Unfolding or aggregation, that is the question

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Cellular processes accompanying protein aggregation are diverse and entangled, making it difficult to investigate the underlying molecular processes in a time-resolved way. Gottlieb, Thompson, and colleagues address this shortcoming using a chemical biology approach to monitor ubiquitination within the first 10 min after the initiation of protein aggregation. Intriguingly, unfolding rather than aggregation seems to trigger the observed events. This work might provide a method to answer open questions regarding the regulation of the proteostasis network upon protein misfolding.

Protein homeostasis is fundamental to maintain a functional proteome. Malfunction of the proteostasis network (PN)[2] can result in protein misfolding and eventually the accumulation of the intracellular protein aggregates that are hallmarks of various proteopathies including Alzheimer's disease and ALS. Untangling the precise relations between the different elements of the PN has been challenging due to the myriad of events that are triggered simultaneously by protein misfolding and aggregation but might provide necessary insights for drug development against these malfunctions. The PN prevents the accumulation of misfolded proteins in part through the ubiquitin proteasome system, which targets proteins for proteasomal destruction (1). However, the kinetic link between consecutive events along the trajectory of the targeted proteins and the interplay between the different elements of the PN at the molecular level are poorly defined. Gottlieb *et al.* (2) now show in their recent work that several proteins—including the proteasomal subunit RPN13—are ubiquitinated within 10 min following acute unfolding of engineered proteins, mediated in part by the recruitment of the E3 ubiquitin ligase UBE3C to the 26S proteasome. These results indicate that the proteasome is responsive to protein unfolding, not just aggregation, and provide new strategies to gain further insights in this area.

To untangle the events occurring immediately after protein unfolding, Gottlieb *et al.* (2) elegantly combined protein destabilizing domains (DDs) and proteomic approaches. To this end, they used a protein termed AgDD engineered by Wandless and colleagues (3). AgDD contains the FK506-binding protein (FKBP1A) mutant that typifies the DDs but is extended on the N terminus with an aggregation-prone peptide and on the C terminus with superfolder GFP (Fig. 1*A*). The fusion protein is stable when bound to the high-affinity synthetic ligand Shield-1 (S1) but readily aggregates and is ubiquitinated upon S1 removal (3).

With these constructs in hand, the authors were able to identify the first proteins that undergo changes in ubiquitination upon S1 removal and consequently AgDD aggregation in a time-resolved manner using ubiquitin remnant immunoaffinity profiling and a quantitative MS approach with tandem mass tagging. Among the 48 identified proteins, including AgDD, members of the proteostasis network were highly enriched and showed increased ubiquitination levels within 2.5–10 min. Interestingly, many ribosomal proteins and ribosome biogenesis factors were identified next to proteasomal subunits, indicating a rapid targeting of key players of the protein synthesis and degradation machineries. Of particular interest is the strong increase in ubiquitination of RPN13 (also known as ADRM1) on residues Lys-21 and Lys-34. Ubiquitination of AgDD and RPN13 upon S1 washout was confirmed by pull-down analysis. Importantly, the authors did not observe any changes in the degradation kinetics of another model substrate targeted by the ubiquitin proteasome system upon aggregation of AgDD, indicating that the observed RPN13 ubiquitination was unlikely to be leading to a general impairment of the 26S proteasome activity. Interestingly, the proteolytic activity itself was increased in these conditions, when measured with a small proteasomal peptide substrate. The E3 ligase UBE3C was previously shown to ubiquitinate a nonaggregating DD reporter and RPN13 (4, 5), and Gottlieb *et al.* confirmed that UBE3C was responsible for AgDD polyubiquitination (*i.e.* attachment of subsequent ubiquitin moieties to form a chain) and RPN13 multiubiquitination (*i.e.* conjugation of ubiquitin to different substrate lysine residues) using an siRNA knockdown approach (2). Importantly, these events occurred rapidly, within minutes of the induced aggregation of AgDD.

As a result of the high aggregation rate, it was not easily possible to distinguish whether the observed events were due to AgDD unfolding or aggregation. Therefore, to discriminate between these two possibilities, the authors returned to a DD variant lacking the N-terminal aggregation-prone peptide, which converts to an unfolded yet soluble molten globule-like state in the absence of S1. The observed results were highly congruent to AgDD within the first 10 min, indicating that indeed unfolding but not aggregation of the model substrate

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[2] The abbreviations used are: PN, proteostasis network; DD, destabilizing domain; S1, Shield-1; Pru, pleckstrin-like receptor for ubiquitin.

