performed to identify cellular proteins conjugated to ISG15.[27,39,40] Zhao, et al, expressed a double-tagged ISG15 protein (6xHis and FLAG N-terminal tags), along with Ube1L and UbcH8, in HeLa cells and then treated the transfected cells with IFN-β. Extracts were prepared 24 hours after IFN treatment and conjugates were purified under denaturing conditions on Ni-NTA resin, followed by purification on anti-FLAG beads. Purification under denaturing conditions assured that conjugated proteins would be identified, as opposed to proteins associated with conjugated proteins. Isolated proteins were separated by SDS-PAGE and regions of the gel were subjected to trypsin digestion, peptides were separated and identified by liquid chromatography and mass spectrometry, as described.[40] This study identified approximately 158 high-confidence targets. In a second study, Giannakopolous, et al, identified ISG15 conjugates in interferon-treated human cells (U937 cells) and in mouse Ubp43-null MEFs.[39] Conjugates were immunopurified using antibody recognizing endogenously expressed ISG15. Mass spectrometry identified a total of 76 mouse and human proteins in the immunoprecipitates. Finally, Wong, et al, stably expressed FLAG-ISG15 in A549 cells and immunopurified conjugates after 48 hours of IFNβ treatment.[27] 174 ISG15 target proteins were identified in this study and together, these three studies identified slightly more than 300 unique proteins as potential ISG15 target proteins.

The identification of ISG15 target proteins was highly anticipated, as it was hoped that this would provide valuable insight into the biologic function of ISG15. This, for example, was the case for identification of targets of Sumo, where target identification revealed a very high percentage of targets to be nuclear proteins involved in regulation of DNA- and RNA-related processes.[41,42] Unfortunately, the identification of ISG15 target proteins was not particularly revealing. The targets were largely abundant constitutively expressed proteins, with the exception of approximately 15 targets that were themselves interferon-induced proteins. The interferon-induced targets included some of the better-characterized antiviral ISGs, including PKR, p56, MxA and RIG-I.[43,44] PKR and RIG-I, as mentioned above, are pathogen recognition receptors. p56 is a translational inhibitor and MxA is an endoplasmic reticulum-associated GTPase. The constitutively expressed proteins included both cytoplasmic and nuclear proteins involved in a diverse array of cellular functions, including cytoskeletal organization, stress responses, translation, transcription, RNA splicing and general metabolism. Therefore, a clear prediction of the biologic function for ISG15 could not be inferred

from this diverse set of target proteins. Finally, it should be noted that a common primary sequence motif has not been identified within this set of target proteins that might serve as a recognition signal for ISGylation and the basis for substrate recognition by the ISGylation machinery remains unknown.

VALIDATION OF ISG15 TARGETS

Validation of ISG15 targets can be performed in several different ways depending on the availability of an antibody to the target, the expression level of the endogenous target protein and the degree to which the target protein is modified with ISG15. In the simplest case, IFNβ can be used to induce the ISG15 conjugation system for 24-48 hours, followed by immunoblotting for the endogenous target protein. The presence of ISG15 conjugates is then seen as the appearance of modified forms of the protein corresponding to the 15 kD Ubl. Multiple conjugates at 15 kD intervals are seen among on some targets. There is no evidence that these represent poly-ISG15 chains and mass spectrometry based mapping of modification sites on individual target proteins strongly suggests that these represent single ISG15 moieties at multiple lysine residues of the target protein (unpublished results). The pattern of modified bands may appear more complicated due to the fact that mono-ISGylated proteins modified at different sites of a target protein may result in slightly different migration on SDS-PAGE gels. In any case, these modified forms can be confirmed to be ISG15 conjugates by siRNA knockdown of one or more of the ISG15 conjugation enzymes. Importantly, only a small number of target proteins have been validated by direct western blotting of IFN-treated cells and these have primarily been those that are IFN-induced and highly expressed, such as p56 and MxA.[38] This may primarily be due to the fact that ISGylation is an inefficient process. Typically, the most robust conjugation observed will be on the order of 10-20% of the total pool of the target and in most cases it is much lower. Alternatively, immunoprecipitation of a target protein, followed by immunoblotting for ISG15 (IP-westerns), may serve to enrich for the target protein and result in more sensitive detection of conjugates. ISG15 conjugates can also be concentrated by immunoprecipitating total conjugates and then immunoblotting for an individual target protein.

