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Stress-induced ubiquitin-rich aggregates in the yeast *Saccharomyces cerevisiae*

Roberta Sarah Torinesi

Registration number: 780313

Tutors: Prof. Ivan Orlandi and Prof. Rita Grandori (University of Milan Bicocca)

Supervisors: Prof. Ivan Orlandi (University of Milan Bicocca)

Dr. Taras Y. Nazarko (Georgia State University)

Coordinator: Prof. Paola Branduardi (University of Milan Bicocca)

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Abstract

Protein misfolding and aggregation are major issues in neurodegenerative disorders. The large inclusions of misfolded proteins can interfere with the proper proteostasis of the cell, possibly leading to failures in cellular functions and loss of neuronal activity in the brain (Hipp *et al.*, 2019). Nevertheless, in healthy eukaryotic cells, reversible protein aggregates are formed in response to external stimuli and represent well-organized deposition sites, which help the cell protecting its proteome in the waiting for the restoration of the physiological condition (Tyedmers *et al.*, 2010). Among these reversible protein aggregates, Dendritic cell Aggresome-Like Induced Structures (DALISs) are transient deposits for ubiquitinated defective proteins and antigens, formed during dendritic cell maturation in response to several stresses (Pierre, 2005). No investigation has ever been conducted to identify these aggregates in other eukaryotic cells, but since they act as deposition sites for ubiquitinated misfolded proteins, we can expect to locate them in other cellular models as well.

The yeast *Saccharomyces cerevisiae* is widely used for the research on cellular aggregates, as it displays a vast range of different dynamic deposition sites, such as P-bodies and stress granules, that are demonstrated to be conserved between different species (Saarikangas and Barral, 2016). We identified four stress conditions under which the yeast *Saccharomyces cerevisiae* manifests the formation of large cytoplasmic bodies, that are heat shock temperatures at 42°C, severe ethanol stress, nitrogen depletion and glucose starvation, and one stress condition which induces the formation of DALISs in mammalian cells, namely puromycin treatment (Lelouard *et al.*, 2004). Therefore, we subjected yeast cells to these stress conditions and investigated the formation of ubiquitin-rich protein aggregates.

We demonstrated the formation of ubiquitin-rich protein aggregates in wild type *S. cerevisiae* cells following the exposure to severe ethanol stress and under prolonged nitrogen starvation, and in the puromycin-sensitive strain following the treatment with puromycin. We further investigated the cellular localization of ubiquitinated aggregates of wild type *S. cerevisiae* cells by fluorescence microscopy. We observed that severe ethanol stress induces the accumulation of many small, round-shaped ubiquitin-rich aggregates, which are disseminated in the cytosol and not colocalized with any cellular structure. Similar round-shaped, disseminated dots were observed under nitrogen starvation conditions, with the difference that under this stress condition the ubiquitinated aggregates appeared larger in size and less numerous in the cell. The ubiquitin-rich structures we observed in yeast cells reflect the characteristic morphology of DALIS reported in dendritic cells (Lelouard *et al.*, 2004).

We then decided to investigate how the ethanol-induced ubiquitin-rich protein aggregates are cleared inside the yeast cell. Whereas the nitrogen starvation condition is known to stimulate the formation of protein aggregates that are cleared by aggrephagy, the selective autophagy of protein aggregates (Lamark and Johansen, 2012), it is not known what mechanisms are involved in the clearance of protein aggregates under severe ethanol stress. We demonstrated that the accumulation of ubiquitin-rich aggregates is persistent in *atg1Δ* mutant cells, whereas in wild type cells the level of ubiquitinated proteins decreases a few hours after the exposure to the stress. This suggests that autophagy is implied in the clearance of ubiquitinated aggregates, as Atg1 is a kinase which plays a crucial role in the regulation of this process (Papinski *et al.*, 2014). On the other hand, we observed that *cue5Δ* mutant cells displayed a minor accumulation of ubiquitin throughout the whole treatment with ethanol, compared to wild type cells. The yeast protein Cue5 is a ubiquitin-binding

protein that collaborates with Ubc4/Ubc5 ubiquitin-conjugating enzymes and the Rsp5 ubiquitin ligase for the conjugation of ubiquitin to its substrate (Lu *et al.*, 2014). Since we observed a lower presence of ubiquitin in *cue5Δ* mutant cells, we propose that Cue5 might have a role in the conjugation of ubiquitin itself to the aggregates under severe ethanol stress.

Riassunto

Il misfolding e l'aggregazione proteici sono alla base dei disturbi neurodegenerativi. Gli accumuli di proteine non correttamente ripiegate possono interferire con la corretta proteostasi della cellula, portando a insufficienze nelle funzioni cellulari e alla perdita dell'attività neuronale cerebrale (Hipp *et al.*, 2019). Ciononostante, in cellule eucariote sane, aggregati proteici reversibili si formano in risposta a stimoli esterni e rappresentano dei siti di deposito ben organizzati che aiutano la cellula a proteggere il proprio proteoma nell'attesa del ripristino della condizione fisiologica (Tyedmers *et al.*, 2010). Tra gli aggregati proteici reversibili, i Dendritic cells Aggresome-Like Induced Structures (DALISs) sono dei depositi transienti per proteine difettive ubiquitinate ed antigeni, che vengono a formarsi durante la maturazione delle cellule dendritiche in risposta a diversi stress (Pierre, 2005). Ad oggi, nessuna indagine è mai stata condotta per identificare tali aggregati in altre cellule eucariote, ma poiché fungono da siti di deposito per proteine non correttamente ripiegate ed ubiquitinate, ci si aspetta di incontrarli anche in altri modelli cellulari.

