

Introduction

Neurodegenerative diseases are debilitating pathologies with a common underlying pathogenic mechanism, involving incorrect protein folding processes and protein aggregation¹. In neurodegenerative states, a specific subset of neurons is subjected to inflammatory factors released by the microglia upon activation by triggering signals. The activating cues are produced when cells are exposed to particular stress conditions (e.g. heat stress, oxidative stress, or toxic agents), and lead to the generation of misfolded proteins. Then, misfolded proteins are accumulated into aggregated structures, which can cause damages to the lipid membranes or impair protein degradation, interfering with the proper function of the proteostasis network, and are therefore associated to failures in neuronal functions and cell death. Contrarily to degenerating neurons, in healthy cells, misfolded proteins are directed into dynamic cytoplasmic aggregates which temporarily mediate the storage of proteins, ultimately addressing them for clearance by the ubiquitin proteasome system (UPS) and by autophagy².

Through an integrated and multidisciplinary approach, different biological models, including mammalian dendritic cells, neurons and yeast cells, will be employed to exhaustively evaluate the role of protein aggregates in aging and neurodegenerative diseases. The budding yeast *Saccharomyces cerevisiae* displays a range of different dynamic granules that appear to be related to those found in metazoan models. Indeed, many cytoplasmic aggregates, such as P-bodies and stress granules, are demonstrated to be conserved from yeast to humans, despite the increased complexity in protein composition and function in higher eukaryotes. Nonetheless, yeast cells have been widely employed to study cytoplasmic granules, providing interesting insights into their physiological roles. Moreover, yeast is a well-established and powerful model organism to study metazoan proteins associated to aging and neurodegenerative diseases, since many pathways that are relevant for these conditions in humans, including protein folding, proteostasis and stress response, are conserved in yeast³. Therefore, *S. cerevisiae* was identified as the most suitable starting point.

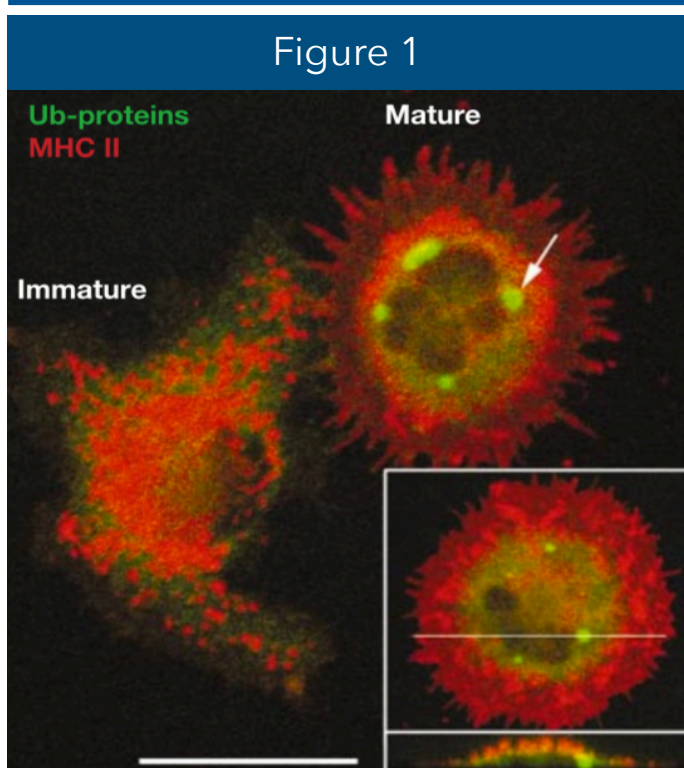


Fig. 1. Ubiquitinated protein accumulation during dendritic cell (DC) maturation. Maturing human DCs aggregate ubiquitinated proteins (green, indicated by an arrow), not observed in immature cells. Z-sectioning revealed the circular shape of DALISs. Size bar, 20 μ m (Lelouard *et al.*, 2002).

