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A new tool in *C. elegans* reveals changes in secretory protein metabolism in *ire-1*-deficient animals

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We recently showed that the *ire-1/xbp-1* arm of the UPR plays a crucial role in maintaining basic endoplasmic reticulum (ER) functions required for the metabolism of secreted proteins even during unstressed growth conditions. During these studies we realized that although *C. elegans* is a powerful system to study the genetics of many cellular processes; it lacks effective tools for tracking the metabolism of secreted proteins at the cell and organism levels. Here, we outline how genetic manipulations and expression analysis of a DAF-28::GFP translational fusion transgene can be combined to infer different steps in the life cycle of secretory proteins. We demonstrate how we have used this tool to reveal folding defects, clearance defects, and secretion defects in *ire-1* and *xbp-1* mutants. We believe that further studies using this tool will deepen the understanding of secretory protein metabolism.

Keywords: UPR, ER stress, ERAD, coelomocytes, *ire-1*, protein folding, DAF-28::GFP, *edem-1*, protein secretion, *C. elegans*

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The ER and the unfolded protein response (UPR)

The ER fulfills many roles in the cell, it is responsible for carbohydrate and drug metabolism,[1] lipid and steroid synthesis,[2] storage and balance of cellular calcium levels,[3] and it is the entry point of the secretory pathway through which secreted and transmembrane proteins are translated and processed.[4] Accordingly, ER homeostasis is essential for proper cellular function and its disruption contributes to many diseases and impairs the development and function of dedicated secretory cells such as plasma cells, insulin secreting cells, and liver cells.[5,6]

The ER has a limited capacity and adjusts itself to accommodate increasing loads of misfolded proteins in the ER. This is achieved by activation of an ER stress response called the UPR. In *C. elegans*, as in higher organisms, the UPR is composed of three parallel pathways: the *ire-1/xbp-1* pathway, the *pek-1* pathway, and the *atf-6* pathway.[7] Each UPR pathway can be activated by the accumulation of misfolded proteins in the ER lumen. When activated, the UPR pathways trigger processes that help restore ER homeostasis by expanding the ER itself,[8] by curtailing processes that further burden the ER[9] and by expressing ER-resident chaperones and ERAD components.[10]

Secretory protein metabolism

Most secretory proteins enter the ER co-translationally through the SEC61 channel.[11] ER signal sequences are recognized by a signal recognition particle (SRP), which also binds to the ribosome.[12] SRP binding inhibits further translation and targets the entire complex (the SRP, ribosome, and growing polypeptide chain) to the rough ER by binding to the SRP receptor on the ER membrane.[12] In the ER lumen, the nascent polypeptide is bound by BiP chaperones to prevent aggregation of unfolded polypeptide chains, Cysteine bridges are created with the aid of PDI chaperones, and the polypeptide enters the calnexin–calreticulin chaperone cycle, until it is properly folded.[13,14] Properly folded proteins are transported to the golgi via COPII-coated vesicles.[15] From the Golgi, secreted proteins are transported in vesicles to the cell membrane, to which they fuse, emptying their content into the intercellular fluid. Polypeptides which fail to fold