Il lievito *Saccharomyces cerevisiae* viene ampiamente utilizzato per la ricerca sugli aggregati cellulari, poiché presenta un vasto range di siti di deposito dinamici, come i P-bodies e gli stress granules, che sono stati dimostrati essere conservati tra diverse specie (Saarikangas and Barral, 2016). Abbiamo identificato quattro condizioni di stress in cui il lievito *Saccharomyces cerevisiae* manifesta la formazione di grossi corpi citoplasmatici, temperature da shock termico a 42°C, stress acuto da etanolo, deprivazione di azoto e carenza di glucosio, oltre che una condizione di stress che induce la formazione di DALISs in cellule di mammifero, ovvero il trattamento con puromicina (Lelouard *et al.*, 2004). Dunque, abbiamo sottoposto le cellule di *S.*

cerevisiae a tali condizioni di stress, esaminando la formazione di aggregati proteici ricchi di ubiquitina.

Abbiamo dimostrato la formazione di aggregati proteici ricchi di ubiquitina in cellule wild type di *S. cerevisiae* a seguito dell'esposizione di stress acuto da etanolo e di deprivazione prolungata di azoto, mentre in un ceppo mutante sensibile alla puromicina abbiamo osservato gli aggregati in seguito del trattamento con questo inibitore. Abbiamo poi esaminato la localizzazione cellulare degli aggregati ubiquitinati in cellule wild type di *S. cerevisiae* grazie a microscopia a fluorescenza, osservando l'accumulo di tanti piccoli aggregati ricchi di ubiquitina dalla forma rotonda a seguito di stress acuto da etanolo. Tali aggregati risultano essere disseminati nel cytosol e non co-localizzati con altre strutture cellulari. Delle strutture simili, di forma arrotondata e disseminate nel cytosol, sono state osservate in condizioni di carenza prolungata da azoto, ma, in questo caso, tali aggregati apparivano più grandi in dimensioni e meno numerosi nella cellula. Ad ogni modo, le strutture ubiquitinate che abbiamo osservato rispecchiano le caratteristiche morfologiche riportate per i DALISs delle cellule dendritiche (Lelouard *et al.*, 2004).

Abbiamo poi deciso di esaminare come gli aggregati ricchi di ubiquitina indotti a seguito di stress acuto da etanolo vengano eliminati all'interno della cellula di lievito. Mentre è risaputo che l'eliminazione degli aggregati indotti in carenza da azoto avvenga per aggrefagia, il processo di autofagia dedicato allo smaltimento di aggregati proteici (Lamark and Johansen, 2012), non è noto quali meccanismi siano coinvolti nella rimozione degli aggregati proteici in condizioni di stress acuto da etanolo. Abbiamo dimostrato che l'accumulo di aggregati ricchi di ubiquitina è più persistente nelle cellule mutanti *atg1Δ* rispetto alle cellule wild type, in cui il livello di proteine ubiquitinate decresce qualche ora dopo l'esposizione allo stress. Ciò suggerisce che

il processo di autofagia sia coinvolto nella rimozione degli aggregati ubiquitinati, poiché Atg1 è una chinasi che possiede un ruolo fondamentale nella regolazione di tale processo (Papinski *et al.*, 2014). D'altro canto, abbiamo osservato che le cellule mutanti *cue5Δ* mostrano un minor accumulo di ubiquitina durante l'intera durata del trattamento con etanolo, rispetto alle cellule wild type. La proteina di lievito Cue5 lega l'ubiquitina e collabora con gli enzimi di coniugazione dell'ubiquitina Ucb4/Ubc5 e con l'ubiquitina ligasi Rsp5 per congiungere l'ubiquitina al suo substrato (Lu *et al.*, 2014). Dato che abbiamo osservato una minor presenza di ubiquitina nelle cellule mutanti *cue5Δ*, suggeriamo che Cue5 possa avere un ruolo nell'ubiquitinazione stessa degli aggregati osservati in condizioni di stress acuto da etanolo.

Introduction

PROTEIN STRUCTURE AND FOLDING

Immediately after being synthesized, proteins fold into their three-dimensional functional conformation, also called native state. The native state of a protein is generally thermodynamically favorable (Anfinsen, 1973), and the information to acquire it is contained within its amino acid sequence (Dobson, 2003).

Protein structure is arranged at four different levels. The primary structure is defined by the amino acid composition and their distribution inside the protein itself, which constitutes the backbone of the polypeptide. The secondary structure refers to local folded structures within the protein due to hydrogen bonds forming between carbonyl and amino groups on the backbone of each peptide, with the two main secondary structures being the α-helix and the β-sheet. The tertiary structure is the three-dimensional shape of the protein, which is due to the interactions between the different R groups of the amino acids present in the chain. The quaternary structure is only present in proteins made up of multiple polypeptide chains, or subunits, and it is defined by the overall organization of the single subunits (Stollar and Smith, 2020).

Protein folding is, therefore, delineated through sidechains and backbone connections via many weak interactions, such as van der Waals forces, hydrogen bonds, electrostatic or hydrophobic interactions, that promote each protein's unique conformation and finalize its functions (Stollar and Smith, 2020). Protein folding is fundamental, as it results in a broad variety of highly specific structures that promote a wide array of biological activities inside the cell, which are crucial for the life of the cell, and the organism itself. Indeed, proteins are the workhorses of all living cells: they

can exert structural functions, act as enzymes to catalyze chemical reactions, shuttle molecules to specific cellular locations, regulate cellular differentiation and growth, and many more essential functions (Gao *et al.*, 2020).

The stability of proteins is affected by high temperatures, extreme pH, mechanical forces, and chemical denaturants, which can cause the polypeptide to unfold or denature. These processes might be reverted with the help of molecular chaperons; however, this is not always possible, and defective proteins may lead to cellular malfunctions, with the consequent onset of different diseases (Horwich, 2002).

