1

Orchestra for assembly and fate of polyubiquitin chains

Kirsten Kuhlbrodt, Julien Mouysset and Thorsten Hoppe[1]

Centre for Molecular Neurobiology (ZMNH), University of Hamburg, Falkenried 94, 20251 Hamburg, Germany

Abstract

Selective protein degradation by the 26 S proteasome usually requires a polyubiquitin chain attached to the protein substrate by three classes of enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). This reaction can produce different polyubiquitin chains that, depending on size and linkage type, can provide distinct intracellular signals. Interestingly, polyubiquitination is sometimes regulated by additional conjugation factors, called E4s (polyubiquitin chain conjugation factors). Yeast UFD2 (ubiquitin fusion degradation protein-2), the first E4 to be described, binds to the ubiquitin moieties of preformed conjugates and catalyses ubiquitin-chain elongation together with E1, E2, and E3. Recent studies have illustrated that the E4 enzyme UFD2 co-operates with an orchestra of ubiquitin-binding factors in an escort pathway to transfer and deliver polyubiquitinated substrates to the 26 S proteasome. Here we propose a model in which E4-dependent polyubiquitination pathways are modulated by different ubiquitin-binding proteins, using ataxin-3 as an example.

[1]*To whom correspondence should be addressed (email thorsten.hoppe@zmnh.uni-hamburg.de).*

Introduction

The ubiquitin–proteasome system (UPS) is the major pathway in eukaryotic cells responsible for selective proteolysis of intracellular proteins. In this system, proteins are marked for degradation by covalent modification with ubiquitin via isopeptide-bond formation between its C-terminus and ϵ-amino groups of internal lysine residues within the substrate protein.

Substrate conjugation generally requires a cascade of enzymes, which includes a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a specific ubiquitin–protein ligase (E3) [1]. Most often, this reaction results in a polyubiquitin chain. A specific lysine residue of each ubiquitin molecule in the extending chain serves as a site for further ubiquitination. Ubiquitin itself contains seven internal lysines (Lys^{6}, Lys^{11}, Lys^{27}, Lys^{29}, Lys^{33}, Lys^{48} and Lys^{63}), all of which have been shown to be used for ubiquitin-chain formation *in vitro* [2]. Most polyubiquitinated substrates with a Lys^{48}-linked ubiquitin chain of four or more ubiquitin moieties are targeted to degradation by the 26 S proteasome, a multicatalytic protease complex that degrades modified substrates into small peptides and amino acids. In contrast, Lys^{63}-linked polyubiquitin chains appear to play non-proteolytic functions in DNA-repair pathways, endocytosis, signal transduction, transcriptional regulation and ribosomal function [3]. Recent studies provide evidence that different fates of polyubiquitinated substrates are determined not only by the size and linkage of polyubiquitin chains, but also by specific ubiquitin conjugate binding factors (for review, see Chapter 4). In yeast, for example, the proteins Rad23 and Dsk2, are known to be involved in targeting polyubiquitinated substrates for proteasomal degradation [4,5].

Polyubiquitin-chain assembly is a processive reaction, which is typically catalysed by E1, E2 and E3 enzymes alone. However, efficient polyubiquitination of certain substrates has been shown to be regulated by additional ubiquitin-chain assembly factors, termed E4s (polyubiquitin chain conjugation factors) [6]. One founding member of this enzyme class is the yeast protein UFD2 (ubiquitin fusion degradation protein-2), which binds to the ubiquitin moieties of preformed conjugates and catalyses polyubiquitin-chain elongation in collaboration with E1, E2 and E3. E4-dependent polyubiquitination appears to be tightly regulated by a set of different ubiquitin-binding proteins. This regulation results not only in the restriction of the number of ubiquitin molecules added by UFD2 to the growing polyubiquitin chain, but also in the subsequent targeting of the substrate to the proteasome [5]. Such an escort mechanism can provide another layer of regulation and specificity in the ubiquitin system. This review discusses the potential role of the ubiquitin conjugate binding factor, ataxin-3, to illustrate how alternatively used ubiquitin-chain-binding proteins can provide even more complexity in this pathway.

Ubiquitin-chain linkage: stamp for functionally distinct processes

Originally, it was assumed that polyubiquitinated proteins are exclusively targeted to the 26 S proteasome for degradation. However, recent studies have shown that the polyubiquitin signal is more versatile than expected. Different lysine residues of ubiquitin can be used for the linkage between ubiquitin monomers, which leads to polyubiquitin chains that are functionally distinct (Figure 1). Basically, two different types of polyubiquitin chains can be generated. In homopolymeric ubiquitin chains, several ubiquitin molecules are all linked to each other using the same donor lysine residue, and these have already been described for Lys^{48}-, Lys^{63}- and Lys^{6}-linked chains. In contrast, heteropolymeric chains contain more than one type of linkage [6]. There is, however, presently no experimental evidence for specific properties of these sporadically described heteropolymeric chains.

