effectively blocked the increased activated Rac levels and formation of lamellipodia. In addition, expression of constitutively active STEF, but not constitutively active STEF lacking a Par-3 interaction domain, was able to induce an increase in Rac-GTP levels and lamellipodia. When the authors used RNAi to ablate endogenous Par-3 levels, Cdc42-induced lamellipodia and Rac1-GTP accumulation was blocked. Next the authors applied these findings to the neuronal polarization model. In concordance with previous data regarding localization of the Par-3/Par-6/aPKC complex, they demonstrate that STEF is at the tip of the axon. Moreover, disruption of the Par-3/Par-6/aPKC–STEF complex through overexpression of various mutants resulted in impaired axon specification. From these data, the authors propose a model whereby Cdc42 is required for the initial signalling step in neuronal polarization, and, following axon specification, Rac activated by the Par–STEF complex works to control axonal growth. Unfortunately, the authors did not present any RNAi studies in these neuronal cells, which would perhaps further delineate the spatial and temporal activity of the Par-3/Par-6/aPKC complex with regard to axonal specification.

The role of the Par-3/Par-6/aPKC complex has long been known to regulate microtubules by mechanisms that include: reorientation of microtubule-organizing centres; phosphorylation of microtubule-associated proteins, controlled by the Par1 polarity protein; and microtubule-based transport in association with the kinesin II complex. The results from these two papers provide a new role for the Par-3/Par-6/aPKC complex in cell polarization: control of actin dynamics. However, the results are conflicting with respect to the regulation of Tiam1/STEF activity. Chen and Macara see negative regulation of Tiam1 by Par-3, in contrast to the results seen by Kaibuchi and colleagues.

One explanation could be that whereas Kaibuchi and colleagues showed that STEF and Tiam1 could both associate with Par-3, they focused their biochemical studies on the activation of Rac by STEF and not by Tiam1. These two GEFs, although being similar in domain structure, may be regulated differently by Par-3. Another consideration is cell context, such that Tiam1/STEF might be regulated in a different manner in epithelia versus neurons. This possibility may be a result of different Par-3 binding partners in the two cell types. In neurons, the Par-3 bound to Tiam1/STEF may be in a complex with activated Cdc42 and Par-6. By contrast, the Par-3 bound to Tiam1/STEF in epithelia may not be bound by these proteins.

Another potential issue is signalling feedback. Par-6 was initially discovered to bind to both Cdc42 and Rac, so it is possible that there could be positive feedback from the Par-3/Par-6/aPKC complex such that Rac activated by Tiam1/STEF could bind to Par-6, perpetuating the initial signal. Kaibuchi and colleagues reported that they did not see a physiological interaction of Rac-GTP with Par-6, but they studied this *in vitro*. Furthermore, they tested only one of the Par-6 isoforms (Par-6C), and preference for G proteins may differ between isoforms. So far, whereas other GEFs have been shown to interact with the Par-3/Par-6/aPKC complex (for example, ECT2)[15], little is understood about the role of GTPase-activating proteins (GAPs) and signalling by the Par-3/Par-6/aPKC complex. GAP activity must be required to negatively regulate Par-3/Par-6/aPKC signalling. Indeed, to fully understand the polarization process we need to greatly enhance our understanding of the functional interactions between Par proteins and the Rho family GTPases. ☐

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Cdc48–Ufd2–Rad23: the road less ubiquitinated?

Omar A. Bazirgan and Randolph Y. Hampton

Targeting substrates to the proteasome is generally thought to depend on long polyubiquitin chains that form on the substrate. Recent work has led to the provocative proposal that some substrates may be targeted to the proteasome through smaller chains that contain only four to six ubiquitin molecules.

The ubiquitin–proteasome pathway has assumed its rightful place as a major mode of protein destruction and control. The basic process, now in the textbooks and widely known to biologists, entails a polymer of multiple ubiquitins being added to a target protein and this directing the protein to proteasomal destruction. Ubiquitination involves covalent addition of ubiquitin to a lysine residue in the target, and the subsequent assembly of a polyubiquitin chain, in which each added ubiquitin becomes the acceptor for the next. This reaction is mediated by a series of enzymes called E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase), which recognize the substrates. Once at the 26S proteasome, the resulting 'herringbone' polyubiquitin structure is recognized by the 19S subunit, unfolded, and the target protein is delivered to the 20S core where proteolysis occurs. But we are not yet out of the woods in terms of understanding the molecular mechanisms that underlie this process.