PROTEIN AGGREGATION IN DISEASES

Only correctly folded proteins are stable enough to pursue their biological functions. For this reason, the inability to fold protein correctly lies behind many different pathological conditions. A large number of newly synthesized proteins (up to 30%) is not functional, mainly due to errors during transcription, incorrect post-translational folding or modification, flawed mRNAs, translational frameshifting or environmental stress conditions (Pierre, 2005; Tyedmers *et al.*, 2010; Antón and Yewdell, 2014). Sometimes, the aggregated status is more thermodynamically favorable and stable than the native one (Fig. 1), as misfolded intermediates may present hydrophobic domains on their surfaces, making the assembly, and thus aggregation, a more energetically feasible process (Ajmal, 2023). In other cases, aggregation-prone proteins have increased aggregation rates in the presence of many proteins (Dasuri *et al.*, 2012).

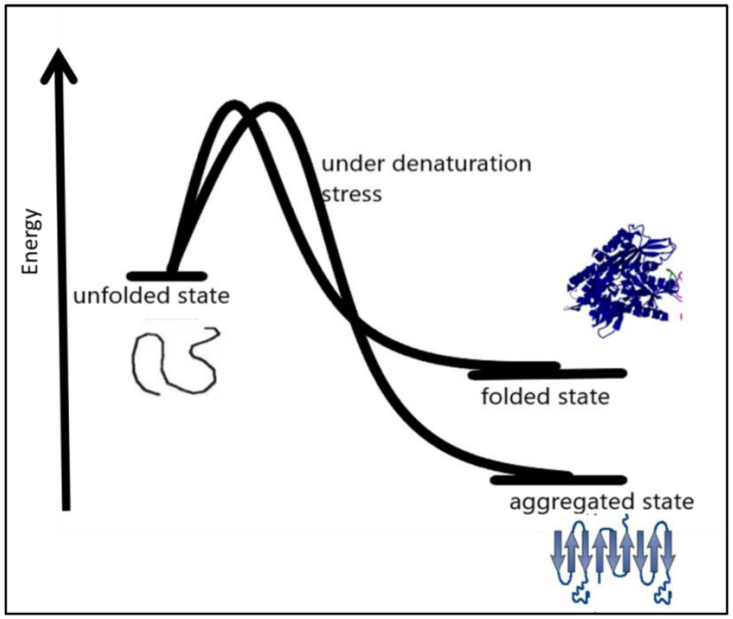

Figure 1. Under certain denaturation conditions, a protein moving into an aggregated state is more energetically favorable than the native state (Ajmal, 2023).

In any case, cells must employ several mechanisms to maintain and retrieve cellular proteostasis, using molecular chaperones able to rescue the newly synthetized Defective Ribosomal Products (DRiPs) and the damaged pre-existing ones, preserving polypeptide function through a mechanism of binding and release (Chen *et al.*, 2011; Kim *et al.*, 2013). However, misfolded protein recovery is not always possible, and when a massive amount of these proteins is produced in a short period of time, the proteolytic machinery becomes overloaded, not being able to properly rescue all the defective polypeptides (Goldberg 2003; Hartl and Hayer-Hartl, 2009; Tyedmers *et al.*, 2010; Chen *et al.*, 2011). The massive production of defective polypeptides can lead to protein aggregation, which might also be incremented by deficiencies in the proteasome, or inhibition of the autophagy systems (Klionsky *et al.*, 2021).

Many neurodegenerative diseases and disorders are characterized by a common pattern of abnormal misfolded proteins generation, which tend to accumulate and aggregate, therefore, representing the major issue for the onset of the disease (Koss *et al.*, 2022). For instance, age-related diseases are characterized by the presence of large inclusions of misfolded proteins which cause the loss of neuronal function in the brain (Hipp *et al.*, 2019). Most of these proteins belongs to the class of intrinsically disordered proteins (IDPs), which can be entirely unstructured, or hybrids of structured domains and long stretches of intrinsically disordered regions (Tsoi *et al.*, 2023). IDPs are common in eukaryotic cells and play important roles in cellular processes, therefore, a mutation in their structure may result in misfolding and aggregation (Oldfield and Dunker, 2014). In degenerating neurons, failures in inaccurately folded protein recovery and clearance are potentially toxic because they may generate amorphous aggregates and amyloid fibrils. Such aggregates and fibrils may induce damages to the lipid membranes and impair protein degradation, interfering with the proper function of the proteostasis network, and can possibly lead to cell death (Labbadia and Morimoto, 2015; Chiti and Dobson, 2017; Klaips *et al.*, 2018; Hipp *et al.*, 2019; Kurtishi *et al.*, 2019). Each inclusion, or aggregate, is identified by a peculiar protein composition, solubility, stability, and cellular localization. For example, Lewy bodies, the hallmark of Parkinson's disease, are composed mainly of misfolded and truncated α-synuclein, causing synaptic dysfunction and neuronal loss (Dickson, 2018; Brás and Outeiro, 2021). On the other hand, protein inclusions of Alzheimer's disease consist of aggregated amyloid-β (Aβ) peptide, which accumulate and deposit in extracellular plaques that interfere with neuronal communication in the brain (Reiss *et al.*, 2018), and neurofibrillary tangles (NFTs), mainly composed of the intrinsically disordered protein Tau, a microtubule-associated protein, which plays a key role in

modulating neuronal physiology (Tabeshmehr and Eftekharpour, 2023). Finally, TAR DNA-binding protein 43 (TDP-43) aggregation is the hallmark of amyotrophic lateral sclerosis (ALS) in neuronal cells, where it accumulates in the cytoplasm, causing axonal swelling and mobility impairment (Gambino *et al.*, 2023).