The single most successful way to validate ISGylation of targets has been to transiently express the target protein along with the conjugation components (ISG15, Ube1L, UbcH8, with or without Herc5) in non-IFN-treated cells. Prior to the identification of Herc5 as the major ISG15 E3, modification of targets was in some cases validated by co-expression of the target with ISG15, Ube1L and UbcH8.[39,40] In retrospect, this combination of components was likely sufficient for modification of some proteins because of a basal level of expression of Herc5.[15] That is, while conjugation can be detected in HEK293T or HeLa cells by co-expression of ISG15, Ube1L and UbcH8, an siRNA against Herc5 dramatically decreases the level of conjugates, while co-expression of Herc5 boosts the level of conjugation.[15] Figure 3 shows the modification of four epitope-tagged target proteins (V5-Ran, FLAG-p56, V5-moesin and TAP-Hsc70) in the presence of a triple (ISG15, Ube1L, UbcH8) or quadruple (ISG15, Ube1L, UbcH8, Herc5) combination of the conjugation components. This demonstrates that modification can in some cases be detected with only basal levels of Herc5 expression, but that it in all cases it is strongly enhanced by co-expression of Herc5.

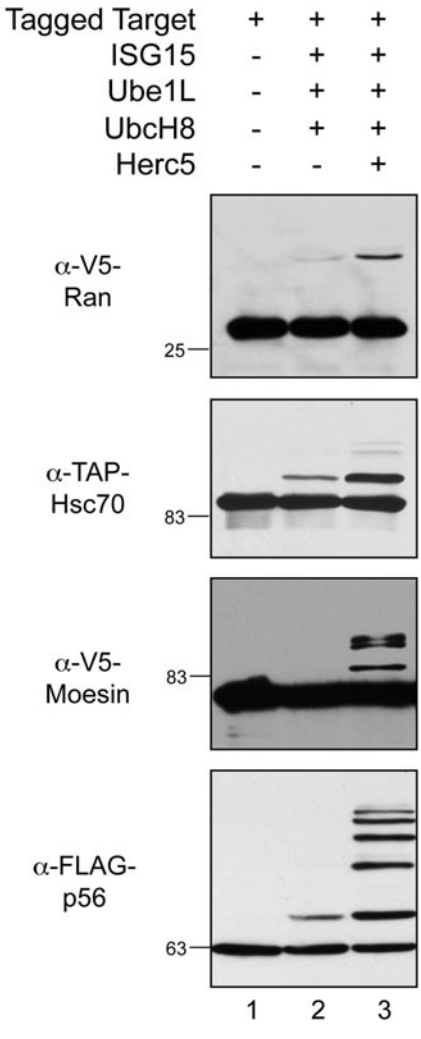

Figure 3. ISG15 conjugation to exogenously expressed targets is enhanced by the co-expression of Herc5. HEK293T cells were transfected with a plasmid expressing an epitope-tagged target protein either alone (lane 1), or combined with Ube1L, UbcH8 and ISG15 (lane 2), or combined with Ube1L, UbcH8, ISG15 and Herc5 (lane 3). Cell extracts were prepared 48 hours posttransfection and analyzed by immunoblotting using the indicated antibodies.

THE EFFECTS OF ISGYLATION ON TARGET PROTEIN FUNCTION

As described above, there are a number of cell-based approaches can be used to generate ISGylated proteins and validate modification of individual proteins. However, it is important to note that an in vitro ISGylation system has not yet been established. Without such a system, it is not possible to easily generate purified ISGylated target proteins for biochemical analyses. This is a key reason why only modest progress has been made

on understanding the biochemical function of ISG15 conjugation. Nevertheless, a small number of studies have examined modification of different target cellular target proteins and described biochemical consequences of modification. The four targets discussed here are Ubc13, filamin B, PP2Cβ, and 4EHP.