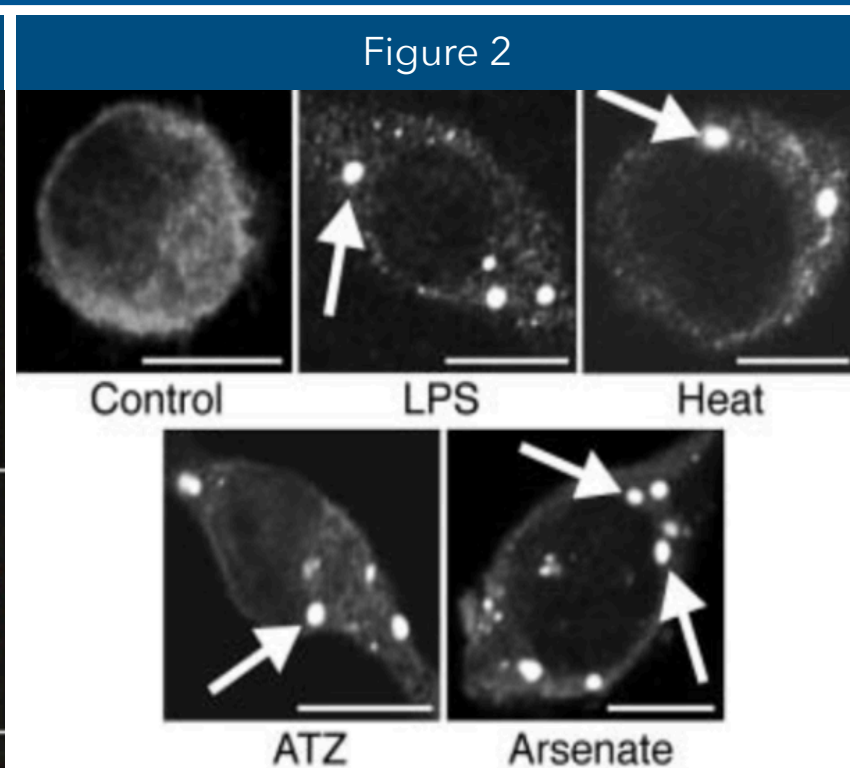


Fig. 2. Stress induces aggresome-like induced structures (ALIS) formation in macrophages. Representative confocal z-sections of RAW 264.7 murine macrophages stained for ubiquitinated proteins with the FK2 mAb after the indicated treatment for 8 h. Ub+ structures (ALIS) are indicated with arrows. Size bar, 10 μ m (Szeto *et al.*, 2006).

DALIS

(Dendritic cell Aggresome-Like Induced Structure)