Protein aggregation is also at the basis of non-neurological diseases, such as cystic fibrosis. Cystic fibrosis is a hereditary disease that affects lungs and the digestive system, it stimulates the production of thick and sticky mucus that can clog the lungs and obstruct the pancreas, preventing proper food processing. The massive production of mucus is a consequence of protein misfolding, due to mutations at critical loci, which leads to defects in protein function of the cystic fibrosis transmembrane conductance regulator (CFTR), resulting in a life-threatening disease (Cheung and Deber, 2008).

In general, the common pattern that brings together these diseases is the abnormal generation of misfolded proteins which tend to accumulate and aggregate due to the many previously mentioned factors.

PROTEIN AGGREGATES IN HEALTHY CELLS

Alterations in the cellular physiological environment are quite common, therefore, cell's adaptation to the new condition is essential to a successful survival. Stress responses are highly conserved through different eukaryotic organisms and range from the activation of pathways that help the cell fighting the insult and to recover the physiological status, to the stimulation of cell death to eliminate the damaged cells. Furthermore, changes in the proteome are among the most common and conserved stress responses (Crawford and Pavitt, 2019), in particular, the coalescence of

proteins into large multimeric intracellular bodies is widely adopted by both prokaryotic and eukaryotic cells (Villaverde *et al.*, 2015; Rueda *et al.*, 2016; Riggs *et al.*, 2020). Indeed, the perturbation of the cellular environment is one of the factors that generate misfolded proteins. Protein aggregation is an organized process frequently employed by healthy cells to control misfolded proteins and to avoid them from becoming toxic to the cell (Saarikangas and Barral, 2016). Protein aggregates function as deposition sites, with no specific compartmentalization, in which different classes of proteins accumulate (Tyedmers *et al.*, 2010). Hence, the majority of these aggregates, rather than being a manifestation of cell damage, represent an adaptive response that helps the cell to protect its proteome from proteins which cannot promptly be rescued by the overloaded proteolytic machinery (Gallardo *et al.*, 2021; Muscolino *et al.*, 2021).

A particular feature of these species of inclusion bodies is that most of them can be reactivated by chaperones during the recovery phase. For example, when the proteasomes are overloaded, ubiquitylated misfolded proteins can be reversibly stored into the juxtanuclear quality-control compartment (JUNQ), in the waiting of becoming substrates for proteasomal degradation (Kaganovic *et al.*, 2008). Another interesting characteristic is that different types of aggregates can coexist within a single cell, sometimes interacting with each other (Kroschwald *et al.*, 2015; Jain *et al.*, 2016), and occasionally even controlling the formation of other inclusion bodies. For instance, the inhibition of processing bodies in yeast cells prevents the formation of stress granules, whose components gather around preassembled processing bodies (Buchan *et al.*, 2008). Moreover, they are demonstrated to interact with each other, establishing a process, called mRNA cycle (Fig. 2), wherein mRNAs are exchanged between polysomes and the two deposition sites (Kedersha and Anderson, 2009; Decker and Parker, 2012 Grousl *et al.*, 2021).

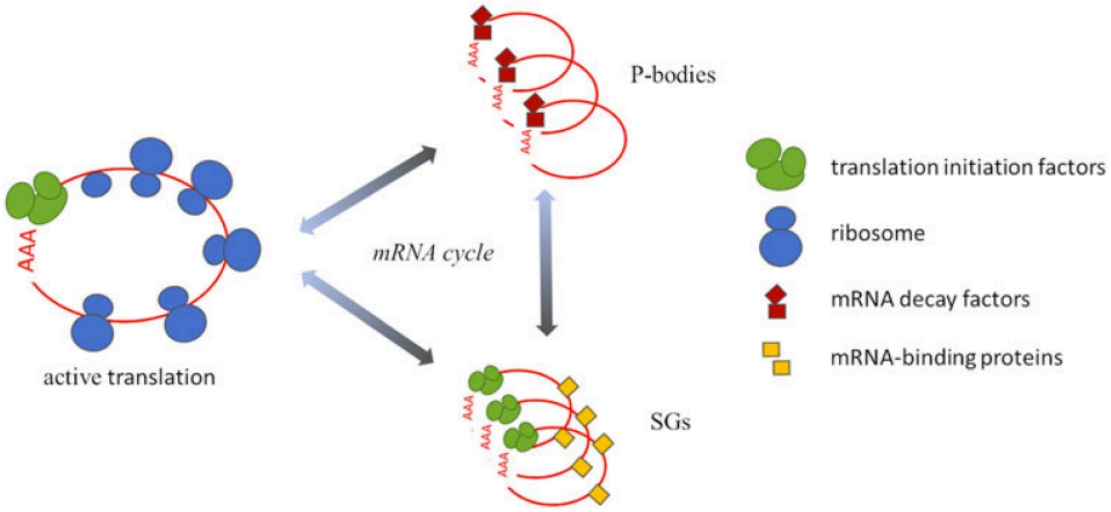

Figure 2. The mRNA cycle model (Grousl *et al.*, 2021).

The organization of proteins into the deposition sites usually begins with changes in the surrounding environment or in intracellular activities (Weber and Brangwynne, 2012). Depending on the signal, the proteins involved can vary from one or a few species, to hundreds, which merge together into one or more aggregated bodies. This entire process is assisted by the activation or inactivation of cellular processes, such as gene expression, through a vast co-ordination of intracellular events, which allows cells to adapt and face unfamiliar conditions (Berchowitz *et al.*, 2015; Ho and Gash, 2015; Weber and Brangwynne, 2012).