Ubc13 is a ubiquitin E2 enzyme that functions in the generation of K63-linked polyubiquitin chains. Ubc13 was identified as a cellular target of ISGylation in one of the three proteomic studies discussed above.[40] Two subsequent reports showed that Ubc13 is ISGylated at lysine residue K92, which lies near the active-site cysteine residue (C87) of the protein. Both studies showed that the ISGylated form of Ubc13 was defective for ubiquitin thioester formation.[45,46] Given the proximity of C87 to K92 in the structure, the inhibition of thioester formation is likely to be simply a result of steric occlusion of C87 by the conjugated ISG15 molecule. The downstream effects of this inhibition have been suggested to result in prevention of NFkB activation,[47] as K63-linked polyubiquitination catalyzed by the Ubc13/Mms2 complex is a critical component of the signaling pathway that leads to NFkB activation. NFkB activation, in turn, is critical for transcriptional activation of genes involved in the innate immune response, including IFNβ. An unresolved problem with this proposed mechanism is that only a small fraction of Ubc13 is ISGylated and it is not clear how this would lead to a significant inhibition of overall Ubc13 activity.

Filamin B is one of three related actin binding proteins that are critical for crosslinking of cortical actin filaments[48] and it was identified in one of the three large-scale ISG15 proteomics projects discussed above.[40] At early time points after interferon stimulation, filamin B tethers RAC1 and a MAP kinase module, which promotes activation of JNK and JNK-mediated apopotosis. The ISGylation of filamin B was shown to lead to release of RAC1, MEKK1 and MKK4 from the scaffold, preventing JNK activation.[49] A model was proposed whereby ISGylation of filamin B, at relatively late time points after interferon stimulation, leads to the inactivation of JNK.

PP2Cβ is a protein phosphatase identified in two ISG15 proteomics studies.[40,50] PP2Cβ dephosphorylates the TAK1 and IKK kinases, leading to inhibition of NFkB signaling and it was shown that ISGylation of PP2Cβ inhibited its activity. Finally, in a case where ISGylation activates, rather than inactivates, a target protein, the 4EHP mRNA cap binding protein was reported to have increased cap binding activity following ISGylation.[51] The biochemical basis of this enhanced binding is unknown.

CONCLUSION

A clearer picture has been to emerge of the enzymes required for ISGylation, the identity of the cellular targets of ISGylation and the spectrum of viruses sensitive to the antiviral effects of ISGylation. Some of the major gaps in our understanding concern (1) the basis for enzyme-substrate recognition in the conjugation process (that is, how a single major E3 enzyme target over 300 cellular proteins?), (2) how the ISGylation of the known targets are directly related to antiviral activities and (3) the precise biochemical effect of ISG15 on target proteins. The establishment of an in vitro ISGylation system will be important for addressing all of these problems, as will a better understanding of the precise steps that ISG15 inhibits in the course of the life cycles of relevant viruses.

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CHAPTER 19

FAT10
Activated by UBA6 and Functioning in Protein Degradation

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Abstract: The ubiquitin-like modifier FAT10 (HLA-F adjacent transcript 10) is the only ubiquitin-like modifier known, which apart from ubiquitin, directly targets proteins to proteasomal degradation. The covalent linkage of ubiquitin or other ubiquitin-like modifiers (ULM) to specific substrates is achieved by adjoining them to target proteins with an enzyme cascade using three enzymes: E1, E2 and E3. The first enzyme activates the ULM, the second enzyme serves a conjugating enzyme and the third enzyme ligates the ULM to its target. More recently, the first enzyme in the FAT10 conjugation machinery was characterized. It turned out that the novel E1 activating enzyme UBA6, which serves as a second E1 for ubiquitin in higher eukaryotes, additionally has the ability to activate FAT10. In this chapter the activation of FAT10 and ubiquitin by UBA6 as well as the role of FAT10 in protein degradation will be discussed.

