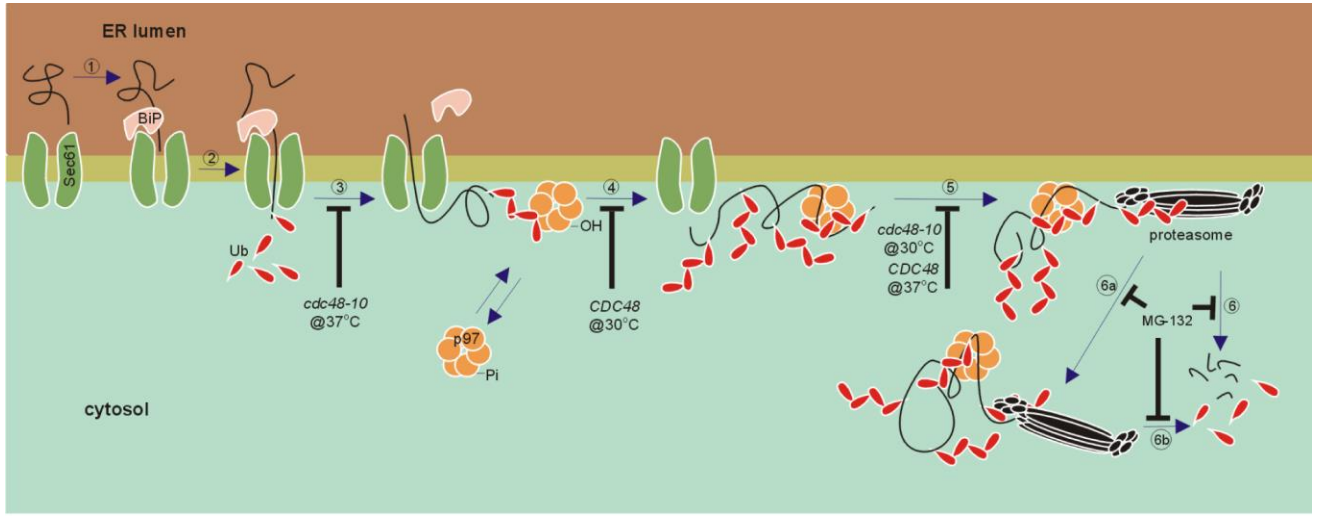
Prof. Shoshana Bar-Nun's Research Interests

Protein quality control mechanisms are the central focus of our research. These mechanisms play a key role in maintaining proteostasis and alleviate proteotoxic stresses. In the secretory pathway, nascent proteins are translocated across the endoplasmic reticulum (ER) membrane. Within the ER, N-glycans are attached, disulfide bonds are formed and proteins acquire secondary and tertiary structure and assemble into oligomeric complexes. Quality control mechanisms monitor folding and assembly, and only proteins that acquire native conformation exit the ER via vesicles and are transported to their final destinations along the secretory pathway.



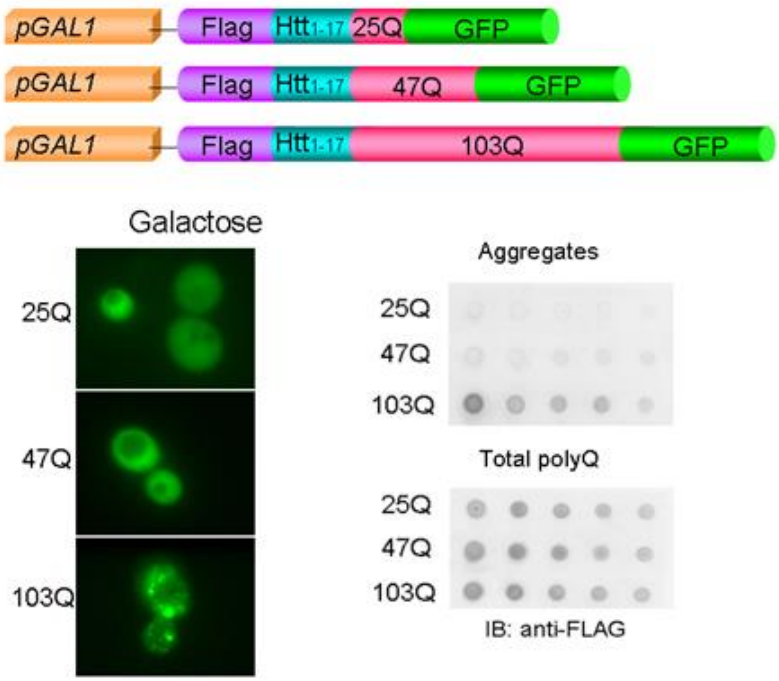
Aberrant proteins are retained in the ER, dislocated back to the cytosol and eliminated by the ubiquitin-proteasome system. This pathway is known as ER-associated protein degradation (ERAD) (Bar-Nun, 2005). Impaired quality control mechanisms underlie the molecular basis of diverse human diseases, including amyloidosis and neurodegenerative disorders such as Alzheimer's, Parkinsons's, Huntington's and polyglutamine and prion diseases. These disorders are in fact 'conformational diseases' caused by protein misfolding and aggregation.