DENDRITIC CELL AGGRESOME-LIKE INDUCED STRUCTURES (DALISs)

Dendritic cell Aggresome-Like Induced Structures (DALISs) are mammalian protein aggregates which function as transient deposition sites for ubiquitinated defective proteins in dendritic cells in response to several stresses, including exposition to microbial products (e.g., bacterial lipopolysaccharide), fever-like temperatures, nutritional stress, and treatment with protein synthesis inhibitors (Pierre, 2005). Their

main components are newly synthetized defective ribosomal products (DRiPs), other than ubiquitin and ubiquitin enzymes (Lelouard *et al.*, 2004). They are dynamic structures, as they can freely interact with each other and with other cytoplasmic elements (Lelouard *et al.*, 2004; Szeto *et al.*, 2006), and their clearance is regulated by autophagy and by the proteasome (Szeto *et al.*, 2006; Wenger *et al.*, 2012). The formation of DALISs in the cytosol of dendritic cells overlaps with their maturation and immune activation (Herter *et al.*, 2005). Only a little is known about the functions of these aggregates, but they have been proposed to delay antigen degradation and presentation by activated dendritic cells (Faßbender *et al.*, 2008; Antón and Yewdell, 2014; Argüello *et al.*, 2016), as they protect DRiPs, a major source of self and viral antigenic peptides for MHC-I-restricted presentation (Yewdell, 2002), from proteasomal degradation, guaranteeing them a longer half-life compared to their non-aggregated counterparts (Lelouard *et al.*, 2004; Argüello *et al.*, 2016). DALISs have been detected also in macrophages (Fig. 3) and in non-immune cells following the exposure to several environmental stresses (Szeto *et al.*, 2006), however, their role in these cellular types is still shrouded in mystery.

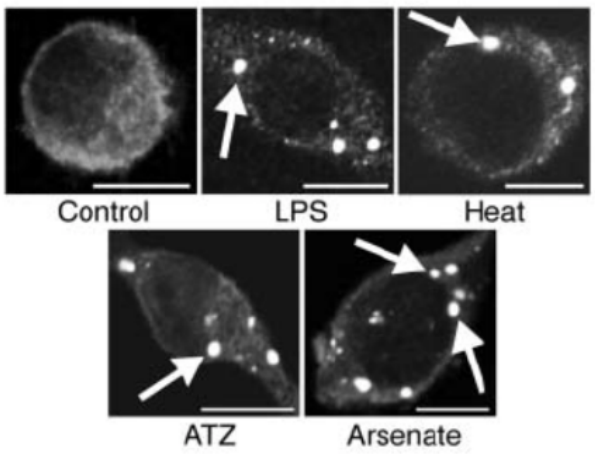

Figure 3. Stress induced aggresome-like induced structures (ALISs) in murine macrophages. ALIS are indicated by the white arrow, size bar 10 μm (Szeto *et al.*, 2006).

Until today, no investigation has been conducted on other eukaryotic models to explore the physiological functions of DALISs. We propose the yeast *Saccharomyces cerevisiae* as a suitable model.

YEAST AS A USEFUL MODEL TO STUDY PROTEIN AGGREGATION

The formation of protein aggregates is a conserved process engaged by eukaryotic cells in response to external stimuli that alter the physiological environment. The yeast *Saccharomyces cerevisiae* is widely used for the research on cellular aggregates, and it is recognized as an ideal system to investigate fundamental processes, being employed in the fields of functional genomics, systems biology and molecular medicine, with the enormous potential of unravelling the complex mechanisms and pathways underlying aging and neurodegenerative disorders (Miller-Fleming *et al.*, 2008; Di Gregorio and Duennwald, 2018). The conservation degree between mammalian and yeast cells, regarding genomic and amino acid sequences and protein function, is high enough to establish that information can be transferred from a eukaryotic species to another (Nielsen, 2019). Indeed, many mammalian genes have a yeast homolog, making it possible to study the gene function directly, otherwise, heterologous expression of the human gene in yeast cells allows the study of other mammalian genes (Tenreiro and Outeiro, 2010). Moreover, several important features of neurodegenerative disorders, including protein aggregation, are faithfully reproduced in yeast, enabling researchers to identify novel candidates of therapeutic targets and drugs for pathologies such as Huntington's and Parkinson's diseases (Outeiro and Muchowski, 2004; Outeiro and Giorgini, 2006; Menezes *et al.*, 2015;

Tenreiro *et al.*, 2017). Therefore, the employment of this model system allowed to accelerate the identification of several conserved proteins, as well as to shed light into the physiological roles of many cellular granules, including stress granules and processing bodies (Sheth and Parker, 2003; Buchan *et al.*, 2008; Grousl *et al.*, 2013; Wang *et al.*, 2018), which are ubiquitously present in all eukaryotic cells (Buchan *et al.*, 2008; Kedersha and Anderson, 2009).