INTRODUCTION

Ubiquitin serves a posttranslational signal changing the fate, function and localization of proteins. The 76-residue protein forms an isopeptide bond between its free C-terminal glycine residue and the ε-amino group of lysines in target proteins. Three sequential enzymes are responsible for conjugating ubiquitin to specific substrates. In the first step, the ubiquitin-activating enzyme (E1) uses ATP to form a thioester bond between the C-terminal glycine of ubiquitin and its active site cysteine residue.[1,2] In the second step a conjugating enzyme (E2) accepts the ubiquitin molecule also forming a thioester

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linkage in its active site. The final step involves a ubiquitin protein ligase (E3), which mediates the transfer of ubiquitin to the substrate.[3] E3 enzymes can either function as adaptor proteins between the E2 and the substrate and are then known as RING (really interesting new gene) E3 ligases or they can act as carrier proteins, receiving ubiquitin from the E2 forming a thioester and transferring it onto a substrate. These E3s are recognized as HECT (homologous to E6-AP C-terminus) E3 protein ligases.[4,5] The conjugation of additional ubiquitin moieties to lys-48 of ubiquitin leads to chain formation and subsequent degradation of target proteins by the 26S proteasome. Lys-63 linked chains as well as monoubiquitylation are involved in kinase activation, DNA repair and endocytosis.[6]

Until now, many ubiquitin-like proteins (UBLs) have been identified that share the typical "ubiquitin-fold" or "β-grasp fold" structure with ubiquitin; however, many of them do not show significant sequence similarity.[7] UBLs can be divided into two subgroups. Firstly, the ubiquitin-binding proteins (UBPs), which possess ubiquitin-like and ubiquitin-binding domains and noncovalently interact with ubiquitylated proteins.[8] Secondly, the ULMs, which become covalently linked to a lysine in a specific target protein via their C-terminal glycine residue by a unique E1-E2-E3 enzyme cascade. To date, 13 ULMs have been described (Table 1). The conjugation pathways for ubiquitin-like modifiers, e.g., NEDD8, SUMO and ISG15, are well characterized, each possessing their exclusive E1 activating enzyme.[9] For almost thirty years it was believed that also ubiquitin uses a single E1 enzyme.[5] However, the hierarchical model of ubiquitin conjugation, which was thought to be comprised of a single E1 enzyme, several E2 enzymes and hundreds of E3 enzymes, must be revised since a second ubiquitin-activating enzyme designated UBA6 was recently discovered in vertebrates and sea urchin.[10-12] Unexpectedly, UBA6 can also activate FAT10.[12] However, the complete FAT10-conjugation pathway and also FAT10-specific substrates are so far unknown. Since two E1 activation systems exist now for ubiquitin and one more E1 can activate two different modifiers in higher organisms, the ubiquitin and ubiquitin-like modifier systems gain more complexity and many questions about the regulatory events and transfer specificity arise.

Table 1. The 13 ubiquitin-like modifiers

ULM	E1
Ubiquitin	UBA1, UBA6
NEDD8	APP-BP1-UBA3
SUMO1-3	AOS1-UBA2
ISG15	UBA7 (UBE1L)
FAT10	UBA6
UFM1	UBA5
URM1	UBA4
ATG8	ATG7
ATG12	ATG7
MNSFβ	?
HUB1	?