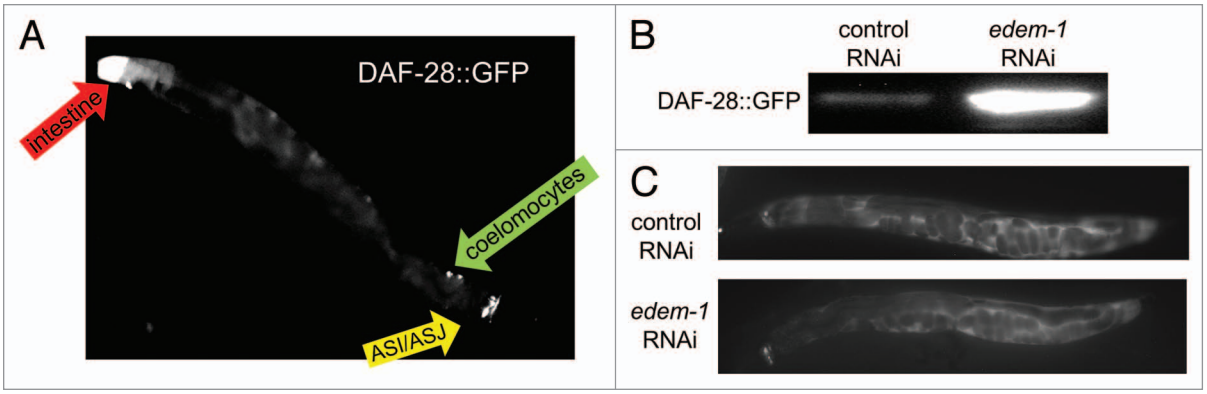

Figure 1. Features of DAF-28::GFP that render it suitable as a secretory protein metabolism marker. (**A**) The expression pattern of DAF-28::GFP differentiates between pre-secreted and post-secreted DAF-28::GFP. Representative fluorescence micrograph of a day 3 animal harboring an integrated DAF-28 transgene fused to GFP. Pre-secreted DAF-28::GFP fluorescence is detected in the producing cells (the ASI/ASJ head neurons [yellow] and in the hindgut [red]). Post-secreted DAF-28::GFP fluorescence is detected in the coelomocyte cells (green), which clear material from the pseudocoelom. (**B**) DAF-28::GFP is subject to ERAD in wild-type animals. Using western blotting, increased levels of DAF-28::GFP are detected upon ERAD inactivation using *edem-1* RNAi. (**C**) ERAD inactivation, which results in the accumulation of misfolded secretory proteins, does not increase DAF-28::GFP fluorescence levels, although it does increase DAF-28::GFP total protein levels (see **B**). Representative fluorescence micrograph of a day 3 *cup-4* mutants harboring an integrated DAF-28 transgene fused to GFP, treated with control or *edem-1* RNAi.

properly in the ER are cleared from the ER via ER-associated degradation (ERAD). In this process, misfolded proteins in the ER are tagged by one of three EDEM proteins, which cleave mannose sugar residues, thus marking misfolded proteins for degradation.[16,17] Mannose-trimmed misfolded proteins are ubiquitylated and transported from the ER to the cytosol, where they are degraded by the proteasome.[18]

Discovery of DAF-28::GFP translational fusion as a tool to study secretory protein metabolism

Many conditions induce protein misfolding in the ER and consequently activate the UPR. These include chemicals that disrupt protein folding (such as tunicamycin and dithiothreitol), environmental conditions (such as high temperatures), and the expression of mutant proteins, prone to misfolding. Whereas the UPR pathways fulfill crucial functions required for embryonic development and viability in *Drosophila*, Xenopus, and mice;[19-21] in *C. elegans*, UPR mutants are viable under physiological conditions. Several studies exploited this to demonstrate that the UPR does affect important physiological processes such as sensitivity to pathogens[22] and lifespan[23] under normal growth conditions. Although these studies demonstrate that UPR genes are important under normal growth conditions as well, it is unclear what they are directly required for.

One of the main functions of the ER is to serve as an entry point into the cell's secretory pathway. Thus, we asked whether UPR genes are important for the life cycle of proteins passing through the secretory pathway under standard growth conditions. To address this, we followed the protein levels and fluorescence pattern and levels of several fluorescently labeled proteins that pass through the secretory pathway, including the insulin-like *Pdaf-28::daf-28::gfp* transgene. To our surprise, in some cases, the DAF-28::GFP fluorescence levels and the DAF-28::GFP protein levels detected by western blotting did not correlate. After in depth dissection of this phenomena we realized that the discrepancy between DAF-28::GFP fluorescence and protein levels occurred when the clearance of misfolded DAF-28::GFP was abrogated. For example, ERAD inactivation increased DAF-28::GFP protein levels but not fluorescence levels[24] (see **Fig. 1B and C**). This suggested that misfolded DAF-28::GFP does not fluoresce as well as properly folded DAF-28::GFP. Thus, although the discrepancy between DAF-28::GFP protein levels and fluorescence levels seems contradictory and confusing at first glance, it is actually very informative as it allows the uncoupling between the detection of the properly folded population of DAF-28::GFP (detected by fluorescence levels of DAF-28::GFP) and the total population of the protein, irrespective to its folding state (detected by western blotting).

The differential fluorescence between the folded state of the protein and the unfolded state of the protein was not common to all reporters. For example, in the case of the mutated Pro-Cathepsin L reporter CPL-1::YFP (W35A and Y35A), both its fluorescence and its total protein levels increase upon ERAD inactivation.[25] Thus, although CPL-1::YFP is extremely suitable to track ERAD function, it is not informative in terms of the folding state of the protein. In contrast, in the case of *Pmyo-3*::secreted GFP, neither its fluorescence nor its total protein levels increased upon ERAD inactivation. Thus, although *Pmyo-3*::secreted GFP is extremely suitable to track secretory proteins within the animal,[29] it is not informative in terms of protein folding state or as a reporter of ERAD function.

