

Ubiquitin versus misfolding: The minimal requirements for inclusion body formation

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Ubiquitin-containing inclusion bodies are characteristic features of numerous neurodegenerative diseases, but whether ubiquitin plays a functional role in the formation of these protein deposits is unclear. In this issue, Bersuker et al. (2016. *J. Cell Biol.* <http://dx.doi.org/10.1083/jcb.201511024>) report that protein misfolding without ubiquitylation is sufficient for translocation into inclusion bodies.

A large number of sporadic and familial neurodegenerative diseases that differ in their age of onset and manifestation share striking pathological features at the cellular level, suggesting that a common etiology may be responsible for the demise of neurons. Most notable is the aggregation of improperly folded proteins in affected neurons in these so-called protein misfolding diseases that include Alzheimer's, Parkinson's, and Creutzfeldt-Jakob disease, as well as amyotrophic lateral sclerosis and other motor neuron diseases. Protein aggregates are inherently toxic for cells, underscoring their candidate status as a common denominator in these diseases (Bucciantini et al., 2002). A causative role for aberrant protein conformations is further strengthened by the existence of a family of rare, inheritable neurodegenerative disorders, which are a direct consequence of expansions of polyglutamine repeats that render the mutant proteins prone to aggregation. Given that neuronal cells often must last an organism's lifetime with little opportunity to dilute protein waste through cell division, it is not hard to imagine that they are particularly susceptible to the gradual accumulation of aberrant proteins that favor precipitation in insoluble protein aggregates.

Neurons and other cells have three major lines of defense to minimize the damage that aggregation-prone proteins can cause to cellular homeostasis (Fig. 1). The first two are based on a seek-and-destroy strategy in which the two main intracellular proteolytic systems play complementary roles. Although monomeric aberrant proteins are efficiently targeted for hydrolysis in proteasomes, these proteolytic complexes are unable to process oligomeric protein aggregates (Verhoef et al., 2002). Destruction of proteins in proteasomes requires complete unfolding of the deemed proteins, which may be hard, if not impossible, in the case of tightly associated misfolded proteins. Macroautophagy, however, is a proteolytic pathway that is able to process oligomeric misfolded proteins, as it involves the capturing of cytosolic constituents, including macromolecular complexes like protein aggregates, in double-membrane vesicles that fuse

with lysosomes (Ravikumar et al., 2004). As such, macroautophagy complements proteasomal degradation in keeping the cellular environment free from toxic protein species.

In the unfortunate case that the production of aggregation-prone proteins exceeds the capacity of both the proteasomal and lysosomal systems, a potential catastrophic situation arises as misfolded proteins may precipitate in large, insoluble aggregates. In these cases, a third protective mechanism can come to the rescue and primarily provides damage control, as it intercepts protein aggregates and sequesters them in dedicated subcellular structures, thereby minimizing the harm that the aberrant proteins may cause (Johnston et al., 1998). It is this process that is responsible for the formation of the characteristic inclusion bodies that are typically observed in affected neurons and known under different names depending on the neurodegenerative disorder in which they occur, such as Lewy bodies in Parkinson's disease, Bunina bodies in amyotrophic lateral sclerosis, and intranuclear inclusions in several polyglutamine disorders (Alves-Rodrigues et al., 1998). Although these structures were originally considered as a potential cause for the cellular pathology, a large body of evidence suggests that they actually lessen the cellular damage caused by toxic proteins (Arrasate et al., 2004). Although their presence may not be without negative consequence for the cells, the controlled formation of waste deposits may be the best possible option for the cell when facing excessive amounts of aggregated proteins.