Our research is aimed to understand the molecular mechanisms that underlie age-related neurodegeneration in general and aggregation of polyglutamine proteins in Huntington's disease in particular. Relying on the high degree of conservation of the quality control mechanisms and ERAD from yeast to man, our work utilizes molecular cell biology and biochemical techniques, both in cultured mammalian cells and in yeast. The power of yeast genetics and the simplicity of our assays allow identification of age-related pro- or anti-aggregation genes and conditions and the discovery of anti-aggregation drugs. These tools also allow us to study *cis*-acting signals that confer ER retention and ERAD onto otherwise stable and secreted proteins, as well as cellular*trans*-acting factors that target these proteins to degradation by the ubiquitin-proteasome system. As a model for *cis*-acting signals, we investigate elements derived from the immunoglobulin (Ig) molecule that include C_H1, the first constant region in Ig heavy chains, and mstp, the conserved glycosylated C-terminus of the ms heavy chain of secretory IgM. The cellular ERAD components that we focus on, addressing their role in ERAD and their regulation mode, include the AAA-ATPase p97/Cdc48 and the proteasome subunits.

Yeast as a model system to for neurodegenerative disorders

Quality control mechanisms function in the frontline of cellular response to aberrant proteins. A major problem inherent to aberrant proteins is their tendency to form aggregates that are toxic to cells, and many human diseases are caused by such aggregations. For example, the incurable Huntington's disease (HD) is a progressive neurodegenerative disorder caused by genetic mutations that generate extended polyglutamine (polyQ) tracts in huntingtin, leading to its aggregation and eventual death of neurons. Aggregation diseases are multi-factorial and caused by disturbed homeostasis between aggregation-prone proteins load and cellular capacity to handle them. Molecular chaperones are key cellular components that handle such proteins and indeed, the molecular chaperone p97 and its yeast homologue Cdc48 were identified as polyQ-interacting proteins and human p97 was implicated in inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia.

Age is a key factor in every aggregation disease. The age of HD onset is inversely correlated with the number of Q repeats, and even a mid-size polyQ tract may lead to HD at a later age. We hypothesize that polyQ aggregation is aggravated by a combination of aging and at least one additional insult such as dwindling of or accumulation of incapacitating mutations in key cellular components, as well as growth conditions that affect cellular metabolism. Since aging and protein aggregation are multi-factorial complex processes, we exploit the excellent genetic tools afforded by *Saccharomyces cerevisiae* and comprehensive experimental approaches for a combinatorial study of



polyQ aggregation and aging.