The differential fluorescence of DAF-28::GFP when folded and when misfolded renders it useful to track secretory protein folding. The increase in DAF-28::GFP levels upon ERAD inactivation, detected by western blotting, makes it a useful reporter to follow ERAD function as well. By devising a set of genetic manipulations for the analysis of DAF-28::GFP expression pattern, expression levels, and fluorescence levels, we demonstrate how the unique properties of DAF-28::GFP can be utilized to differentiate between different

steps in its metabolism as a secretory protein. In the next sections, we explain how to use this new molecular tool to analyze secretory protein metabolism. Finally, we will demonstrate its efficacy in revealing changes in secretory protein metabolism in *ire-1* mutants, whose ER homeostasis is disrupted.

DAF-28::GFP expression pattern

DAF-28 is one of more than 40 insulin-like peptides in *C. elegans*.[26] The DAF-28::GFP translational fusion transgene, which contains a signal peptide, is translated and processed in the ER and deposited in secretory granules, from which it is released into the body cavity of the animal.[27] In wild-type animals, fluorescent DAF-28::GFP is detected in its producing cells (the ASI and ASJ neurons and in the posterior intestine) and in the coelomocytes[28] (**Fig. 1A**). Coelomocytes are macrophage-like scavenger cells that take up secreted material from the body cavity and degrade it.[29] In animals with functional coelomocytes, proteins secreted into the animals' body cavity are rapidly cleared into the coelomocytes, where they can be detected until they are degraded.[29]

In order to:	Do:	Our observations in wild type and *ire-1* mutants:	Conclusions:
Follow secretion levels	Insert mutation in *cup-4* gene	**wt**: increased fluorescence ***ire-1***: unchanged low fluorescence	*ire-1* mutants secrete less DAF-28::GFP
Compare folding state	Compare DAF-28::GFP fluorescence levels to western-blot levels	**wt**: high fluorescence & low western-blot levels ***ire-1***: low fluorescence & high western-blot levels	Folding state of DAF-28::GFP in *ire-1* mutants is worse than in wild type
Compare translation levels	Block ERAD function and compare DAF-28::GFP western-blot levels	**wt**: high levels after ERAD block ***ire-1***: lower levels (than wt) after ERAD block	*ire-1* mutants translate less DAF-28::GFP
Follow ERAD function	Compare DAF-28::GFP western-blot levels before and after blocking ERAD	**wt**: blocking ERAD increases DAF-28::GFP levels ***ire-1***: blocking ERAD does not affect DAF-28::GFP levels	In *ire-1* mutants ERAD does not function

Figure 2. How to use the DAF-28::GFP transgene to follow secretory protein metabolism. The table summarizes the combination of experimental techniques and genetic manipulations that should be done in order to use the DAF-28::GFP transgene to follow distinct steps in secretory protein metabolism. These include protein secretion, protein folding state, translation/production levels, and ERAD function. The observations and the corresponding interpretation of each of these settings, in wild-type animals (wt) and in *ire-1* mutants are presented.

Estimating protein secretion and coelomocyte function

The fluorescence of GFP-labeled proteins detected in the coelomocytes is a standard tool used to estimate protein secretion.[30,31] However, this estimate is sensitive to additional processes that take place in the coelomocytes, such as the rate at which the coelomocytes take up material from the body cavity as well as the degradation rate of the cargo once it enters the coelomocyte. To circumvent these, coelomocyte function can be sabotaged by introducing a mutation in the *cup-4* coelomocyte-specific gene.[29,32] This prevents the clearance of secreted material from the body cavity of the animal into the coelomocytes and enables measurement of the secreted fluorescent material in the body cavity of the animals instead.

When using this approach, we assume that the *cup-4* mutation will significantly elevate the fluorescence in the body cavity of animals with an active secretory pathway and with functional coelomocytes. In contrast, we predict that the *cup-4* mutation will not increase the level of fluorescent proteins in the body cavity of animals with a severe secretion defect, whose body cavity fluorescence will remain low. We further predict that in actively secreting animals with dysfunctional coelomocytes, the *cup-4* mutation will not further increase the level of fluorescent proteins in the body cavity of animals, whose body cavity fluorescence will be high to begin with (see summary table in **Fig. 2**).