The role of the different lysine residues used for the assembly of polyubiquitin chains has been investigated by genetic approaches in budding yeast [3]. These experiments revealed that Lys^{48} of ubiquitin is essential for growth, which is directly linked to its central role in proteolysis. However, recent work by Flick et al. [7] demonstrated an additional proteolysis-independent role for Lys^{48}-linked chains. Polyubiquitination through Lys^{48} mediates inactivation of Met4 (transcriptional activator of sulphur metabolism), a transcription factor for genes involved in the biosynthesis of sulphuric amino acids.

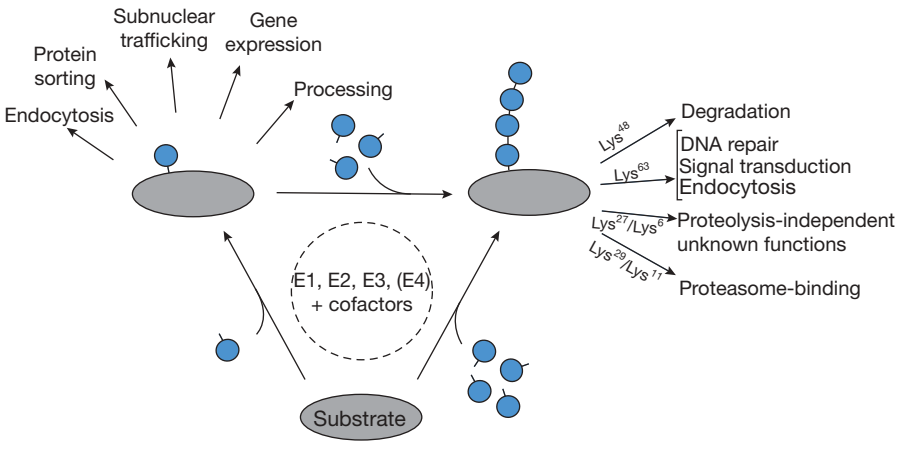

Figure 1. Ubiquitination serves as a versatile post-translational signal

Ubiquitination is performed by E1, E2, E3 and sometimes E4 enzymes together with additional cofactors, and leads to either mono- or poly-ubiquitinated substrates. Mono-ubiquitination, in most cases, regulates the activity of the substrate protein, providing signalling function in diverse processes. Throughout polyubiquitination, distinct ubiquitin chains can be formed depending on the lysine linkage used between the single ubiquitin molecules, which determines the fate of the substrate.

Surprisingly, in this case the polyubiquitin chain attached to Met4 stabilizes the protein, instead of inducing its proteasomal turnover. It has been suggested that the chain might be masked by interacting proteins and is therefore not recognized by the proteasome [7].

As well as Lys^{48}-linked chains, atypical chains linked through Lys^{29} and Lys^{11} could be similarly competent proteasomal targeting signals, since both are able to bind to the 26 S proteasome [3]. In contrast, Lys^{63}-linked chains act as non-proteolytic signals in several intracellular pathways, such as DNA repair, transcriptional regulation, protein trafficking and ribosomal protein synthesis. One example is the polyubiquitination via Lys^{63} of PCNA (proliferating-cell nuclear antigen) during error-free repair of damaged DNA, which controls its activity [8]. Lys^{63}-linked chains are also important for various regulation steps in inflammatory signalling pathways, induced by the transcriptional activator NF-κB (nuclear factor κB). In these steps, Lys^{63}-linkage-based polyubiquitination of the IKK [IκB (inhibitory κB) kinase complex] is necessary for the activation of the complex, which then phosphorylates IκB proteins that sequester NF-κB transcription factors into the cytosol. It has been shown that such modifications promote various protein–protein associations (for review, see [9]).

The less frequently used Lys^{27}-linked and Lys^{6}-linked polyubiquitin chains additionally provide functions that are distinct from proteasomal targeting. Attachment of Lys^{27}-linked polyubiquitin chains to the co-chaperone and ubiquitin domain protein, BAG-1 (Bcl-2-associated athanogene), does not induce proteasomal degradation, but rather promotes the association of BAG-1 with the proteasome ([10], but see [10a]). This enables BAG-1 to act as one of the coupling factors between molecular chaperones and the proteasomal complex. As yet, Lys^{6}-linked polyubiquitin chains have only been reported to affect the activity of DNA repair factors.

Taken together, the generation of these different polyubiquitin chains provides an important level of complexity. However, the molecular mechanism by which the different chain linkages are determined remains elusive. Perhaps linkage formation is regulated by specialized E3s. Indeed, the formation of Lys^{6}-linked ubiquitin chains is catalysed by an unusual E3 activity provided by a heterodimer consisting of BRCA1 (breast-cancer susceptibility gene 1), a breast and ovarian tumour suppressor, and BARD1 [BRCA1-associated RING (really interesting new gene) domain 1] [11,12].