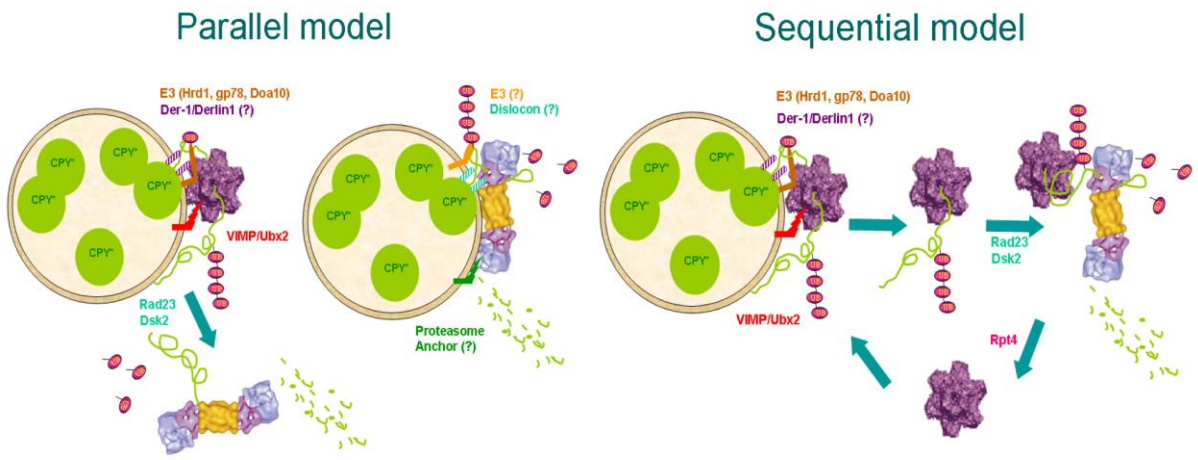
Aggregation can be easily monitored by fluorescent microscopy or a simple filtration assay. The involvement of Cdc48 in polyQ aggregation is addressed by screening for known and novel cofactors, substitutes and regulators of Cdc48 that ameliorate aggregation of the toxic long polyQ (103Q) huntingtin fragment. The non-toxic mid-size polyQ (47Q) huntingtin fragment is used as a sensitive biosensor for the identification of age-related pro- or anti-aggregation genes and conditions and for the discovery of anti-aggregation drugs.

The Discovery of p97/Cdc48 and its Role in ERAD

In 2000 we discovered that the p97, and its conserved yeast homologue Cdc48, is an essential component of ERAD that takes part in the elimination of virtually all ERAD substrates (Rabinovich et al., 2002). The p97/Cdc48 is a cytosolic homo-hexameric AAA-ATPase that functions as a molecular machine. Initially, we found p97 in association with a luminal ERAD substrate and subsequently demonstrated the role of its homologue Cdc48 in the

degradation in yeast of two well-established ERAD substrates, membrane 6myc-Hmg2 and the luminal CPY* (Rabinovich et al. 2002). To date, p97/Cdc48 is a hallmark of ERAD, although it also participates in several other cellular functions (Bar-Nun, 2005). Being an ATPase that resides in the cytosol, p97/Cdc48 provides the driving force for dislocating ERAD substrates from the ER back to the cytosol, as the stabilized luminal ERAD substrate CPY* remained trapped within the ER lumen in the temperature-sensitive *cdc48-10* mutant (Elkabetz et al., 2004).

Interrelations between Cdc48 and proteasomal AAA-ATPases: The 26S proteasome complex is composed of a 20S proteolytic chamber and a 19S regulatory cap. Several AAA-ATPases (known in yeast as Rpt1-6) form a hetero-hexameric ring at the base of this 19S regulatory particle. We have shown that the proteolytic activity of the proteasome is dispensable for the dislocation a luminal ERAD substrate (Elkabetz et al., 2004) but two of the 19S regulatory particle subunits, Rpt2 and Rpt4, are essential for ERAD. While Rpt2 is required for degradation of every proteasomal substrate, since it gates the entry of substrates into the proteolytic chamber, Rpt4 is essential for the elimination only of ERAD substrates and is dispensable for degradation of cytosolic proteins (Lipson et al., 2008). Interestingly, Rpt4 is involved in dislocation of ERAD substrates, a role already assigned to Cdc48 (Elkabetz et al., 2004). Further experiments on the interrelations between these two AAA-ATPases suggest that Cdc48 extracts the substrate from the ER, while Rpt4 is required for transferring the substrate from Cdc48 to the proteasome (Lipson et al., 2008). We also studied the gating of the 20S catalytic particle and showed that degradation of cytosolic and ERAD substrates was similarly accelerated upon truncation of the N-termini of the 20S subunits α3 and α7 known to gate the proteolytic chamber (Rabinovich et al., 2006).