E1 ENZYMES AND THE ACTIVATION MECHANISM

The activation of ubiquitin goes back to its bacterial ancestors ThiS and MoaD, which comprise the "ubiquitin-fold" and are activated by a similar mechanism. The activation step of these two proteins requires the E1-like enzymes ThiF and MoeB, which are involved in the thiamine and molybdopterin biosynthesis, respectively.[13,14] Both enzymes possess an ATP-binding domain facilitating the adenylation of the ubiquitin-fold proteins and formation of a thioester linkage at an active site cysteine in the case of MoeB and disulfide-linked thiocarboxylate in the case of ThiF. However, in prokaryotes ThiS and MoaD are modified by the attachment of a sulphur atom at the C-terminus, which produces thiocarboxylates. Consequently, they are required for sulphur transfer in the thiamine and molybdopterin biosynthetic pathways, but they are not serving as signal molecules.[15]

Up to now there are eight E1 enzymes identified in higher eukaryotes covering the activation of 11 different ULMs (Table 1).[16] For some of these enzymes the protein structure is available and the activation mechanism was investigated especially for ubiquitin, NEDD8 and SUMO.[17-20] UBA1, UBA6 and UBA7 form a single polypeptide chain,[15,16,21] whereas the SUMO and NEDD8 E1 enzymes, AOS1-UBA2 and APP-BP-1-UBA3, respectively, show a heterodimeric structure consisting of two polypeptide chains corresponding to the N- and C-terminal parts of the full-length E1s.[22,23] Structural analyses of the E1 enzymes revealed that they basically comprise three structural elements. In the N-terminal region two MoeB/ThiF homology domains are located, of which the second domain is involved in ATP binding containing the consensus motif *GXGXXGCE*.[14,19-21] A second element bears the active site cysteine within the consensus sequence *PYCTXXXP* positioned in the second adenylation domain.[21,24] The C-terminal end of an E1 holds the third element, the ubiquitin-fold domain (UFD), responsible for the E2 recruitment.[20,25,26] More distantly related E1s like UBA4, UBA5 and ATG7 form homodimers and are structurally more closely related to MoeB and ThiF, however hold also distinct domains, which might give unique features.[27-30]

The activation step is mechanistically best characterized for the ubiquitin E1 and starts as follows: the E1 enzyme requires ATP to adenylate the ubiquitin-like modifier at its C-terminal glycine.[2,31,32] This is followed by a nulceophilic attack of the sulfhydryl group of the E1 active site cysteine on the anhydride bond, forming a high energy thioester bond between the ubiquitin-like modifier and the E1, releasing AMP.[1,2,31-33] Then a second ubiquitin-like modifier molecule is adenylated in the ATP-binding site of the enzyme and the E1 enzyme is fully loaded, which leads to the binding of an E2 conjugating enzyme.[31,32] Mechanistically, this process is driven by the relative affinity of the enzymes to each other with the loaded E1 having a higher affinity to free E2s.[34] This is thought to be a general process for all ubiquitin-like modifiers.

UBA6: AN ACTIVATING ENZYME OF UBIQUITIN AND FAT10

In 2007 three groups independently identified a novel E1 enzyme called UBA6 (UBE1L2, E1-L2), which is a second ubiquitin-activating enzyme as well as the FAT10-activating enzyme.[10-12] It has approximately 40% amino acid sequence identity with UBA1 forming a single polypeptide chain. Two different groups identified UBA6 as a specific, second ubiquitin-activating enzyme in vertebrates and sea urchin.[10,11] Chiu et al[12] however used a smaller His tagged FAT10 and found that under these conditions

FAT10 could also be activated assuming that the much larger GST (glutathione S-transferase) tag, which was used by the other groups, masked the binding site of FAT10 and UBA6. It is now clear that UBA6 activates both ULMs ubiquitin and FAT10. The multiple charging specificity has so far only been described in the autophagy system where ATG7 serves as an activating enzyme for two different modifiers, ATG8 and ATG12 in yeast and also several ATG8 family members in higher eukaryotes[35-37] and in the mammalian SUMO pathway, where the activating enzyme APP-BP-1-UBA3 can activate SUMO-1, -2 and -3.[38,39] Recently, it was furthermore described that SUMO-2 can be activated by the UFM1-activating enzyme UBA5.[40] The essential function of UBA6 however might be charging of ubiquitin as UBA6 knock-out mice are lethal but FAT10 knock-out mice are viable and fertile.[41] However, it seems possible that FAT10 plays a key role in cytokine-induced environments of specific cells types in the immune response.