Polyubiquitination regulated by E4s

A new family of conjugation factors has been identified, which seems to regulate polyubiquitination at the level of ubiquitin-chain elongation [6]. Such factors, able to catalyse an extension of the polyubiquitin chain in collaboration with E1, E2, and E3, have been termed E4s (Figure 2). The first E4 to be described was the yeast protein UFD2, which is required for efficient

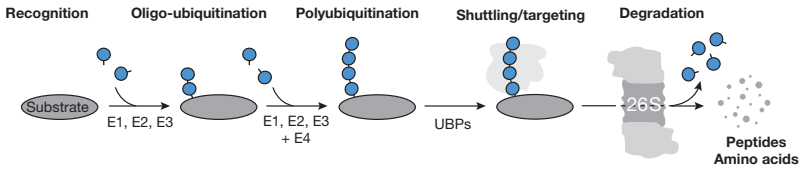

Figure 2. E4-dependent polyubiquitination and targeting
Substrate proteins are recognized for proteasomal targeting and are polyubiquitinated by the sequential activity of E1, E2 and E3 enzymes. In some cases, the additional function of E4 enzymes is required for efficient polyubiquitination. Chains with four to six ubiquitin moieties are an optimal length for interacting with UBPs (ubiquitin-binding proteins) that shuttle polyubiquitinated substrates to the 26 S proteasome for subsequent degradation into peptides and amino acids. Concomitantly with degradation, ubiquitin moieties are recycled.

polyubiquitination of ubiquitin-fusion-degradation-model (UFD) substrates and the transcription factor SPT23 [13]. Genetic and biochemical studies have revealed that UFD2 binds to substrates conjugated with one to three ubiquitin molecules, and catalyses the addition of further ubiquitin moieties in the presence of E1, E2 and E3s, yielding polyubiquitinated substrates that are targets for the 26 S proteasome. UFD2 defines the first identified family of E4s, which is characterized by a conserved C-terminal U-box (modified RING motif without the full complement of Zn^{2+}-binding ligands), a UFD2-homology domain, of approx. 70 amino acids, that is structurally related to the RING finger motif found in RING finger E3s (Figure 3). This UFD2 family includes human members and homologues in mouse, fission yeast, *Dictyostelium*, *Arabidopsis* and *Caenorhabditis elegans*, suggesting that alternative E4-dependent degradation pathways exist.

Another member of this family is CHIP [C-terminus of the Hsp (heat-shock-protein)-70-interacting protein], previously identified as a negative regulator of Hsp70 ATPase activity. In addition to the U-box, CHIP bears three tandem TPR (tetratricopeptide repeat) motifs at its N-terminus (Figure 3) that bind to the chaperones Hsp70 and Hsp90. Several studies revealed that CHIP displays E3 activity and mediates the ubiquitination of a variety of chaperone-bound substrates. Thus CHIP is likely to be involved in regulating the cellular balance between protein folding and degradation. However, CHIP has been shown to display E4 function, by regulating the ubiquitination activity of the E3 Parkin [14].

Our recent work identified a novel functional interaction between the *C. elegans* orthologues of CHIP and UFD2, CHN-1 and UFD-2, respectively, and the myosin chaperone UNC-45. Both UFD-2 and CHN-1 alone, in collaboration with E1 and E2, conjugate one to three ubiquitin moieties to the substrate UNC-45. Therefore, in *C. elegans*, both CHN-1 and UFD-2 work independently as E3 enzymes in this pathway. However, in combination, CHN-1 and UFD-2 are able to increase the ubiquitination of UNC-45. These

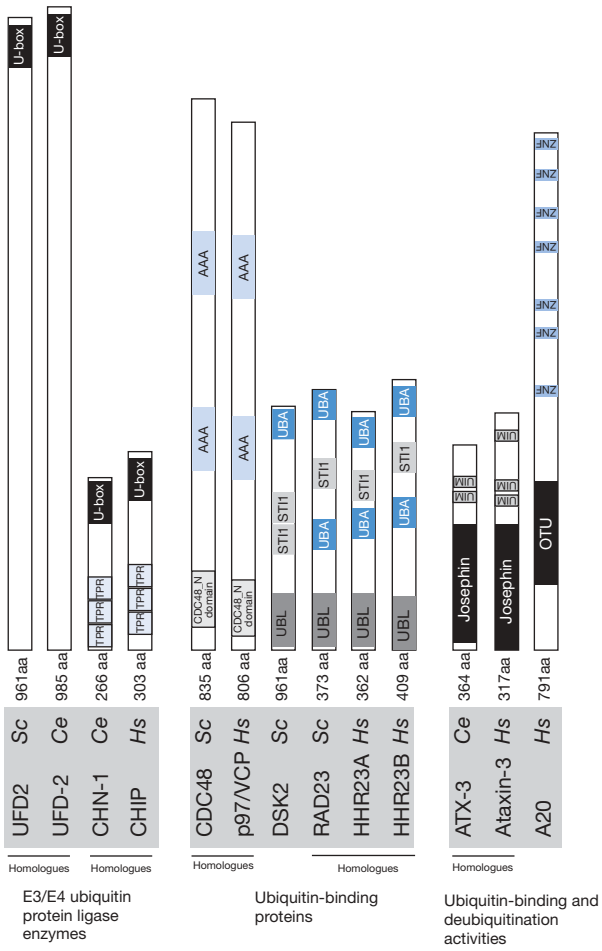

Figure 3. Schematic drawing of the primary structures of escort-pathway components and homologues