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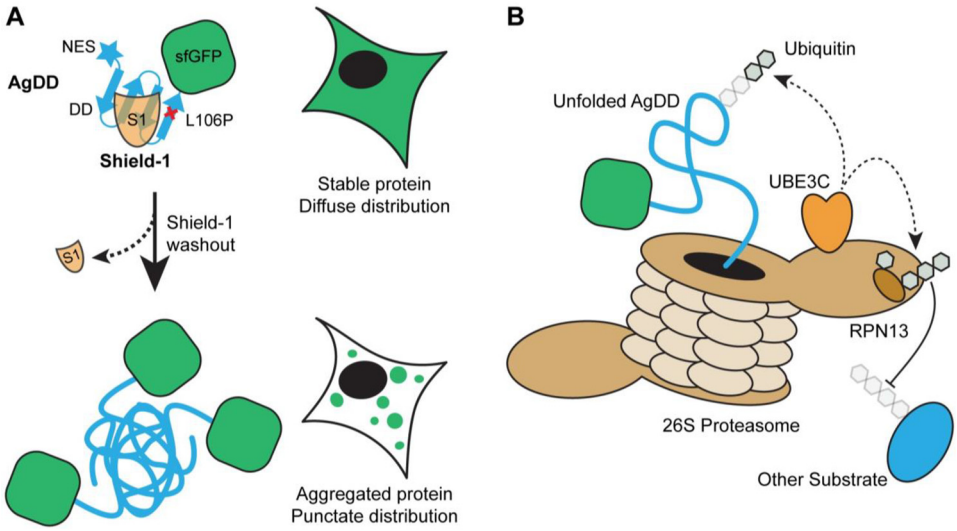

Figure 1. *A*, AgDD remains soluble in the presence of S1 but readily aggregates upon S1 washout. *B*, AgDD unfolding stimulates UBE3C recruitment to the 26S proteasome, leading to increased ubiquitination of AgDD, perhaps to increase substrate processivity, and increased RPN13 ubiquitination, to potentially prevent the recruitment of other proteasome substrates to the engaged particle. When the cell is not overloaded with misfolded proteins, these other substrates would then be degraded by other—nonengaged—proteasome particles. *NES*, nuclear export signal; *sfGFP*, superfolder GFP.

was triggering UBE3C recruitment, and RPN13 and AgDD ubiquitination, although aggregation seems to accelerate the response (2). Notably, the observed time resolution that goes along with the chemical biology approach presented by Gottlieb *et al.* offers a unique view of the chain of events that take place when the PN is challenged.

So, what is the role of RPN13 ubiquitination in the context of protein misfolding? Previous work from Goldberg and colleagues (4) showed that RPN13 ubiquitination increases upon proteasome inhibition as well as other conditions that lead to a general increase of the cellular ubiquitination levels, such as heat shock and arsenite treatment— conditions that all lead to an accumulation of misfolded proteins. Importantly, UBE3C-mediated ubiquitination of RPN13 reduces the capacity of the proteasome to bind to polyubiquitinated substrates (4), consistent with the fact that the RPN13 ubiquitination sites (Lys-21 and Lys-34) are adjacent and within the RPN13-Pru domain that binds ubiquitin (6). UBE3C was previously shown to ubiquitinate the nonaggregating DD reporter to promote its degradation by increasing proteasomal processivity (5). Yet recruitment of UBE3C to the proteasome is also enhanced in the presence of ubiquitinated substrates (7). One model that could reconcile these findings is that UBE3C is recruited to the proteasome to promote the complete degradation of misfolded proteins by conjugating additional ubiquitin moieties, increasing their affinity to the proteasome. The concomitant ubiquitination of RPN13 would then prevent the recruitment of additional proteasome substrates, because RPN13-conjugated complexes would appear to be substrate-engaged particles with potentially "more difficult" proteins to process (Fig. 1*B*), similar to what Goldberg and colleagues proposed (4, 7). The molecular mechanism that mediates UBE3C proteasomal recruitment remains to be determined in future investigations. More generally, the present study demonstrates that changes at the proteasomal level rapidly occur in the cell in the presence of a single pool of unfolded proteins (*i.e.* AgDD or DD), in conditions where the overall activity of the ubiquitin proteasome system does not appear to be impaired. The future challenges will be to determine whether this mechanism is specific to proteasome substrates that are potentially more difficult to process (*e.g.* due to misfolding) and whether it plays a role in proteopathies more broadly.

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