| DALIS in dendritic cells | ALIS in macrophages and non-immune cells | DALIS are cytosolic protein aggregates detected in dendritic cells after DC activation and maturation in response to microbial products (e.g. LPS) or after exposure to heat shock temperatures ³ . Equivalent structures, called aggresome-like induced structure (ALIS), can be found in macrophages and non-immune cells, where they are triggered by TLR stimulation, fever-like temperatures, oxidative stress and starvation ⁴ . DALIS are storage compartments for polyubiquitinated defective ribosomal products (DRiPs), hence their formation and maintenance are dependent upon protein synthesis ^{1,5} . DALIS do not need microtubules and actin networks to assemble ^{3,5} and they are not caged with vimentin ³ . DALIS have a dynamic, transient structure, which allows them to freely interact with each other and with other cytoplasmic elements in the hours subsequent to their formation ^{4,5} . Proteasome-mediated degradation is implicated in the clearance of these cytosolic aggregates ⁴ . DRiPs are the major source of self and viral antigenic peptides for MHC-I-restricted presentation. The transient aggregation of DALIS delays antigen degradation and presentation by activated APCs, hence regulating antigen presentation ^{1,5} . In non-immune cells, ALIS may act as short-term processing/ degradation platforms for misfolded proteins able to prevent cytotoxicity and cell death, as observed in degenerating neurons ⁶ . |
|--|---|---|
| PAMPs stimulation Fever-like temperature | PAMPs stimulation Fever-like temperature Oxidative stress Starvation | |
| Composed by newly synthesized defective proteins (DRiPs) | | |
| Formation and maintenance dependent on protein synthesis | | |
| No need of microtubule and actin networks to assemble | | |
| Vimentin-free structures | | |
| Dynamic structure (freely interact with cytoplasmic elements and other ALIS) | | DALIS are cytosolic protein aggregates detected in dendritic cells after DC activation and maturation in response to microbial products (e.g. LPS) or after exposure to heat shock temperatures ³ . Equivalent structures, called aggresome-like induced structure (ALIS), can be found in macrophages and non-immune cells, where they are triggered by TLR stimulation, fever-like temperatures, oxidative stress and starvation ⁴ . DALIS are storage compartments for polyubiquitinated defective ribosomal products (DRiPs), hence their formation and maintenance are dependent upon protein synthesis ^{1,5} . DALIS do not need microtubules and actin networks to assemble ^{3,5} and they are not caged with vimentin ³ . DALIS have a dynamic, transient structure, which allows them to freely interact with each other and with other cytoplasmic elements in the hours subsequent to their formation ^{4,5} . Proteasome-mediated degradation is implicated in the clearance of these cytosolic aggregates ⁴ . DRiPs are the major source of self and viral antigenic peptides for MHC-I-restricted presentation. The transient aggregation of DALIS delays antigen degradation and presentation by activated APCs, hence regulating antigen presentation ^{1,5} . In non-immune cells, ALIS may act as short-term processing/ degradation platforms for misfolded proteins able to prevent cytotoxicity and cell death, as observed in degenerating neurons ⁶ . |
| Transient (4-24h after stress) | | |
| Ubiquitination areas for DRiPs & antigen storage during DC maturation | | |

DALIS and Puromycin

Puromycin is an antibiotic erroneously incorporated by the ribosome at the C-terminus of elongating polypeptides at the place of the correct amino acid. For this reason, its incorporation during translation is a chain terminating event, which generates truncated proteins⁷. Studies revealed that drugs stimulating defective protein synthesis, such as puromycin, function as DALIS enhancers⁵.

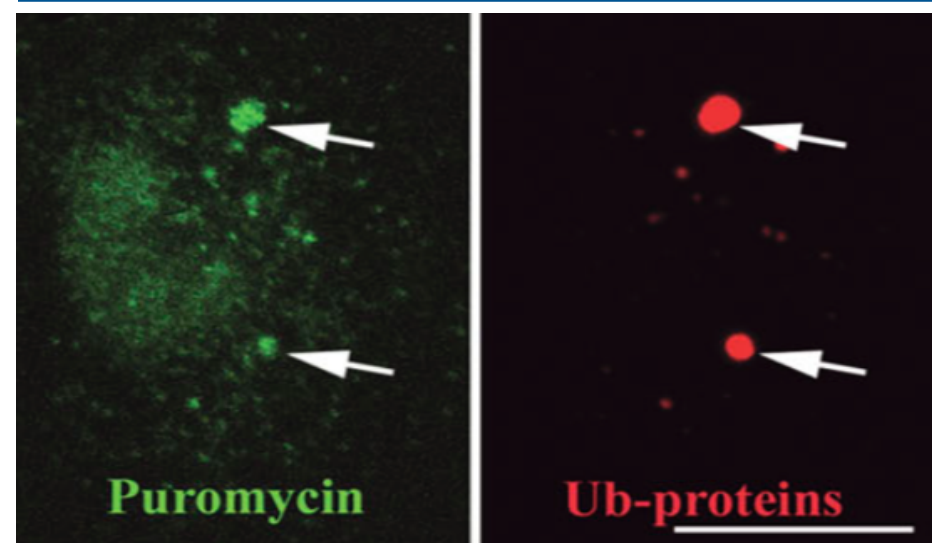


Fig. 3. Puromycin-labeled protein (green) incorporation in the central area of DALIS (red, arrows) is visualised as soon as 5 min after puromycin addition. This suggests a specific structural organisation with a center extremely active in defective protein recruitment (Lelouard *et al.*, 2004).