Functionally distinct domains are marked by boxes: Josephin domain, OTU domain, UBA domain, UBL domain, U-box, UFD2 homology domain (a modified RING motif without the full complement of Zn^{2+}-binding ligands). aa, amino acid; CDC48_N domain, N-terminal domain of CDC48; *Ce*, *C. elegans*; *Hs*, *Homo sapiens*; *Sc*, *S. cerevisiae*; STI1, heat-shock chaperonin-binding motif; ZnF, A20-like zinc finger.

results suggest a novel mechanism in which two E3 enzymes 'team up' to achieve E4 function. The assembly of such an E3/E4 complex in multicellular organisms could be controlled by developmentally-regulated co-expression of both enzymes in specific tissues. Furthermore, it is conceivable that different

combinations of E3 enzymes could result in alternative substrate-specific complexes with E4 activity [15].

However, E4 activity does not necessarily depend on the existence of a U-box domain. In contrast to UFD2 and CHIP, a different type of E4 enzyme is represented by p300, previously known as a transcriptional cofactor and histone acetyltransferase. p300 has been shown to polyubiquitinate mono-ubiquitinated species of the tumour suppressor p53 in collaboration with the E3 enzyme MDM2 (murine double minute clone 2 oncoprotein), whereas p300 alone is not able to conjugate ubiquitin to p53. The regulation of the GAP1 (general amino acid permease) of *Saccharomyces cerevisiae* again shows that E4 activity is not exclusively restricted to single proteins. In this case, the BUL1/BUL2 complex possesses E4 activity, necessary for ubiquitin-chain elongation of mono-ubiquitinated GAP1. p300 and the BUL1/BUL2 complex are the only E4 enzymes without a U-box that have been identified so far [16].

It is intriguing to speculate that E4 might regulate the selection of lysine residues used for ubiquitin–ubiquitin linkages during polyubiquitin-chain assembly. Indeed, as has been shown by Saeki et al. [17], yeast UFD2 catalyses a linkage switch from Lys^{29}, used for mono-ubiquitination, by further elongation of the ubiquitin chain through Lys^{48}.

Modulation of the polyubiquitin chain

Such a linkage-type switch, mediated by the yeast E4 enzyme, offers another level of control in polyubiquitin-chain assembly. An additional example of such a mechanism is the switch from mono- to poly-ubiquitination by the yeast proteins RAD5, MMS2, and UBC13, which appears to control the activity of PCNA during DNA repair [8]. Representing another fine-tuning mechanism, modulation of polyubiquitination can be reversed by the action of DUBs (deubiquitinating enzymes). This defines ubiquitination as a post-translational modification that is regulated by feedback mechanisms comparable to signals in other regulatory processes, such as phosphorylation [9,18]. Polyubiquitin-chain attachment and removal act as on- and off-switches at several points along signal-transduction cascades, as has been illustrated for the regulation of the TNF (tumour necrosis factor)-induced signal transduction pathway that leads to activation of the NF-κB family of transcription factors (for review, see [19]).

Intriguingly, the NF-κB inhibitor A20 has been discovered to be an enzyme with dual ubiquitin editing functions in the NF-κB signalling pathway. The N-terminal OTU (ovarian tumour) domain of A20, which is characteristic of DUBs of the OTU family, disassembles Lys^{63}-linked ubiquitin chains from RIP (receptor-interacting protein), an essential mediator of the TNF receptor 1 signalling complex. RIP deubiquitination works as a prerequisite for the subsequent attachment of Lys^{48}-linked chains mediated by the C-terminal E3 activity of A20, targeting RIP for proteasomal degradation. These two opposing activi-

ties of deubiquitination and ubiquitination are united within the same protein (Figure 3) in order to change the linkage of the polyubiquitin chain [19].

The ubiquitin-binding protein ataxin-3

Another ubiquitin-binding protein that comprises two distinct catalytic activities that are functionally related to the ubiquitin system is ataxin-3 [20]. Mutations in the human *ataxin-3* gene have been associated with the neurodegenerative disease spinocerebellar ataxia type 3 (SCA3) [21]. This disorder, also known as Machado–Joseph disease (MJD), is the most common dominantly inherited ataxia and one of at least nine neurodegenerative polyQ (polyglutamine) diseases, including Huntington's disease. PolyQ diseases are caused by the pathological expansion of a polyQ region in the disease protein, which leads to a misfolding process and subsequent accumulation of insoluble protein aggregates. In this context, it is very interesting that a variety of genes of the ubiquitin pathway seem to be implicated in the development of neurodegenerative diseases. Genes implicated in Parkinson's disease, for example, encode the E3 enzyme Parkin and the ubiquitin C-terminal hydrolase L1 (UCH-L1). In the case of SCA3/MJD, the disease pathology is also attributed to alterations in ubiquitin-pathway functions, since ataxin-3 has been shown to interact with ubiquitinated substrates [20,22,23].