Cdc48 Suppressors: Ssz1 and the link between ERAD and PDR: In its role in ERAD,Cdc48 collaborates with Ufd1 and Npl4, forming a Cdc48-Ufd1-Npl4 complex. In genetic screens for suppressors of *cdc48* temperature-sensitive mutants, we have identified *SSZ1* and show that it upregulates Cdc48 via the pleiotropic drug resistance (PDR) network. A p*SSZ1* plasmid restored the impaired ERAD of the membrane substrates 6myc-Hmg2 in yeast cells carrying mutations in *CDC48*, *UFD1* and *NPL4*, while deletion of the *SSZ1* gene had no effect. Ssz1p activates Pdr1p, the PDR master regulator. Indeed, plasmids of *PDR1* or its target gene *RPN4* increased the levels of mutant Cdc48 protein and restored ERAD in the *cdc48-10* temperature-sensitive mutant. Rpn4 regulates transcription of proteasome subunits but also of*CDC48*, thus *RPN4* deletion abolished ERAD. However, the diminished proteasome level in *Δrpn4* was sufficient for degrading a cytosolic substrate, whereas the impairedERAD-M was the result of diminished levels of Cdc48 and indeed, ERAD was restored by expression of p*CDC48.* The restored ERAD-M in the hypomorphic strains of the Cdc48 partners *ufd1-2* and *npl4-1* by the p*CDC48* plasmid, and in *cdc48-10*temperature-sensitive mutant by the p*cdc48-10* plasmid, combined with the finding that neither p*SSZ1* nor p*cdc48-10* restored ERAD-L of CPY*-HA, support our conclusion that Ssz1 suppressing effects is brought about by upregulating Cdc48(Bosis et al., 2009). These findings uncover a regulatory link between PDR, which induces membrane transporters for efflux of cytotoxic compounds, and ERAD, which eliminates damaged proteins generated by such compounds, and extend our knowledge on the coordination of cellular networks that are responsible for coping with stress.

ERAD Signals

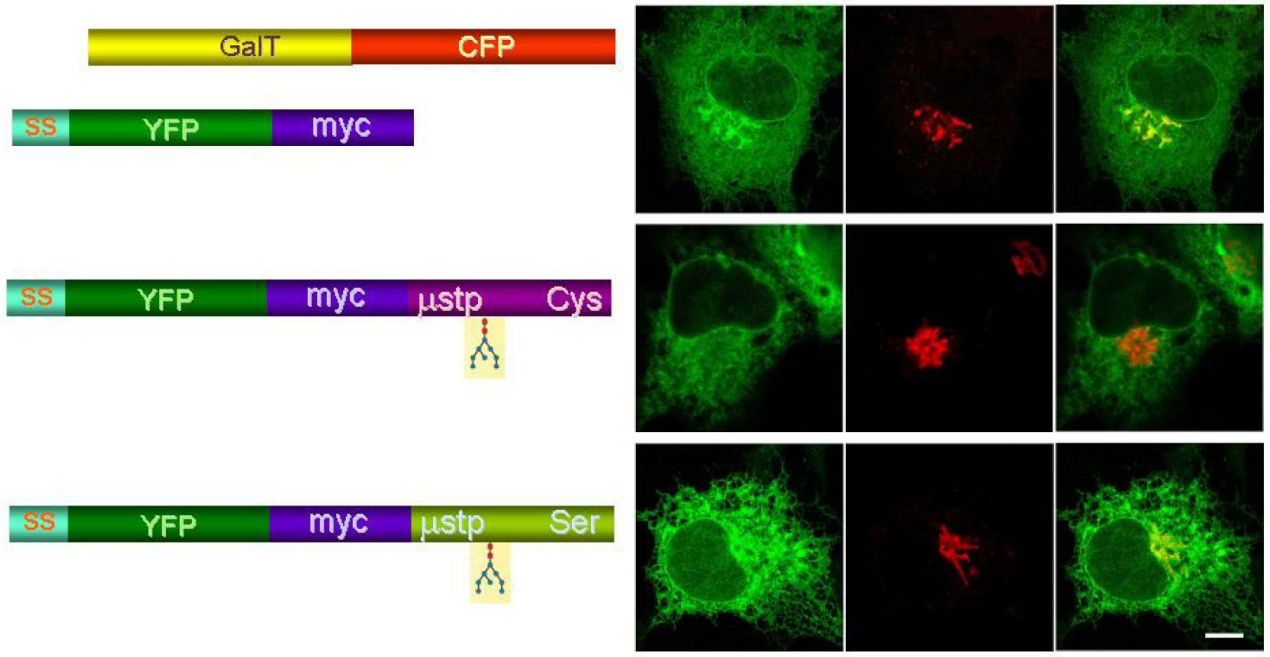
Immunoglobulins and sIgM in B-Cells: Secretory IgM (sIgM), the first antibody secreted from young plasma cells, is an excellent model to study folding, assembly and secretion of proteins. Antibodies are synthesized in the secertory pathway and assemble into well characterized complexes composed of two light chains and two heavy chains linked via inter-chain disulfide bonds. sIgM is produced but retained and rapidly degraded B-cells, whereas the same B-cells express on their surface a membrane form of IgM. The ms heavy chain of sIgM and the mm of the membrane IgM differ only in their C-termini. The unique C-terminus of ms, designated the mstp, contains 20 amino acids, including a penultimate Cys (Cys575 in mouse ms) and an additional N-glycosylation site (Asn563 in mouse ms). The distinct intracellular fates ofms and mm are dictated by the mstp and its Cys575 in particular. The sIgM retained and degraded in B-cells was assembled into ms_2k_2 monomer that were degraded prior to the *trans*-Golgi, whereas sIgM pentamerization in plasma cells took place in or beyond this compartment (Shachar et al., 1992).