Measurement of protein folding

To estimate DAF-28::GFP folding state, both its fluorescence levels and its total protein levels need to be determined. This is because the fluorescence of DAF-28::GFP reflects only the properly folded population of DAF-28::GFP, whereas DAF-28::GFP levels detected by western blotting reflect the total population of the protein, irrespective to its folding state. As described above, because functional coelomocytes constantly remove properly folded proteins that have been secreted into the body cavity, blocking coelomocyte function is imperative for the measurement of the cumulative material that has been produced and secreted. By comparing protein fluorescence levels and total levels by western blot, in animals whose coelomocytes have been inactivated, an estimate of the ratio of correctly folded and misfolded protein can be achieved.

Using this approach, we predict that conditions, such as ERAD inactivation that disrupt the clearance of misfolded proteins protein, will increase DAF-28::GFP protein levels without affecting its fluorescence (see **Fig. 1** and summary table in **Fig. 2**). In contrast, we predict that conditions that improve protein folding, such as increased chaperone levels, will mainly affect DAF-28::GFP fluorescence levels.

Measurement of ERAD function

The outcome of ERAD function is degradation of misfolded secretory proteins. Thus, the extent of ERAD function can be assessed by blocking ERAD (i.e., via *edem-1* RNAi treatment), and following the increase in the levels of potential ERAD substrates as a result of ERAD inactivation. By comparing the accumulation of ERAD substrates upon ERAD inactivation between different strains, their relative ERAD function can be assessed.

For example, in animals with a functional ERAD, *edem-1* inactivation is expected to significantly increase DAF-28::GFP levels detected by western blotting without affecting DAF-28::GFP fluorescence. (Note that the increased levels of DAF-28::GFP protein levels, detected upon ERAD inactivation in wild-type animals, were not associated with a corresponding increase in DAF-28::GFP fluorescence levels, because of the misfolded nature of the proteins that were intended for ERAD.) In contrast, ERAD inactivation in animals whose ERAD is compromised to begin with, should not result in an increase in DAF-28::GFP total protein levels (see summary table in **Fig. 2**).

Measurement of secreted protein production

Western blots assess the steady-state levels of proteins. Since secreted proteins are produced in the ER but are also degraded via ERAD, simple measurement of proteins levels by western blotting represents the balance between protein production and protein degradation. In order to compare secreted protein production rate between strains, protein levels must be uncoupled from ERAD function. This can be achieved by western blotting for secretory protein levels in animals in which ERAD function has been blocked (for example, using *edem-1* RNAi). The importance of blocking ERAD function to assess secretory protein production rate is especially important when assessing the production of metastable proteins, such as DAF-28::GFP, which have a high turnover rate via the ERAD system. In such cases, when the ERAD system is left functional, a major fraction of the produced protein is misfolded, and thus, rapidly cleared from the system. Consequently, although a relatively low level of the protein can be detected under normal conditions, when ERAD function is blocked, significantly higher levels of the produced protein are revealed.

In principle, a change in the production rate of the protein may be due to altered transcript levels or due to altered translation levels. These two processes can be further uncoupled when transcript levels are comparable (see summary table in **Fig. 2**).

Discussion and Conclusions

The ER is the site of synthesis of secreted and cell membrane proteins. Many of the proteins which are synthesized in the ER mediate the crosstalk of the cells with their environment. Despite the fact that *C. elegans* has been a useful and powerful system to study the genetics of many cellular processes; thus far it has not been used to track the metabolism of proteins synthesized in the ER. This is in spite of their fundamental roles in the cell and in the organism. This may be due, at least in part, to the lack of well-characterized easily tractable ER substrates in *C. elegans*. Here, we described a new tool that enables the tracking of secretory protein metabolism, at the cell and organism levels in *C. elegans*. This is achieved by combining genetic manipulations (i.e., ERAD and coelomocyte inactivation), microscopy, and western blotting, to track the folding state and expression pattern of a DAF-28::GFP translational fusion protein in *C. elegans*. Two features of the DAF-28::GFP translational fusion protein render it suitable for this task. (1) it is a meta-stable protein prone to be misfolded and whose misfolded form is a substrate for ERAD degradation. (2) Unlike properly folded DAF-28::GFP, misfolded DAF-28::GFP does not fluoresce. This, in turn, enables the differentiation between the two folding states of the protein.