Ataxin-3 possesses two different functional domains [24,25]: an N-terminal deubiquitinating Josephin domain and two to three UIM (ubiquitin-interacting motif) domains at the flexible C-terminal tail, which also contains the polyQ region expanded in SCA3/MJD (Figure 3). It has recently been shown that ataxin-3 binds ubiquitinated proteins [20,22]. This interaction is mediated by the UIMs, which contain a conserved cluster of amino acids, including a nearly invariant serine and a conserved leucine, which is often essential for ubiquitin binding [24]. The Josephin domain of ataxin-3 includes highly conserved amino-acid stretches reminiscent of the catalytic residues of deubiquitinating cysteine proteases [25]. Cysteine proteases in this context are also known as DUBs and have been divided into four families: USPs (ubiquitin-specific proteases), UCHs and two novel ubiquitin-specific families, comprising the Jab/Csn5 and MPN (Mpr1p Pad1p N-terminal) domain-containing proteins [the JAMM (JAB1/MPN/Mov34) group of hydrolases] and a family of cysteine proteases that contains an OTU domain (for review, see [26]). Structural studies have confirmed that ataxin-3 is evolutionarily related to deubiquitinating cysteine proteases of the UCH family. This has been substantiated by *in vitro* experiments in which ataxin-3 was able to remove ubiquitin from polyubiquitin chains [20]. Cys^{14} has been identified as the major catalytic amino acid essential for the deubiquitination activity, since the abolition of protease activity by mutation of Cys^{14} inhibits the cleavage of ubiquitinated proteins. Interestingly, the Josephin domain, which harbours this ubiquitin-specific protease activity, also mediates protein–protein interactions [27], specifically binding to the two

ubiquitin chain recognition factors HHR23A and HHR23B, the human homologues of the yeast protein RAD23.

Ataxin-3 might modulate E4-dependent polyubiquitin-chain assembly

A recent study has described that the yeast proteins RAD23 and DSK2 bind preferentially to UFD2-assembled polyubiquitin chains, which are restricted in size to an average of four to six ubiquitin molecules by the concerted action of a CDC48 complex [5]. The AAA (ATPase associated with various cellular activities)-ATPase CDC48, which binds to ubiquitin via its N-terminus (Figure 3), exerts its function in co-operation with the cofactors NPL4 and UFD1, forming the specialized CDC48–UFD1–NPL4 complex. It seems that CDC48 is a substantial component of a pathway which shuttles ubiquitin conjugates, together with additional interacting factors, to the 26 S proteasome [5]. In this escort pathway, oligo-ubiquitinated substrates are recognized by the substrate-recruiting cofactors of CDC48, relocated on to the bound E4 enzyme UFD2 for polyubiquitination, and subsequently handed over to RAD23 and DSK2, which finally mediate the delivery of ubiquitinated substrates to the 26 S proteasome.

Supporting this model, it has become evident from previous studies on Lys^{48}-linked chains that the affinity to the proteasome is influenced by the length of the ubiquitin chain. Binding affinity and chain length are related in a non-linear manner, since affinity increases more than 100-fold as the chain is lengthened from two to four ubiquitins, in contrast with a further increase of only 10-fold when eight more ubiquitin moieties are added to the chain [28]. Chains of four ubiquitin molecules act as a minimum signal for high-affinity binding to the 26 S proteasome and efficient degradation [20,28]. These chains contain enough ubiquitin molecules for optimal binding to the C-terminal UBA (ubiquitin-associated) domains of HHR23A and HHR23B (see Figure 3 and [29]).

Interestingly, *in vitro* ataxin-3 binds preferentially to Lys^{48}-linked tetra-ubiquitin chains [30] and to different components of the escort pathway. Doss-Pepe et al. [22] reported that ataxin-3 interacts with both HHR23B and p97/VCP (valosin-containing protein), the mammalian CDC48 homologue. Specifically, the Josephin domain of ataxin-3 interacts with the UBL (ubiquitin-like) domain of HHR23B, and the C-terminal region of ataxin-3 interacts with the N-terminal domain of p97/VCP. The authors propose a model in which ubiquitinated substrates are transferred from HHR23B to ataxin-3, the latter of which may function as a transiently associated polyubiquitin-chain recognition subunit of the 26 S proteasome. This implies that the activity of p97/VCP seems to be required for a concerted delivery of ubiquitinated substrates to the proteasome. Thus it is likely that ataxin-3 might play a role in the escort pathway.