Transport-dependence: sIgM degradation was non-lysosomal and occurred prior to the medial- Golgi (Amitay et al., 1991), yet required vesicular export from the ER (Amitay et al., 1992). In pre-B cells that do not express any Ig light chains, the free μs was also rapidly degraded but this degradation was transport-independent (Rabinovich et al., 1993). We reconstituted the compartmentalized sIgM degradation along the secretory pathway *in vitro*, in permeabilized cells (Winitz et al., 1996) and pioneered the concept of transport-dependent ERAD-L. We demonstrated in pre-B cells that differentiated into light chain-expressing B-cells shifted ms degradation from vesicular transport-independent process into a mechanism that required budding of vesicles from the ER (Elkabetz et al., 2003).

IgG assembly: Moving from endogenous sIgM in B-cells to non-lymphoid mammalian cells allowed us to address quality control processes operating in Ig production. The γ2b heavy chain of mouse IgG2b is retained but not degraded. When expressed in non-lymphoid COS-7 cells, we found intriguing interrelations between Cys residues involved in γ2b folding and IgG2b assembly (Elkabetz et al., 2005). We identified unique active antibody composed of two light chains associated non-covalently with the heavy chains homodimer, which formed between them an unusual S-S bond (Elkabetz et al., 2008). Interrelations between assembly and secretion and a role played by Cys were also discovered in our collaborative study on human acetylcholine esterase (Kerem et al., 1993).

sIgM is an ERAD substrate: We found that ALLN, now known as a proteasome inhibitor, blocked sIgM degradation (Amitay et al., 1992). Subsequently, we demonstrated that several specific proteasome inhibitors blocked sIgM degradation and in their presence, poly-ubiquitin was conjugated to the accumulating sIgM, hence sIgM is a *bona fide* luminal ERAD substrate (Elkabetz et al., 2003).

ERAD signals – the mstp: We established the mstp as an ERAD signal that conferred ER retention and targeted otherwise stable proteins to the ubiquitin-proteasome system. The μstp was fused to two reporter secretory proteins, a truncated secreted version of thyroid peroxidase (TPO) and yellow fluorescent protein led to the secretory pathway by a signal sequence (ssYFP). When expressed in several non-lymphoid cells, μstpCys fusion proteins were retained in the ER, conjugated to poly-ubiquitin and degraded by the proteasome, whereas those fused to μstpSer were stable proteins that were efficiently secreted (Shapira et al., 2007). These data are consistent with the contribution of the penultimate Cys in the μstp to the intracellular retention of μs and with the importance of the oxidizing thiol milieu within the ER lumen (Shachar et al., 1994).