However, why did nature invent two different E1s for ubiquitin? The conventional gene knock-out of UBA6 in mice leads to embryonic lethality before day E10.5, so a redundancy can not be the reason. Additionally, the amino acid sequence identity of UBA1 and UBA6 is only ~40%. UBA7[42], the ISG15-activating enzyme, for example is even more closely related to UBA1 with ~46% amino acid conservation.[11,16] In some species like *Arabidopsis thaliana* and wheat two or three UBA1 orthologs have evolved which display a ~90% identical sequence.[43,44] Therefore the conservation in these duplicates or triplicates is much higher and a redundancy in these is more likely since previous studies could not show a difference in function of the plant E1s.[43] The question arises how UBA1 and UBA6 differ from each other. One reason might be the use of diverse E2 enzymes. It has been shown that UBA6 can interact with several E2s, but most of them can also accept ubiquitin from UBA1.[11] A UBA6-specific conjugating enzyme called USE1 (UBE2Z)[45] was identified and as the name implies it specifically accepts ubiquitin from charged UBA6.[11] This suggests that also specific E3s and substrates may be involved in the UBA6-USE1 pathway. These findings suggest that the ubiquitin-conjugation pathway is gaining more complexity, at least in higher organisms.

FAT10: A MEMBER OF THE UBIQUITIN-LIKE PROTEIN FAMILY

FAT10 possesses two ubiquitin-like domains in tandem array and its gene is located at the telomeric end of the major histocompatibility complex (MHC) class I region close to the human leukocyte antigen (HLA)-F locus leading to its designation of HLA-*F a*djacent *t*ranscript *10* (FAT10).[46,47] However, the gene was characterized as a MHC nonclass I gene, showing no sequence similarity to the classical or nonclassical MHC class I genes.[46] Expressed only in mammals, FAT10 contains a diglycine motif at the C-terminus as most ubiquitin-like modifiers resulting in the attachment to target proteins.[12,48] The crystal structure of FAT10 has not been revealed, however, structural modeling implicates a close similarity to the structure of ISG15, which also consists of two UBL domains but, in contrast to the Type II interferon induced FAT10, is inducible by Type I IFNs.[49] Unlike for ISG15, FAT10-specific substrates have not been identified so far. Until now the physiological function of FAT10 is poorly understood. Apart from the localization of the *fat10* gene in the genome, several other facts point towards a possible function in the immune system. The ubiquitin-like modifier is for example synergistically inducible by the cytokines IFN-γ and TNF-α in almost all cell types and it is constitutively expressed

in mature dendritic cells and B cells.[50,51] FAT10 mRNA is abundant in lymphoid organs like spleen, gut, lymph nodes and especially the thymus as tested in different mouse and human tissues.[41,52,53] Furthermore, FAT10-deficient mice show hypersensitivity for lipopolysaccharide.[41] Another study found that FAT10 inhibited hepatitis B virus expression in a hepatoblastoma cell line.[54] The involvement of FAT10 in the regulation of $CD4^+$ T-cell anergy, acting as a downstream factor of FOXP3 was also described[55] as well as the induction of FAT10 expression during dendritic cell (DC) maturation with poly(I:C), LPS or CD40 ligand.[52,56] In monoyctes, conversely, FAT10 expression was not induced by triggering Toll-like receptors with LPS and CpG oligonucleotides.[52] It was further reported that an important component of the FAT10-conjugation pathway, the E1 activating enzyme UBA6 as well as the interacting partner NUB1 were also induced in DCs upon stimulation with maturation agents.[56] Moreover, it was shown that FAT10 expression does not change the MHC class I surface expression on cells and the MHC class I antigen presentation pathway is not altered.[48]

FAT10 overexpression causes caspase-dependent apoptosis in a murine tetracycline repressible fibroblast cell line, which seems to be in part due to FAT10 conjugation since a mutated from of FAT10 lacking the diglycine motif at its C-terminus was a much weaker inducer of apoptotis.[48] Additionally, Ross et al found apoptosis induction in human renal tubular epithelial cells, which upregulated FAT10 after HIV-1 infection.[57] There is also evidence that FAT10 plays a role in TGF-β induced cell death, since it is upregulated in TGF-β treated hepatoma cells.[58] One could speculate that FAT10 is either involved in the apoptosis pathway of TNF signalling since it can be induced by this cytokine, or that it can conjugate to anti-apoptotic proteins leading to an inhibition of survival. However, the role of FAT10 in apoptosis induction needs yet to be clarified.