Interestingly, the protein modifier ubiquitin, a posttranslational modification covalently linked to lysine residues of target proteins, appears to be somehow involved in each of these three protective mechanisms. Polyubiquitylation, or conjugation of a chain of ubiquitin molecules, targets proteins for proteasomal degradation and is likewise also critical for proteasomal destruction of misfolded proteins (Kleiger and Mayor, 2014). Even though macroautophagy was originally seen as a nonselective catabolic pathway, more recent studies have suggested that it also involves a high level of specificity with ubiquitin chains being an important substrate recruitment signal (Kraft et al., 2010). In sharp contrast to the well-defined targeting function of ubiquitin in these proteolytic mechanisms, its possible role in the formation of inclusion bodies has been less clear. This is somewhat ironic, given that the initial observations of ubiquitin-positive inclusions in neurodegeneration date back almost three decades (Mori et al., 1987) and have been among the main findings that sparked the interest in a possible role of

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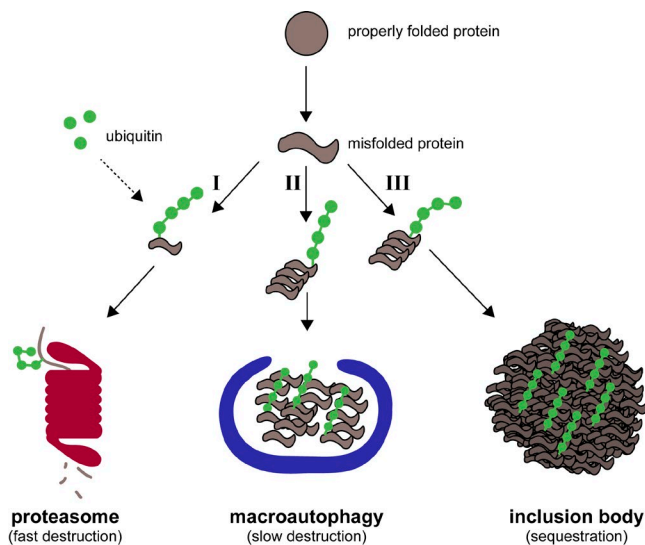


Figure 1. Three lines of defense against misfolded proteins. There are three protective mechanisms that are involved in minimizing the toxicity of misfolded proteins: proteasomal degradation (I), macroautophagic clearance (II), and inclusion body formation (III). Ubiquitin is linked to each of these processes, as it can target proteins for proteasomal and macroautophagosomal degradation and is enriched in inclusion bodies.

dysfunctional ubiquitin-dependent proteasomal degradation in neurodegenerative disorders (Cummings et al., 1998).

In this issue, Bersuker et al. revisited this important question using an elegant system that allowed them to follow specifically designed reporter proteins that could be switched from folded to misfolded states by administration of cell-permeable ligands. Using this approach, they confirmed that introducing a misfolded state resulted in rapid clearance of the reporter proteins by ubiquitin-dependent proteasomal degradation, the first line of defense against misfolded proteins. Consistent with the prevailing model, they also found that the misfolded reporters accumulated in inclusion bodies when they increased the load of aggregation-prone proteins by simultaneously expressing a fragment of mutant huntingtin containing an expanded polyglutamine repeat, the protein responsible for Huntington's disease. Interestingly, chemical inhibition of the ubiquitin activase, an enzyme that is critical for ubiquitin conjugation, showed that translocation of the reporter proteins to inclusion bodies did not require ubiquitylation, arguing that the misfolded state is sufficient to reach the final destination.

If ubiquitin is not needed for targeting misfolded proteins to inclusion bodies, why then do these proteinaceous deposits contain such large amounts of ubiquitin? The fact that ubiquitin is not required for the recruitment of misfolded proteins to inclusion bodies does not exclude the possibility that ubiquitylation targets properly folded proteins to inclusion bodies. Thus, a possible scenario is that inclusion bodies, once they have been seeded by the ubiquitin-independent sequestration of misfolded proteins, will start to gather soluble polyubiquitylated proteins that typically accumulate under conditions of disturbed protein homeostasis. The authors investigated this possibility by expressing a reporter substrate that contained a degradation signal and was therefore efficiently targeted for ubiquitin-dependent proteasomal degradation. Interestingly, even though these substrates accumulated in a ubiquitylated form when proteasomal degradation was obstructed, they did not localize to the inclusion

bodies that otherwise gathered misfolded reporters. This suggests that ubiquitin chains—at least those that target substrates for proteasomal degradation—are not sufficient to autonomously target proteins to inclusion bodies and, at the same time, excludes the possibility that their presence is due to a general sequestration of ubiquitylated proteasome substrates.