GalT
CFP
SS
YFP
myc
SS
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myc
µstp
Cys
SS
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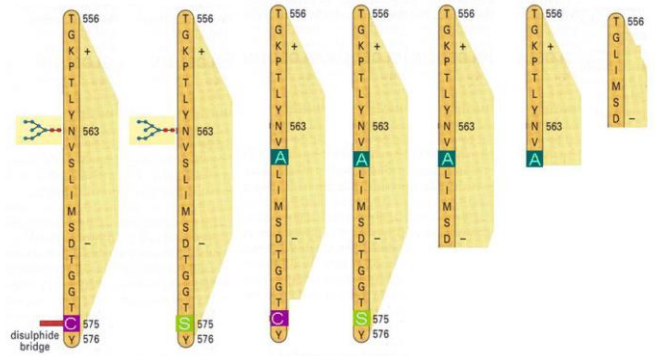
The μstp is highly conserved in evolution, including its penultimate Cys and its N-glycan. To address the role of N-glycan in regulating protein folding and degradation, we generated unglycosylated μstp. We found accelerated degradation of the unglycosylated mutants and ssYFP-μstpSer turned from secreted protein into an ERAD substrate (Shapira et al., 2007). The effect of the N-glycan could not be attributed to limiting amounts of processing enzymes or lectins acting as chaperones or cargo receptors. A refined characterization of the mstp revealed that its last 15 residues were sufficient to confer ER retention and rapid degradation. This dual function was hampered upon linking N-glycan or if a predicted b-strand was shortened or broken. A b-strand structure, rather than the actual sequence of the 15 residues, was essential for tight retention and rapid degradation, as both functions were conferred by a b-strand constructed from different residues and hampered if this b-strand was broken. The initial distinction between glycosylated and unglycosylated ssYFP was by the ER chaperone BiP, which strongly preferred the unglycosylatedspecies and could account, in part, for their tighter retention and accelerated degradation. However, BiP could not act single-handedly, as all unglycosylated species were associated equally well with BiP, irrespective of their targeting to secretion or degradation. It appeared that a b-strand structure was responsible for executing the tight retention and rapid degradation (Shapira et al., in preparation).



Retention	yes tight	no secreted	yes tight	yes tight	yes tight	no secreted	no secreted
Degradation	slow	no	fast	fast	fast	no	no

Glucosidases: The importance of the N-glycan and of chaperones/lectins and enzymes that recognize and process the N-glycan led to a theoretical study. Based on *in vitro*studies published by our collaborators, we constructed a mathematical model that tested key steps in the N-glycan-dependent quality control mechanisms. Our model predicted that glucosidase II was inhibited by its end product, the unglucosylated N-glycan and that the inhibition was more effective if the end product was also trimmed by mannosidase. This physiologically significant prediction was confirmed experimentally by our collaborators. In addition, our model showed that the N-glycan dissociation from the lectin/chaperone calreticulin was spontaneous and independent of glucosidase II, while this enzyme prevented the re-association of the unglucosylated N-glycan with calreticulin (Bosis et al., 2008).

Competition for ERAD substrates between dislocation and secretion: Using yeast mutants and invertase fused to μstp, we revealed in the ER lumen competition between secretion and ERAD. The μstpCys, unlike μstpSer,

conferred retention onto invertase. The penultimate Cys contribution was corroborated by improved secretion of invertase-μstpCys upon incubation with β-mercaptoethanol or deletion of Eug1, identifying this ER-resident thiol oxidoreductase as a key player in the μstpCys-dependent retention. The retained invertase-μstpCys dislocated to the cytosol in a process involving the E3 ubiquitin ligase Doa10 and the AAA-ATPase Cdc48 and Rpt4 and was degraded by the ubiquitin-proteasome system involving the E2 Ubc7 and the proteasomal Rpt2. Hence, the μstpCys was recognized as an ERAD signal also in yeast. Coupling between retention and dislocation of invertase-μstpCys was deduced from improved secretion under conditions of blocked dislocation. However, while the proximal dislocation step competed with secretion, the distal ERAD steps of ubiquitination or proteasomal degradation were dispensable for either retention or dislocation of invertase-μstpCys (Nadel et al., submitted).