Moreover, recent studies indicate that UFD2a, the human homologue of yeast UFD2, in conjunction with p97/VCP forms a ternary complex with ataxin-3 [23]. Another factor implicated in binding to ataxin-3 is the ubiquitin–protein ligase CHIP [31], whose *C. elegans* orthologue CHN-1 acts in concert with *C. elegans* UFD-2 to form an E3–E4 polyubiquitination complex [15]. Since ataxin-3 interacts both with CHIP and UFD2a, it is intriguing to speculate that ataxin-3, together with CHIP and UFD2a, is involved in regulating the ubiquitination status of specific substrates. Indeed, the conservation of this complex in *C. elegans* (K. Kuhlbrodt and T. Hoppe, unpublished work) leads to the attractive idea that *C. elegans* ataxin-3 (ATX-3), in collaboration with CHN-1 and UFD-2, could be directly involved in the regulation of the myosin assembly chaperone UNC-45, a known substrate of the E3–E4 complex formed by CHN-1 and UFD-2 [15].

Taken together, it is tempting to speculate that ataxin-3 is not mainly a substrate for ubiquitination, but that it appears to be involved in an escort pathway. Moreover, the combination of deubiquitination activity and ubiquitin-binding properties indicates that ataxin-3 plays a general regulatory role in the ubiquitin system. To down-regulate its activity, ataxin-3 seems to be ubiquitinated and degraded by the proteasome *in vivo*, which is not unusual, as it has already been shown for E3 enzymes such as TRAF (TNF-receptor-associated factor)-2 and TRAF-6 in the NF-κB pathway [32].

Conclusion

In the UPS pathway, polyubiquitination regulated by E4 enzymes is required for efficient targeting of certain protein substrates for proteasomal degradation. Ubiquitin-binding proteins help to shuttle E4-catalysed polyubiquitinated chains to the 26 S proteasome. This course of events has recently been described in yeast, providing evidence for the existence of an escort pathway associated with a size-restriction mechanism [5]. It is conceivable that such an escort pathway might be a general mechanism for shuttling polyubiquitinated substrates to the proteasome in a regulated manner. Consequently, size-restriction of polyubiquitin chains could be a widespread mechanism accomplished by several ubiquitin-binding proteins, which subsequently escort the size-restricted ubiquitin conjugates for degradation. Richly et al. [5] proposed that a loose ubiquitin number code might exist for the size of a polyubiquitin chain, which, depending on the different binding properties of these escort factors, directs specificity and efficiency in proteasomal targeting.

It is an attractive idea that diversity in the escort pathway is created by modulating polyubiquitin-chain signals. A particularly striking example could be provided by the function of ataxin-3. Here, we propose a model in which ataxin-3 regulates the ubiquitination status of proteins destined for degradation. Ataxin-3 might act antagonistically to the escort complex by

reversing the transfer direction of UFD2-polyubiquitinated protein substrates. Its deubiquitination activity would provide a feedback mechanism, enabling fine-tuning of the polyubiquitin signal, which otherwise would be the concomitant signal for degradation (Figure 4A). Alternatively, ataxin-3 might assist the CDC48 complex in restricting the polyubiquitin-chain length to ensure maximum efficiency for proteasomal targeting. In this scenario, a proofreading mechanism for size-restricted ubiquitin chains would be provided by ataxin-3 (Figure 4B). Other potential functions of the ataxin-3 protease activity could include editing functions that regulate the polyubiquitin-chain linkage.

It is tempting to speculate that each substrate is accompanied by an orchestra of distinct escort components and modulating factors, contributing to courses of events which might occur directly at the proteasome. An important future challenge will be to unravel the prominence of such regulat-