Although this basic model is almost universal, various other factors can be involved

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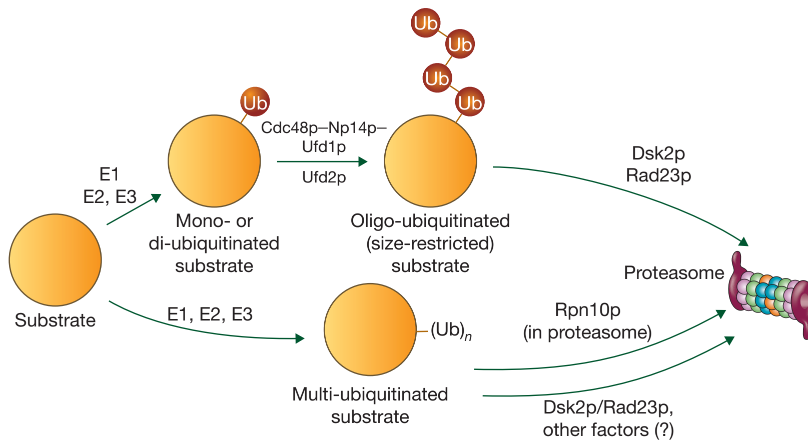

Figure 1 Two roads to the proteasome? In the two-route pathway (top pathway) proposed by Richly *et al.*[1], a substrate is first mono- or di-ubiquitinated by E1, E2 and E3. Then Ufd2p adds further ubiquitins by virtue of its U-box ligase activity. Cdc48p (in complex with its binding partners Npl4p and Ufd1p) cooperates with Ufd2p to restrict chain length, resulting in an 'oligo-ubiquitinated' substrate with comparatively short chains. This oligo-ubiquitinated substrate is then ferried to the proteasome by Dsk2p or Rad23p. In the alternative, canonical pathway (bottom pathway), E3 polyubiquitinates the substrate, which is then recognized by the proteasome subunit Rpn10p. Dsk2p and Rad23p, and other factors, may also contribute to this branch.

between ubiquitination and the proteasome. Richly *et al.*[1] have recently studied a number of these factors. Their new proposed pathway unites molecules from two walks of ubiquitin life: first, Ufd2p and Cdc48, proteins that are required for ubiquitination and degradation of ubiquitin fusion degradation (UFD) substrates with an in-frame amino-terminal ubiquitin[2,3]; and second, Dsk2 and Rad23, the homologous ubiquitin-recognizing proteins now thought to mediate delivery of a number of ubiquitinated substrates to the proteasome[4–6]. This study explores numerous physical, biochemical and genetic interactions between these molecules, from which the authors propose a specific route to the proteasome. By their model, proteins of the first group would bring about 'oligo-ubiquitination' (four to six molecules) of the target, and physical interactions with the second group would ensure rapid delivery to the proteasome.

It had previously been shown that Ufd2p can increase the length of polyubiquitin chains on UFD substrates, by increasing processivity during chain formation in conjunction with Cdc48p and its binding partners Ufd1p and Npl4p[7]. In this capacity, Ufd2p is described as an 'E4' enzyme to distinguish it from the E3 (Ufd4p) that initiates the modification of UFD substrates. Ufd2p has a U-box, which is a functional variant of the RING domain found in many E3s[8]. The current work shows that the U-box is required for this elongating activity and that Ufd2p binds to Cdc48p only if two other proteins, Npl4p and Ufd1p, are present. The Cdc48p–Npl4p–Ufd1p complex has been previously implicated in the ubiquitin-mediated degradation of numerous proteins[9]. In the new work, Richly *et al.*[1] used *in vitro* ubiquitination reactions to show that Cdc48p, when combined with Ufd2p, has a surprising activity: although Ufd2p alone markedly increases the size of polyubiquitin chains in an *in vitro* reaction, Cdc48p limits these chains to small 'oligo-ubiquitinated' structures of four to six molecules. This *in vitro* activity occurs independently of Cdc48's binding partners, and requires a molar excess of Cdc48 over substrate. Intriguingly, four to six ubiquitin molecules is the minimum threshold required for recognition by the proteasome[10] and by Rad23p[11]. Thus, the authors proposed that Cdc48p governs Ufd2p-mediated elongation, biasing the reaction to modifications of four to six ubiquitins, as opposed to the much longer chains that occur in the absence of Cdc48p.

The second part of the study focuses on the homologous proteins Dsk2p and Rad23p, each containing carboxy-terminal ubiquitin-binding domains (UBA) and an N-terminal domain that interacts with the proteasome (UBL). A model that has sprung from the work of numerous laboratories suggests that these factors shuttle polyubiquitinated proteins to the proteasome, using the UBA domains to bind the ubiquitin chain and the UBL domain to find the proteasome. The authors confirm that Ufd2p interacts with Rad23p[12] and with Dsk2p. Furthermore, Ufd2p binding to Rad23p and Cdc48p occurs through distinct parts of the Ufd2p, allowing the formation of a ternary complex. Taken together, these interaction studies are consistent with a pathway in which Cdc48p–Npl4p–Ufd1p and Ufd2p bring about oligo-ubiquitination of a protein substrate, and then Ufd2p mediates the transfer of this ubiquitinated protein to Rad23 or Dsk2, the proteins that guide them to the proteasome.