STRESS CONDITIONS THAT STIMULATE AGGREGATES FORMATION IN *SACCHAROMYCES CEREVISIAE*

In *Saccharomyces cerevisiae*, the formation of large cytoplasmic bodies is manifested following the exposure to a wide range of external stimuli. Such stress conditions are heat shock temperatures, ethanol addition, nitrogen depletion and glucose starvation. Heat shock temperatures stimulate the termination of translation, with a subsequent accumulation of the components of the translational machinery into cytoplasmic granules (Grousl *et al.*, 2013). Heat shock causes changes in the fluidity of the plasma membrane, by modifying the protein composition and increasing its permeability (Piper, 1995). Temperatures as high as 42°C lead to the generation of misfolded protein, protein aggregation (Grousl *et al.*, 2013), and the synthesis of heat shock proteins, which were shown to concentrate in Q-bodies (Escusa-Toret *et al.*, 2013).
Ethanol stress also alters the fluidity of the plasma membrane, changing its permeability for ions, and generates protein dysfunction, increasing the levels of denatured polypeptides and inducing the accumulation of insoluble proteins (Ma and Liu, 2010; Kato *et al.*, 2019). The treatment with ethanol at sublethal doses (4-10%

(v/v)) is known to cause the denaturation of pre-existing proteins, and the generation of misfolded protein and protein aggregates (Piper, 1995; Escusa-Toret *et al.*, 2013). Nitrogen starvation triggers the reorganization of transcriptional, translational, and post-translational processes. In particular, it causes the activation of GCN2 (General Control Non-derepressible 2), a kinase involved in the phosphorylation of eIF2a. This phosphorylation leads to the stalling of translation initiation, which results in polysome disassembly and in the aggregation of reversible protein assemblies, such as stress granules and processing bodies (Kedersha and Anderson, 2009). The lack of nitrogen sources also leads to the decrease of ribosome biogenesis and to the induction of cell cycle arrest to reduce cellular size (Hoyle *et al.*, 2007). Moreover, it stimulates the turnover of nonessential nitrogen-containing macromolecules to restore intracellular amino acid homeostasis (Rødkær and Færgeman, 2014).

In a similar way, glucose removal inhibits translation initiation, causing initiation factors redistribution into reversible membraneless inclusions (Rødkær and Færgeman, 2014). Glucose depletion induces the accumulation of transient proteasome-storage granules (PSGs), which are dismembered upon glucose restoration, and that act as storage of proteasome subunits (Karmon and Ben Aroya, 2020).

Normally, the protein assemblies formed under these stress conditions are reversible. Their disassembly involves either the disruption of the interactions between the components, or the degradation of the components themselves (Fernandes *et al.*, 2018). In any case, the clearance of the inclusion bodies usually occurs after stress alleviation (Cherkasov *et al.*, 2013) and it is often aided by molecular chaperones (Walters *et al.*, 2015).

STRESS-INDUCED PROTEIN AGGREGATES IN YEAST

In yeast cells, misfolded polypeptides accumulate in many different protein inclusions, such as stress granules, P-bodies, juxtanuclear quality control compartments (JUNQ) and intranuclear quality control compartments (INQ), insoluble protein deposits (IPOD), age-associated protein deposits (APOD), Q-bodies and Whi3 super-assemblies (Fig. 3; Saarinkangas and Barral, 2016). However, only the formation of stress granules (SGs), P-bodies and Q-bodies is induced by stress conditions (Fig. 4).

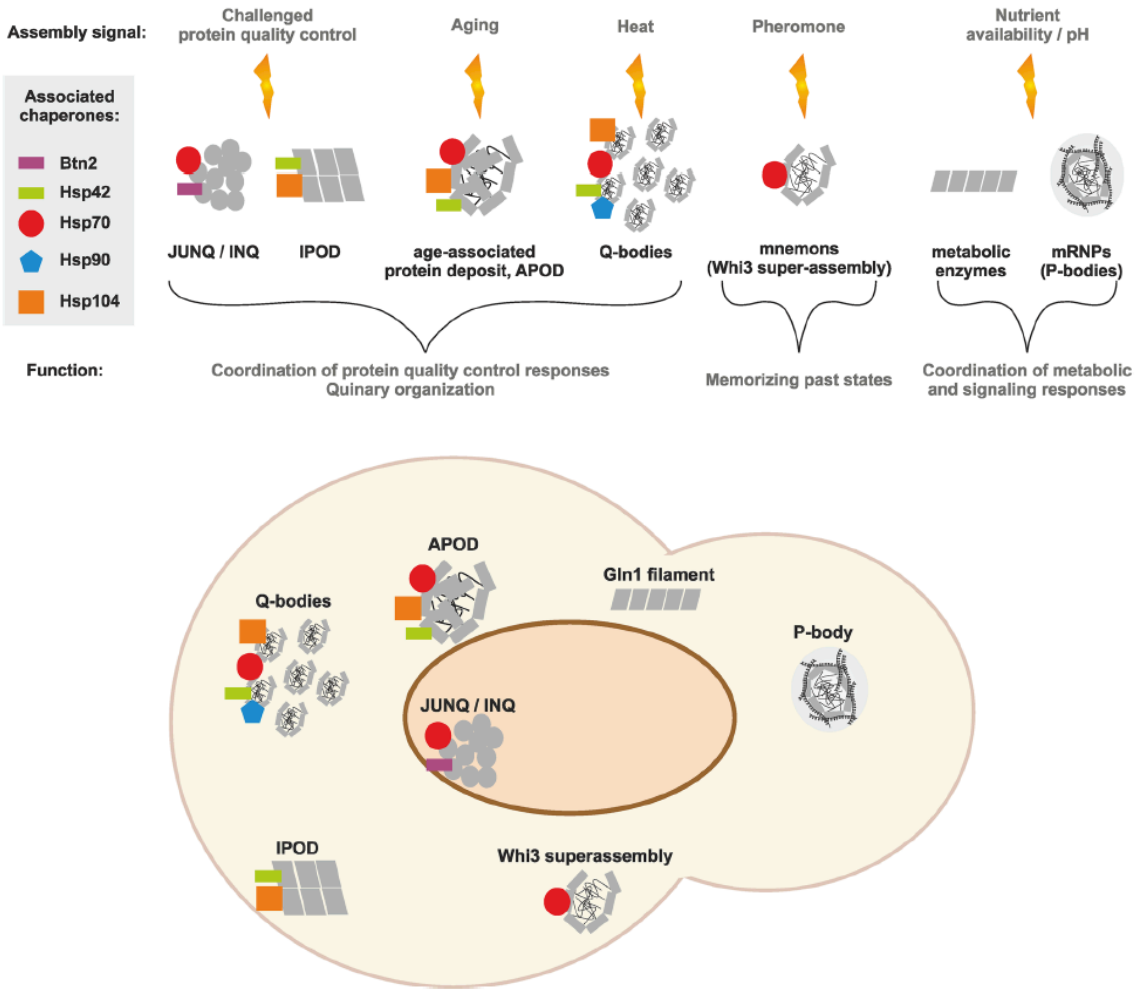

Figure 4. Protein aggregates in yeast and their location inside the cell. Only RNP bodies and Q-bodies are induced by stress conditions (Saarinkangas and Barral, 2016).