Figure 3

References

- Argüello R.J., Reverendo M., Gatti E., Pileri P. (2016). *Immunol Rev.* **272**, 28-38. doi:10.1111/imr.12427
- Longo V.D., Shadel G.S., Kaeblerlein M., Kennedy B. (2012). *Cell Metab.* **16**, 18-31. doi:10.1016/j.cmet.2012.06.002
- Lelouard H., Gatti E., Cappello F., Gresser O., *et al.* (2002). *Nature*. **417**, 177-182. doi:10.1038/417177a
- Szeto J., Kanika N.A., Casadieu V., Norman R., *et al.* (2006). *Autophagy*. **2**, 189-199. doi:10.4161/auto.2731
- Lelouard H., Ferrand N., Marguet D., Bortol J., *et al.* (2004). *J Cell Biol.* **164**, 667-675. doi:10.1083/jcb.200312073
- Pileri P. (2005). *Immunol Rev.* **207**, 184-190. doi:10.1111/j.0105-2896.2005.00300.x
- Aviner R. (2020). *Comput Struct Biotechnol J.* **18**, 1074-1083. doi:10.1016/j.csbj.2020.04.014
- Schindler D., Davies J. (1975). *Methods Cell Biol.* **12**, 17-38. doi:10.1016/0091-679x(08)60949-8
- Gruel T., Ivanov P., Malova L., Pomphrey F., *et al.* (2013). *PLoS One*. **8**, e57083. doi:10.1371/journal.pone.0057083
- Hayle N.P., Castelli L.M., Campbell S.G., Holmes L.E.A., *et al.* (2007). *J Cell Biol.* **179**, 45-74. doi:10.1083/jcb.200707010

Puromycin and Yeast

Yeasts are naturally resistant to puromycin, since they show little or no uptake of the drug⁸. To overcome this problem, a puromycin-sensitive triple mutant (EPP), lacking the ERG6, PDR1 and PDR3 genes, of the BY4741 strain was obtained.

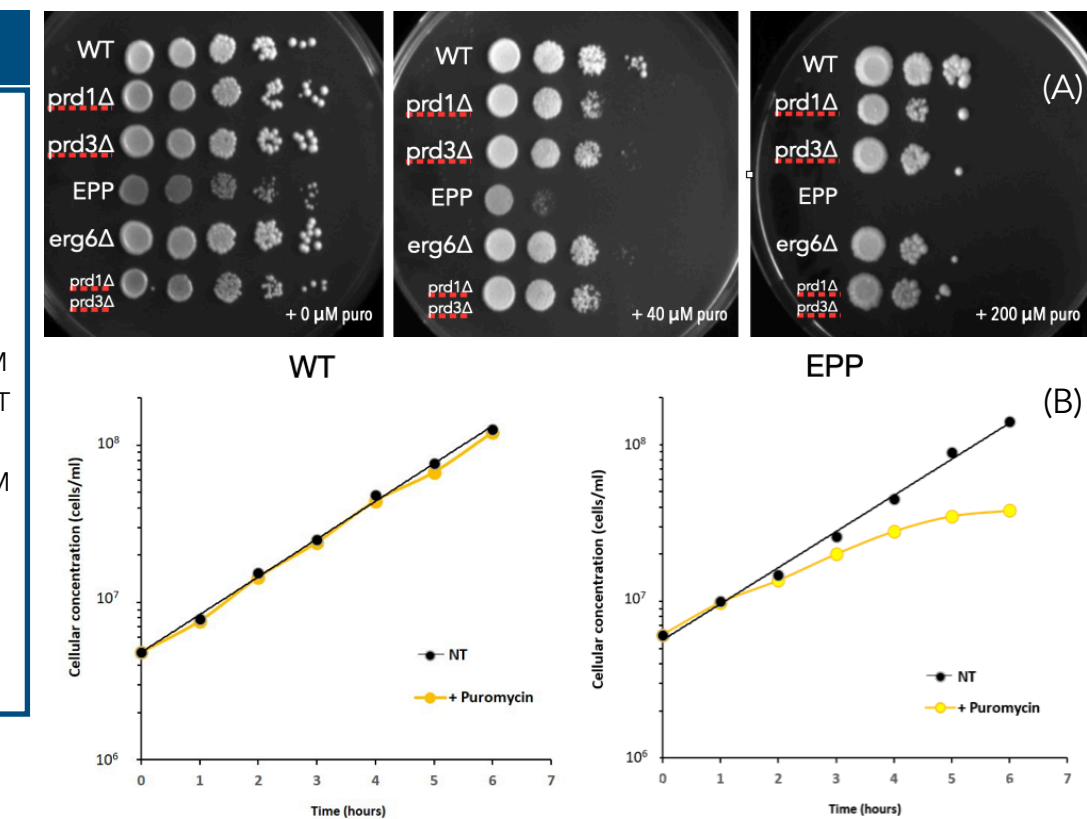
A. ERG6 gene regulates the biosynthesis of membrane ergosterol. Its deletion has been shown to ablate ergosterol production, transforming the membrane into a more permeable barrier for small molecules.