Further binding studies suggest that the short oligo-ubiquitin chains can specify recognition by Rad23 and Dsk2. In co-precipitation experiments, Rad23p and Dsk2p were more efficient at recognizing the short ubiquitin chains than was Rpn10p, a proteasomal ubiquitin-recognizing subunit. All three proteins could bind highly ubiquitinated proteins well. These findings led to the idea that perhaps the Ufd2p–Cdc48p–Rad23p (Dsk2p) route to the proteasome works through generation and recognition of short oligo-ubiquitinated chains, whereas the Rpn10p pathway is more biased towards recognition of long chains, produced by distinct E3s.

Finally, the authors extend their ideas to the *in vivo* case, by doing phenotypic and degradation studies on a number of yeast mutants. They examined processing of the Spt23p transcription factor and degradation of two endoplasmic reticulum-associated degradation pathway substrates, Hmg2p and deg1-Sec61p. These substrates are processed by ubiquitin pathways involving Cdc48p–Npl4p–Ufd1p, but none is a UFD pathway substrate. Although there are many interesting observations in these experiments, two results are important. In most cases, the loss of Ufd2p was not enhanced by further loss of Rad23p or Dsk2p, indicating that Ufd2p may function upstream of them. Furthermore, although null mutants of Ufd2p or Rpn10p inhibited degradation to varying degrees, the double null always had the strongest effect. This would be consistent with the authors' model that Rpn10p and Ufd2p mediate independent routes to the proteasome — the Ufd2p route being the 'size-restricted' ubiquitination pathway, and the Rpn10p route being the more typical polyubiquitinated pathway (Fig. 1).

This new model demands a variety of important experiments. Perhaps the most novel aspect of this work is the notion that Cdc48p can function to restrict ubiquitin chain elongation to small oligo-ubiquitin chains of four to six copies. This conjecture is made from their *in vitro* studies of UFD substrate ubiquitination.

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Because Cdc48p binds ubiquitin, one wonders if this is simply a case of the chains being protected from further modification. In fact, a similar *in vitro* effect has been observed with the ubiquitin-binding domains of Rad23p[13,14]. It will also be important to integrate the proposed action of Cdc48p with its ATPase activity[9,15]. This work does not address the role of ATP hydrolysis, or the possibility that the effects seen are a result of the Cdc48p hexamer not completing its enzymatic cycle. In addition, the fact that the Cdc48p chain-size effect requires its concentration to be equal to or higher than that of the substrate might give some enzymologists pause. Whatever the mechanistic details of this Cdc48p 'restriction' activity, it will be necessary to test this role *in vivo*.

Cdc48p and Ufd2p have a growing number of functions in living cells. The Cdc48p–Npl4p–Ufd1p complex has been implicated in many studies of ubiquitination, and may function at more than one juncture in ubiquitination pathways[15,16]. Thus, it will be important and necessary to evaluate where Cdc48p functions in individual contexts. Because this versatile enzyme has many binding partners[17,18], it could also have different functions in different complexes. Nevertheless, if Cdc48p does control the size of polyubiquitin chains, this would be a new and important action of this widely used protein. The *ufd2* null mutants had striking degradation phenotypes in the featured studies. Are these all owing to loss of the proposed pathway? Earlier studies by the Jentsch group clearly show that Ufd2 can extend ubiquitin chains formed by the HECT domain E3 Ufd4p[7]. However, Ufd2p can also be viewed as a bona fide E3 (refs 19, 20) and it may have many roles as a recruitable E3. Thus, the *in vivo* effect of a null mutation must be interpreted with this versatility in mind.

The proposed pathway posits that Rpn10p might have a preference for heavily ubiquitinated proteins, whereas Rad23p/Dsk2p might be biased towards recognizing proteins with a smaller number of ubiquitin molecules. This 'size channelling' idea implies another layer of regulation and specificity in the ubiquitin pathway, and should be examined in detail. It is clear that four to six ubiquitins are needed for high affinity binding to intact proteasomes or isolated Rad23p[10,11]; however, detailed biophysical studies with Rad23p indicate that affinity only increases with increasing chain size[11], indicating that larger sizes are accepted as well. Additionally, whereas the genetic studies show a convincing synergy between null mutations of *ufd2* and *rpn10*, it is important to recall that null mutations of *rpn10* cause a demonstrable altered structure to the proteasome[21], suggesting that numerous models could account for this interaction. Some of the elegant *in vitro* systems used to examine directly the kinetics of substrate processing by the proteasome will undoubtedly shed light on the importance of chain size in proteasomal delivery[14,22,23].

All of these ifs and buts only highlight the potential importance of this work. There may be two roads to the proteasome, and the one taken might make "… all the difference."[24] □

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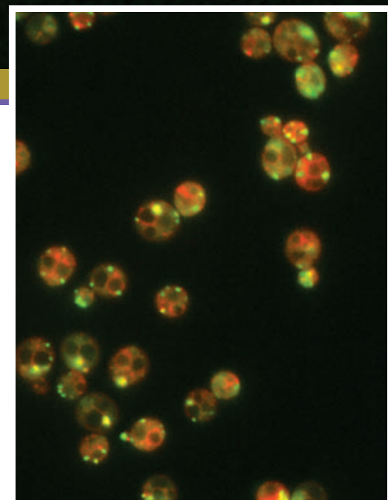

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