On the contrary, the spindle assembly checkpoint protein MAD2 (mitotic arrest-deficient 2) interacts with FAT10 during mitotic phase of the cell cycle leading to a reduction of MAD2 localization at the kinetochores in prometaphase and an abbreviated mitotic phase. This is in line with the observation that chromosome instability occurred in long-term in vitro cultures with highly elevated FAT10 protein levels.[47,59] Furthermore, FAT10 was found to be upregulated in a transcriptome analysis of human fetal cells taken from pregnancies with trisomy 21.[60] All these findings imply a role of FAT10 in cell cycle regulation. It was therefore suggested that FAT10 is also connected with the formation of malignancies, since it was shown to be upregulated in 90% of hepatocellular carcinoma (HCC) and 80% of colon, ovary and uterus carcinomas[53] and also to be involved in gastric carcinogenesis.[61] Another study found that FAT10 is a potential marker for liver preneoplasia, since it was upregulated in hepatocytes of a drug-induced mouse model of chronic liver disease, which is assumed to develop into hepatocellular carcinoma. [62] Furthermore, p53 downregulated the expression of FAT10 in cancer cell lines by binding directly to the FAT10 promoter. This also supported the role of FAT10 in tumor development.[63] More recently, however, Lukasiak et al[52] suggested that FAT10 upregulation in tumours is rather caused by a proinflammatory immune response against the tumour, given that FAT10 expression in HCCs and colon cancers strongly correlated with the expression of the IFN-γ and TNF-α dependent proteasome subunit LMP2 in these cancer tissues. Moreover, FAT10 fails in holding oncogenic properties.[52] Interestingly, the FAT10 promoter region was reported to contain putative transcription factor binding sites for IFN (IRF-1, IRF-2, STAT-1), TNF (NF-κB) and retinoid (MZF-1) responsiveness.[63] Consistently, FAT10 can be induced with IFN-γ, TNF-α[50] or retinoids,[64] respectively.

THE ROLE OF FAT10 IN PROTEIN DEGRADATION

NEDD8 ultimate buster 1 long (NUB1L) was identified as a noncovalent interaction partner of FAT10 in a yeast two-hybrid screen.[65] NUB1L is a 14 amino acid longer splice variant of NUB1, which was first reported to interact with NEDD8 and to target it for proteasomal degradation.[66] On the contrary, Hipp et al only detected interaction of NUB1L with FAT10, not with NEDD8.[65] The binding domain of FAT10 to NUB1L was assigned to the three ubiquitin-associated (UBA) domains in the C-terminal region of NUB1L, whereas only the C-terminal UBL domain of FAT10 bound to NUB1L. Furthermore, the UBL domain of NUB1L as well as both UBL domains of FAT10 could interact with the 26S proteasome.[67] For NUB1 the S5a proteasomal subunit at the 19S regulatory particle was proposed as the acceptor subunit in vitro.[66] For FAT10 the binding site at the proteasome has not yet been identified. These features categorize NUB1L in the group of ubiquitin-domain proteins, which are thought to link polyubiquitinated proteins and the 26S proteasome.[68] However, in the case of NUB1L the UBA domains do not bind to monoubiquitin or polyubiquitin chains.[69]