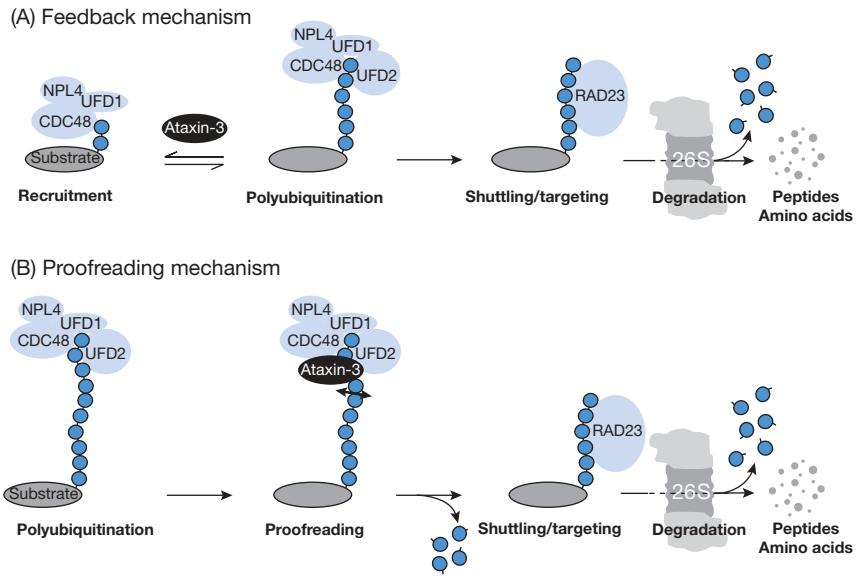

Figure 4. Hypothetical model for a function of ataxin-3 in the escort pathway
UFD2-assembled polyubiquitin chains are regulated to an optimal length of four to six ubiquitin molecules by the concerted action of the CDC48–UFD1–NPL4 complex. Substrates linked to these size-restricted chains are subsequently escorted to the 26 S proteasome for degradation by a succession of interacting factors. Based on its interaction with p97/VCP and UFD2a, the mammalian homologues of yeast CDC48 and UFD2, ataxin-3 might play a role in a conserved escort pathway. Ataxin-3 could be involved in the size-restriction mechanism of this pathway by two alternative modes of action. (**A**) In association with the deubiquitination activity, ataxin-3 could stabilize the substrate by shortening the ubiquitin chain. (**B**) Alternatively, ataxin-3 could support substrate degradation by controlling the efficient length of size-restricted chains in a proofreading mechanism. Moreover, the ability of ataxin-3 to bind to the proteasome [22] suggests that the whole pathway might take place directly at the 26 S proteasome.

ed networks by the identification of additional ubiquitin-binding factors and of specific substrates.

Summary

- *The ubiquitin system usually marks proteins for proteasomal degradation by covalent modification with ubiquitin. This ubiquitination process is mediated by E1, E2 and E3 enzymes.*
- *Each individual lysine residue of ubiquitin can be used for ubiquitin-chain formation (Lys^{6}-, Lys^{11}-, Lys^{27}-, Lys^{29}-, Lys^{33}-, Lys^{48}-, or Lys^{63}-based linkages), resulting in functionally distinct polyubiquitin chains.*
- *Polyubiquitination is sometimes regulated by additional conjugation factors, called E4 enzymes. The yeast E4 enzyme UFD2 co-operates with an orchestra of ubiquitin-binding factors in an escort pathway to transfer and deliver polyubiquitinated substrates to the 26 S proteasome.*
- *The affinity of polyubiquitin chains for the proteasome is influenced by the length of the chain; four to six ubiquitin molecules are enough for optimal binding to RAD23 and DSK2, which finally mediate the delivery of ubiquitinated substrates to the proteasome.*
- *Alternative escort pathways might be involved in modulating polyubiquitin-chain signals. Speculation about a potential role for the ubiquitin-conjugate-binding factor ataxin-3 in this escort mechanism should illustrate how alternatively used ubiquitin-chain-binding proteins can provide more complexity in this pathway.*

We thank Johnny Kim and Heather P. Ostendorff for discussions and comments on the manuscript. We apologize to those whose work could not be cited owing to space limitations.

References

1. Pickart, C.M. (2004) Back to the future with ubiquitin. *Cell* **116**, 181–190
2. Peng, J., Schwartz, D., Elias, J.E., Thoreen, C.C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D. & Gygi, S.P. (2003) A proteomics approach to understanding protein ubiquitination. *Nat. Biotechnol.* **21**, 921–926
3. Pickart, C.M. & Fushman, D. (2004) Polyubiquitin chains: polymeric protein signals. *Curr. Opin. Chem. Biol.* **8**, 610–616
4. Madura, K. (2004) Rad23 and Rpn10: perennial wallflowers join the melee. *Trends Biochem. Sci.* **29**, 637–640
5. Richly, H., Rape, M., Braun, S., Rumpf, S., Hoege, C. & Jentsch, S. (2005) A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. *Cell* **120**, 73–84
6. Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H.D., Mayer, T.U. & Jentsch, S. (1999) A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* **96**, 635–644

7. Flick, K., Ouni, I., Wohlschlegel, J.A., Capati, C., McDonald, W.H., Yates, J.R. & Kaiser, P. (2004) Proteolysis-independent regulation of the transcription factor Met4 by a single Lys^{48}-linked ubiquitin chain. *Nat. Cell Biol.* **6**, 634–641
8. Hoege, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G. & Jentsch, S. (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature (London)* **419**, 135–141
9. Sun, L. & Chen, Z.J. (2004) The novel functions of ubiquitination in signaling. *Curr. Opin. Cell Biol.* **16**, 119–126
10. Alberti, S., Demand, J., Esser, C., Emmerich, N., Schild, H. & Hohfeld, J. (2002) Ubiquitylation of BAG-1 suggests a novel regulatory mechanism during the sorting of chaperone substrates to the proteasome. *J. Biol. Chem.* **277**, 45920–45927

10a Iberti, S., Demand, J., Esser, C., Emmerich, N., Schild, H. & Hohfeld, J. (2003) Erratum. *J. Biol. Chem.* **278**, 15702–15703