B. PDR1 and PDR3 are the pleiotropic drug resistance genes. They regulate the transcription of the multi-drug pump gene PDR5, which encodes an ABC-type protein involved in resistance to drugs.

The EPP mutant was tested both *in vitro* and *in vivo* for their resistance to different puromycin concentrations. The growth of 5-fold serial dilutions of wild type (WT), EPP and single deletion mutant cells was assessed on YPD agar containing 40 μ M and 200 μ M puromycin (Figure 4a). The growth of WT and EPP cells was also analysed in liquid media containing 0 μ M and 40 μ M puromycin. Growth curves were measure using a Coulter Counter at 1-hour intervals at a constant temperature of 30°C (Figure 4b). As expected, while puromycin concentrations as high as 200 μ M had no effect on the growth of wild type cells, puromycin at concentrations of 40 μ M slowed the growth of the EPP strain, with complete growth inhibition at 200 μ M.

Figure 4

Fig. 4. Characterisation of the puromycin-sensitive triple mutant (erg6 Δ pdr1 Δ pdr3 Δ) of the BY4741 strain. (A) Growth of 5-fold serial dilutions of WT, EPP and single deletion mutants cells on YPD agar containing 0 μ M, 40 μ M and 200 μ M puromycin. (B) Growth curves for WT (wild type) and EPP triple mutant cells in liquid media containing 0 μ M (black) and 40 μ M (yellow) puromycin. Growth curves were measure using a Coulter Counter at 1-hour intervals at a constant temperature of 30°C.



Future Perspectives

Saccharomyces cerevisiae strain BY4741 (WT) and its puromycin-sensitive triple mutant (erg6 Δ pdr1 Δ pdr3 Δ) will be used in future experiments. Exponentially growing cells will be subjected to different stresses (see table). Protein ubiquitination will be assessed by Western Blot analysis using the anti-ubiquitin antibody FK2. FK2 is a monoclonal antibody, specific for mono- or polyubiquitinated proteins which does not react with free ubiquitin. Then, cytoplasmic aggregates will be visualized by fluorescent microscopy.

| Stress | Characteristics |
|-------------------------|--|
| Heat Shock at 37°C | Heat shock at 37°C has been shown to induce cytoplasmic granules aggregation in yeast ⁹ . |
| Heat Shock at 42°C | Heat shock at 42°C has been shown to induce DALIS formation in dendritic cells ⁴ and cytoplasmic granules aggregation in yeast ⁹ . |
| Starvation (– nitrogen) | Nitrogen depletion inhibits translation initiation by reducing the association of initiation factors with ribosomes and inducing aggregation into cytoplasmic granules ¹⁰ . |
| Starvation (– glucose) | Glucose depletion inhibits translation initiation, causing initiation factors redistribution into cytoplasmic granules ¹⁰ . |
| Puromycin (40 μ M) | Puromycin functions as DALIS enhancer ⁵ . |