NUB1L has the ability to accelerate the degradation of FAT10, of several N-terminal fusion proteins of FAT10 and also of a covalently linked FAT10 conjugate in a stable mouse fibroblast transfectant with the UBL domain of NUB1L being essential.[61,65] Furthermore, it was shown that the degradation of FAT10 and its fusion proteins is independent of ubiquitin, but requires NUB1L, since in vitro degradation experiments showed, that FAT10-linked dihydrofolate reductase (DHFR) could not be efficiently degraded by the 26S proteasome if NUB1L was missing.[61,70] Therefore, FAT10 is the only ubiquitin-like modifier serving a similar function as ubiquitin in leading target proteins to the proteasome and facilitating their degradation together with the UBL-UBA protein NUB1L, which serves not only as a linker between FAT10 and the 26S proteasome, but also as a facilitator of degradation by the proteasome (Fig. 1).

More recently, histone deacetylase 6 (HDAC6) was also reported to interact noncovalently with FAT10 under proteasomal inhibition, which leads to the localization of FAT10 in aggresomes depending on an intact microtubule network (Fig. 1).[71] This finding links FAT10 to the second major pathway of protein degradation in the cell, because it has been shown that proteins, which are stored in aggresomes, can be eliminated by the lysosomal pathway via autophagy.[72,73]

CONCLUSION AND FUTURE PERSPECTIVES

Since the discovery of FAT10 in 1996 the understanding of the role of FAT10 in proteasomal degradation has expanded considerably. However, to disclose the physiological function of FAT10 it will be important to investigate in the future what specific substrates FAT10 conjugates to and which possible role FAT10 plays in degradation of these substrates. Also what is the link of FAT10 to autophagy? And is there a link to the immune response? Still many questions need further clarification. The further investigation of the FAT10 knock-out mice might give new insight on that matter. The identification of a FAT10-activating enzyme brought us one step closer to identifying FAT10 targets. It will be exciting to examine what E2 and E3 enzymes FAT10 requires. Furthermore, the dual specificity of the E1 enzyme UBA6, activating

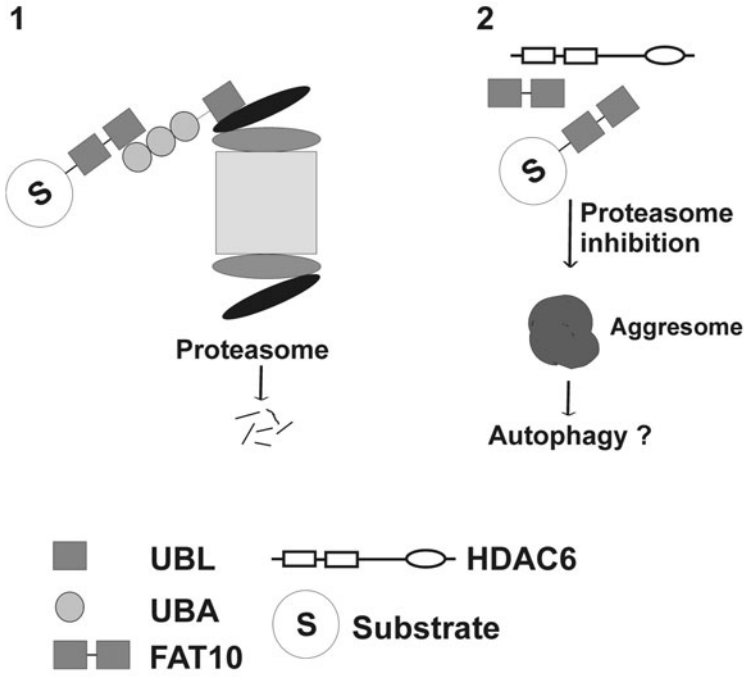

Figure 1. FAT10 is linked in two different ways to protein degradation. 1) FAT10 interacts with the UBA domains of NUB1L and together they target proteins for degradation by the proteasome. 2) Under proteasomal inhibition FAT10 and FAT10-linked proteins colocalize with HADC6 in aggresomes. This might lead to degradation by autophagy.

ubiquitin and FAT10, holds many open questions, which have to be addressed in the upcoming years.

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