Alternatively, ubiquitin in inclusions may reflect an attempt of the cell to get rid of the sequestered protein aggregates once they have reached the inclusion body by targeting them for destruction via ubiquitin-dependent proteolytic systems. Indeed, *in vivo* studies suggest that inclusion bodies are not a dead-end product but can be cleared from affected neurons (Yamamoto et al., 2000). Even though ubiquitin-dependent autophagosomal and proteasomal degradation are primary candidates for facilitating disposal of inclusions (Martín-Aparicio et al., 2001; Wong et al., 2008), it should be noted that it is presently unclear how this would be mechanistically executed. The data presented by Bersuker et al. (2016) show that the pool of ubiquitin in inclusion bodies is rather static, arguing against a direct role in the turnover, if any, of the ubiquitylated proteins present in the inclusions.

Where do these findings leave us? It is fair to say that the functional significance of ubiquitin in inclusion bodies remains somewhat elusive. Following the road of exclusion as in the present study, we can put a solid strike through several trivial explanations for the presence of ubiquitin in inclusions, but further research will be needed to get a more definitive answer about ubiquitin's role in this process or the lack thereof. It also brings up questions about the role of the microtubule-associated deacetylase HDAC6 in this process. Some studies have provided data that support an essential role for this cytosolic deacetylase in transporting aggregates to inclusions by virtue of its ability to simultaneously bind ubiquitin conjugates and the dynein motors that are required for their sequestration (Kawaguchi et al., 2003; Olzmann et al., 2007). However, HDAC6 has also been linked to degradation of aggregation-prone proteins by macroautophagy, suggesting that it may indirectly influence the kinetics of inclusion body formation (Pandey et al., 2007; Lee et al., 2010). Even though these processes are not mutually exclusive and may well be functionally linked, the present findings motivate a closer look at the molecular mechanisms that link HDAC6 to the formation of inclusion bodies. It should be noted that although the presented data demonstrate that the canonical ubiquitin chains that target proteins for proteasomal degradation are insufficient to promote their translocation to inclusion bodies, it does not exclude implication of alternative ubiquitin chains. Ubiquitin modifications come in many different flavors, and, in particular, the K63-linked polyubiquitin chains, which do not target for proteasomal degradation, have been linked to both macroautophagy and inclusion body formation (Lim and Lim, 2011).

The present work also underscores the importance of the exclusive role of protein aggregation in directing misfolded proteins to inclusion bodies. This finding resonates with an earlier study from the same group, in which they reported that targeting of misfolded proteins for autophagy is a direct consequence of their aggregation and does not necessarily require ubiquitylation (Riley et al., 2010). A picture starts to emerge of a general strategy in which the attention of these protective mechanisms is directly drawn to the problematic proteins by the very same virtue that causes their misbehavior, namely their tendency to aggregate. The central role of protein aggregation, as opposed

to ubiquitylation, may also be relevant for the similarities and dissimilarities between the formation of inclusion bodies in the cytosolic and nuclear compartments of cells. Whereas the present study probes into the role of ubiquitin in the generation of cytosolic inclusions, intranuclear inclusions are most notoriously associated with the pathology of neurodegenerative diseases. Even though there are fundamental differences in ubiquitin targeting and transport mechanisms between these compartments, the intrinsic property of the proteins to aggregate applies to both, and it is also feasible that in the nucleus, the misfolded domains suffice to facilitate their translocation to inclusion bodies. The lack of a need for a middleman in this critical process may reflect the archaic nature of this innate response and allow rapid incapacitation of these inherently toxic species. This will also ensure that handling of these proteins is not susceptible to disturbed ubiquitin homeostasis, as often is the case in neurodegenerative disorders.

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