11. Wu-Baer, F., Lagrazon, K., Yuan, W. & Baer, R. (2003) The BRCA1/BARD1 heterodimer assembles polyubiquitin chains through an unconventional linkage involving lysine residue K6 of ubiquitin. *J. Biol. Chem.* **278**, 34743–34746
12. Morris, J.R. & Solomon, E. (2004) BRCA1:BARD1 induces the formation of conjugated ubiquitin structures, dependent on K6 of ubiquitin, in cells during DNA replication and repair. *Hum. Mol. Genet.* **13**, 807–817
13. Hoppe, T., Matuschewski, K., Rape, M., Schlenker, S., Ulrich, H.D. & Jentsch, S. (2000) Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. *Cell* **102**, 577–586
14. Imai, Y., Soda, M., Hatakeyama, S., Akagi, T., Hashikawa, T., Nakayama, K.I. & Takahashi, R. (2002) CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol. Cell* **10**, 55–67
15. Hoppe, T., Cassata, G., Barral, J.M., Springer, W., Hutagalung, A.H., Epstein, H.F. & Baumeister, R. (2004) Regulation of the myosin-directed chaperone UNC-45 by a novel E3/E4-multiubiquitylation complex in C. elegans. *Cell* **118**, 337–349
16. Hoppe, T. (2005) Multiubiquitylation by E4 enzymes: 'one size' doesn't fit all. *Trends Biochem. Sci.* **30**, 183–187
17. Saeki, Y., Tayama, Y., Toh-e, A. & Yokosawa, H. (2004) Definitive evidence for UFD2-catalyzed elongation of the ubiquitin chain through Lys^{48} linkage. *Biochem. Biophys. Res. Commun.* **320**, 840–845
18. Ravid, T. & Hochstrasser, M. (2004) NF-kB signaling: flipping the switch with polyubiquitin chains. *Curr. Biol.* **14**, R898–R900
19. Heyninck, K. & Beyaert, R. (2005) A20 inhibits NF-kB activation by dual ubiquitin-editing functions. *Trends Biochem. Sci.* **30**, 1–4
20. Burnett, B., Li, F. & Pittman, R.N. (2003) The polyglutamine neurodegenerative protein ataxin-3 binds polyubiquitylated proteins and has ubiquitin protease activity. *Hum. Mol. Genet.* **12**, 3195–3205
21. Kawaguchi, Y., Okamoto, T., Taniwaki, M., Aizawa, M., Inoue, M., Katayama, S., Kawakami, H., Nakamura, S., Nishimura, M., Akiguchi, I. et al. (1994) CAG expansions in a novel gene for Machado–Joseph disease at chromosome 14q32.1. *Nat. Genet.* **8**, 221–228
22. Doss-Pepe, E.W., Stenroos, E.S., Johnson, W.G. & Madura, K. (2003) Ataxin-3 interactions with rad23 and valosin-containing protein and its associations with ubiquitin chains and the proteasome are consistent with a role in ubiquitin-mediated proteolysis. *Mol. Cell. Biol.* **23**, 6469–6483
23. Matsumoto, M., Yada, M., Hatakeyama, S., Ishimoto, H., Tanimura, T., Tsuji, S., Kakizuka, A., Kitagawa, M. & Nakayama, K.I. (2004) Molecular clearance of ataxin-3 is regulated by a mammalian E4. *EMBO J.* **23**, 659–669
24. Masino, L., Nicastro, G., Menon, R.P., Dal Piaz, F., Calder, L. & Pastore, A. (2004) Characterization of the structure and the amyloidogenic properties of the Josephin domain of the polyglutamine-containing protein ataxin-3. *J. Mol. Biol.* **344**, 1021–1035

25. Scheel, H., Tomiuk, S. & Hofmann, K. (2003) Elucidation of ataxin-3 and ataxin-7 function by integrative bioinformatics. *Hum. Mol. Genet.* **12**, 2845–2852
26. Guterman, A. & Glickman, M.H. (2004) Deubiquitinating enzymes are IN/(trinsic to proteasome function). *Curr. Protein Pept. Sci.* **5**, 201–211
27. Wang, G., Sawai, N., Kotliarova, S., Kanazawa, I. & Nukina, N. (2000) Ataxin-3, the MJD1 gene product, interacts with the two human homologs of yeast DNA repair protein RAD23, HHR23A and HHR23B. *Hum. Mol. Genet.* **9**, 1795–1803
28. Thrower, J.S., Hoffman, L., Rechsteiner, M. & Pickart, C.M. (2000) Recognition of the polyubiquitin proteolytic signal. *EMBO J.* **19**, 94–102
29. Raasi, S., Orlov, I., Fleming, K.G. & Pickart, C.M. (2004) Binding of polyubiquitin chains to ubiquitin-associated (UBA) domains of HHR23A. *J. Mol. Biol.* **341**, 1367–1379
30. Chai, Y., Berke, S.S., Cohen, R.E. & Paulson, H.L. (2004) Poly-ubiquitin binding by the polyglutamine disease protein ataxin-3 links its normal function to protein surveillance pathways. *J. Biol. Chem.* **279**, 3605–3611
31. Jana, N.R., Dikshit, P., Goswami, A., Kotliarova, S., Murata, S., Tanaka, K. & Nukina, N. (2005) Co-chaperone CHIP associates with expanded polyglutamine protein and promotes their degradation by proteasomes. *J. Biol. Chem.* **280**, 11635–11640
32. Kovalenko, A., Chable-Bessia, C., Cantarella, G., Israel, A., Wallach, D. & Courtois, G. (2003) The tumour suppressor CYLD negatively regulates NF-kB signalling by deubiquitination. *Nature (London)* **424**, 801–805