Stress granules and P-bodies are ribonucleoprotein (RNP) bodies. Their formation is one of the most conserved responses carried out by yeast cells to many stress stimuli (Kedersha and Anderson, 2009). They contain polyadenylated mRNAs stalled in translation initiation (Ivanov *et al.*, 2019), translation initiation factors (Kedersha *et al.*, 2005; Buchan and Parker, 2009), and many RNA and non-RNA binding proteins (Kedersha *et al.*, 2005; Hoyle *et al.*, 2007). Both these aggregates are transient bodies, as they quickly disassemble upon stress removal (Wheeler et al., 2016; Wang et al., 2019), with a distinct composition and structure, but they are spatially, compositionally, and functionally linked. Indeed, they are both implicated in the storage and decay of RNA, playing a crucial role in the control of gene expression (Kedersha *et al.*, 2005; Kedersha and Anderson, 2009; Buchan *et al.*, 2011; Decker and Parker, 2012). Stress granules are typically associated with prolonged and severe forms of stress, such as heat shock, oxidative stress, and disruption of polysomes. On the other hand, P-bodies are formed under glucose depletion, heat shock temperatures and oxidative stress (Kedersha and Anderson, 2009). SGs assembly appears to follow the formation of processing bodies, as their components gather around preassembled P-bodies, moreover, the inhibition of P-bodies prevents the formation of SGs, but not *vice versa* (Buchan *et al.*, 2008). The interaction between P-bodies and stress granules suggests a cytoplasmic mRNP cycle wherein mRNAs are exchanged between polysomes, P-bodies and SGs (Kedersha and Anderson, 2009; Decker and Parker, 2012). Indeed, SGs are thought to function as *triage* sites, where mRNAs are stored and assigned to translation or degradation, hence having a pivotal role in the regulation of RNA homeostasis under stressful conditions (Anderson and Kedersha, 2008; Anderson and Kedersha, 2009; Vanderweyde *et al.*, 2013).

Another species of yeast aggregates are Q-bodies. They are induced by heat shock temperatures and ethanol addition and are described as transient amorphous protein clusters which help the cell deal with prolonged stress exposure (Escusa-Toret *et al.*, 2013). Their composition seems to vary depending on the stress stimulus and the physiology of the cell, but usually they are associated with the endoplasmic reticulum membrane and are degraded by the proteasome (Fang *et al.*, 2014).

Ubiquitin-rich structures equivalent to the mammalian DALISs have never been detected in yeast, however, the conservation degree between mammalian and yeast cells encourages the idea of yeast as an extremely suitable model to conduct a preliminary study on these cytoplasmic protein aggregates.

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Thesis scope and outline

The aim of the present study is to investigate the formation of stress-induced ubiquitin-rich protein aggregates, similar to mammalian DALISs, in the yeast *Saccharomyces cerevisiae*.

In the first section of the thesis, named "*Stress-induced ubiquitin-rich aggregates: identification and visualization in the yeast Saccharomyces cerevisiae*" and realized at the University of Milan Bicocca in Milan, Italy, we focused on the study of four different stress conditions, heat shock temperatures at 42°C, severe ethanol stress, nitrogen depletion and glucose starvation, which were previously shown to stimulate the formation of protein aggregates in the yeast *Saccharomyces cerevisiae*. We also included the treatment with 200 μM puromycin, as this drug was demonstrated to induce the formation of DALIS in mammalian dendritic cells. We demonstrated the formation of ubiquitin-rich protein aggregates in *S. cerevisiae* cells following the exposure to severe ethanol stress, under nitrogen starvation conditions, following the treatment with 200 μM puromycin. Fluorescence microscopy analysis allowed to observe how these stress conditions induce the accumulation of round-shaped ubiquitin-rich aggregates, which are disseminated in the cytosol and not colocalized with any cellular structure.

In the second chapter of the thesis, named "*The role of Atg1 and the ubiquitin- and Atg8-binding protein Cue5 in ethanol-induced ubiquitin-rich aggregates clearance and ubiquitination*" and executed at the Georgia State University in Atlanta, GA, USA, we investigated how ethanol-induced ubiquitin-rich protein aggregates are cleared inside *S. cerevisiae* cells. We demonstrated a durable accumulation of ubiquitin-rich aggregates in *atg1Δ* mutant cells, in contrast to the decrease of wild type cells a few hours following the exposure to the stress. We also showed how *cue5Δ* mutant cells

displayed a minor accumulation of ubiquitin throughout the stress condition, compared to wild type cells. Based on our results, we suggest autophagy is implicated in the clearance of ubiquitinated aggregates, as Atg1 regulates the process, whereas we propose that the yeast protein Cue5 is involved in the ubiquitination of ethanol-induced ubiquitin-rich aggregates.