We have successfully used the DAF-28::GFP tool to investigate the physiological requirement of UPR signaling for the lifecycle of proteins passing through the secretory pathway under standard growth. We focused on the *ire-1* arm of the UPR, since we found that DAF-28::GFP expression pattern was significantly altered in UPR mutants with a defective *ire-1* UPR pathway, but not in *pek-1* or *atf-6* UPR mutants.[24] The increased fluorescence of the DAF-28::GFP protein in the cells that produce DAF-28::GFP, and its corresponding absence in the coelomocytes and in the body cavity of the animals implied that protein secretion is impaired in *ire-1* and *xbp-1* mutants. Consistent with this, disruption of coelomocyte function using a *cup-4* mutation did not increase the fluorescence in the body cavity of these animals, which remained low. Thus, we concluded that protein secretion in *ire-1* and *xbp-1* mutants is less efficient than in wild-type animals (see summary table in **Fig. 2**).[24] This suggested that the UPR plays a crucial role in maintaining ER plasticity and function even in the absence of external ER stress.

We demonstrated that DAF-28::GFP levels are not further increased in *ire-1* or *xbp-1* mutants upon ERAD inactivation.[24] This is in contrast to wild-type animals, where *edem-1* inactivation significantly increased the levels of the metastable DAF-28::GFP protein as detected by western blotting. (Note that the increased levels of DAF-28::GFP detected upon ERAD inactivation in wild-type animals were not associated with a corresponding increase in DAF-28::GFP fluorescence levels, because of the misfolded nature of the proteins that were intended for ERAD.) This suggested that the ERAD machinery in *ire-1* and *xbp-1* mutants is inactive to begin with, providing a plausible explanation for the accumulation of misfolded protein in these animals (see summary table in **Fig. 2**).[24]

We also used the DAF-28::GFP to evaluate how *ire-1* deficiency affects secretory protein production rate. To this end, we blocked ERAD function in wild-type animals and in *ire-1* mutants using *edem-1* RNAi treatment, and compared DAF-28::GFP total protein levels. We found that DAF-28::GFP protein levels in wild-type animals were much higher than in *ire-1* mutants (see summary table in **Fig. 2**).[24] Importantly, performing the same comparison when the ERAD system is left active gave the opposite result—namely, total DAF-28::GFP levels in wild-type animals appeared much lower than in *ire-1* mutants. This suggests that although DAF-28::GFP production rate is higher in wild-type animals compared with *ire-1* mutants, this is masked by a high turnover rate of the proteins via the ERAD system in wild-type animals.

By western blotting, we further found that *ire-1* mutants, and to a slightly lesser extent, *xbp-1* mutants, accumulate more DAF-28::GFP protein than control animals. However, most of this protein turned out to be in a misfolded state, as it did not increase the fluorescence measurements in the whole animals.[24] Nevertheless, *ire-1*

mutants did accumulate higher levels of fluorescent DAF-28::GFP in their producing cells compared with *xbp-1* mutants. This suggests that the secretory defect of *ire-1* mutants is more severe than that of *xbp-1* mutants. This further suggested that *xbp-1*-independent functions of *ire-1* contribute to secretory protein metabolism and ER function in *xbp-1* mutants. We further demonstrated that inactivation of the *C. elegans* Beclin homolog, which is important for autophagosome formation, further disrupted ER homeostasis in *xbp-1* mutants, matching it to that of *ire-1* mutants.[24] This suggests that *ire-1*-mediated autophagy may account for most of the difference in ER homeostasis between *ire-1* and *xbp-1* mutants.

In summary, we have demonstrated that animals expressing the DAF-28::GFP transgene can be useful for the analysis of modifiers of secretory protein metabolism. We have demonstrated how we have used this tool to analyze secretory protein metabolism in UPR mutants. Our data supports the idea that *ire-1* signaling is critical for secretory protein metabolism, not only under conditions of environmental stress, but also under normal growth conditions. Under these conditions, *ire-1* and *xbp-1* are required for the maintenance of basic ER functions, including secretory protein production, protein folding, protein secretion, and degradation of misfolded proteins passing through the secretory pathway. These findings may explain a wide range of physiological defects associated with UPR deficiencies.[19-23,33-38]

By screening for defects in DAF-28::GFP secretion, one can identify additional genes that, similarly to *ire-1*, are required for proper protein secretion and metabolism. Furthermore, DAF-28::GFP-expressing animals can be used for screening for mutations that restore or improve secretory protein metabolism. Further analysis of DAF-28::GFP expression pattern and folding state can provide mechanistic insights to the observed changes in DAF-28::GFP metabolism. Such studies with candidate genes as well as high-throughput screens will further our understanding of secretory protein metabolism.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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