Stress-induced ubiquitin-rich aggregates: identification and visualization in the yeast *Saccharomyces cerevisiae*

INTRODUCTION

By prevailing opinion, protein aggregation represents a dangerous phenomenon, being associated to age-related neurodegenerative disorders, such as Alzheimer's or Parkinson's Diseases (Hipp *et al.*, 2019). Nonetheless, this event is not always a synonym of cellular failure or cell death. Indeed, protein aggregates function as well-organized deposition sites which help the cell to protect its proteome from large amounts of misfolded polypeptides generated in response to external stresses, in the waiting for the restoration of the physiological condition. In this context, Dendritic cells Aggresome-Like Induced Structures (DALISs) function as transient deposits for ubiquitinated defective proteins and antigens (Lelouard *et al*., 2004). However, their function is still not completely understood, and no investigation to identify these aggregates in other eukaryotic cells has ever been conducted.

The yeast *Saccharomyces cerevisiae* is a well-established and powerful model organism to study cytoplasmic aggregates, as it provided interesting insights on their physiological roles (Decker and Parker, 2012). Moreover, many pathways that are relevant for pathological conditions in mammalian cells, including protein folding, proteostasis and stress response, are conserved in yeast (Longo *et al*., 2012).

We identified four main stress conditions under which the yeast *Saccharomyces cerevisiae* manifests the formation of large cytoplasmic bodies, namely heat shock temperatures at 42°C, severe ethanol stress, nitrogen depletion and glucose starvation. Heat shock temperatures stimulate the termination of translation, leading to the generation of misfolded protein and protein aggregation (Grousl *et al*., 2013); ethanol stress causes the denaturation of existing proteins, generating misfolded protein and protein aggregates (Piper, 1995; Escusa-Toret *et al*., 2013); nitrogen starvation triggers the stalling of translation initiation, with the subsequent aggregation of reversible protein assemblies (Kedersha and Anderson, 2009) and induction of cell cycle arrest to reduce cellular size (Hoyle *et al*., 2007); glucose removal inhibits translation initiation, causing initiation factors redistribution into reversible membraneless inclusions (Rødkær and Færgeman, 2014). We also included the treatment with 200 μM puromycin, as this drug was demonstrated to induce the formation of DALIS in mammalian dendritic cells (Lelouard *et al*., 2004).

In the present study, we examine the formation of stress-induced ubiquitin-rich protein aggregates following the exposure to the previously mentioned stress stimuli, with the purpose of investigating whether DALIS-equivalent structures form in *Saccharomyces cerevisiae* cells.

Strains and Media

Yeast strains with a BY4741 background were used in this study (Table 1).

Mutant	Strain	Background	Genotype	Source
WT	BY4741	BY4741	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachman *et al.*, 1998
pdr1Δ	YAD336	BY4741	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pdr1Δ::KanMX4*	Cary *et al.*, 2014
pdr3Δ	YAD521	BY4741	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pdr3Δ::KanMX4*	Cary *et al.*, 2014
erg6Δ	YAD522	BY4741	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 erg6Δ::KanMX4*	Cary *et al.*,2014
pdr1Δ pdr3Δ	YAD271	BY4741	MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pdr1Δ::Nat pdr3Δ::hphMX*	Cary *et al.*, 2014
EPP	14339	BY4741	MAT**a** *met15Δ0 leu2Δ0 met15Δ0 ura3Δ0 erg6Δ::LEU2 pdr1Δ*::natMX *pdr3Δ*::hphMX	Cary *et al.*, 2014

Table 1. *S. cerevisiae* strains used in this study

Cells were grown in batches in Yeast Extract Peptone Dextrose (YPD) medium (2% glucose, 1% yeast extract, 2% peptone) or in minimal medium (6.7 g/L Difco Yeast Nitrogen Base (YNB), 2% glucose, 200 mg/L Histidine, 200 mg/L Uracil, 400 mg/L Leucin, 400 mg/L Lysin, 400 mg/L Methionine) at 30°C at 150 rpm.

Cell growth was monitored by determining cell number using a Coulter Counter-Particle Count and Size Analyser, as described (Vanoni *et al*., 1983). Duplication time was obtained by linear regression of the cell number increase over time on a semi-logarithmic plot. Puromycin (EMD Millipore Corp.) was prepared by dissolving the puromycin dihydrochloride powder in sterile water to a concentration of 20 mM and added to growth media to achieve the final concentrations listed. Puromycin was added to YPD agar plates prior to pouring.

Exponentially growing cells in YPD medium were supplemented with ethanol 10% (v/v) for 3 hours or incubated at 42°C for 6 hours. For nitrogen depletion, mid-log phase cells were harvested, medium was discarded by centrifugation and pellet resuspended in starvation-medium (2% glucose, 1.7 g/L Difco YNB w/o ammonium sulphate and amino acids) and incubated at 30°C at 150 rpm for 24 hours. For glucose starvation, exponentially growing cells were harvested, medium was discarded by centrifugation and pellet resuspended in starvation-medium (6.7 g/L Difco YNB without amino acids, 200 mg/L uracil, 200 mg/L histidine, 400 mg/L leucin, 400 mg/L lysin, 400 mg/L methionine) and incubated at 30°C at 150 rpm for 24 hours. At designated time-points, aliquots of the yeast cultures were harvested and centrifuged, cell pellets collected were frozen at −20°C until protein extraction was performed.

All experiments were done at least in triplicate.

Insoluble Aggregates Extraction

Insoluble aggregated proteins were analyzed adapting the protocol of Kato *et al.* (2019). Briefly, cells were treated with Zymolyase 20T (2.5 mg/mL) at room temperature for 20 minutes and disrupted by vortexing with glass beads in Lysis Buffer (50 mM PBS, 1.0 mM EDTA, 5% glycerol, pH 7.0) containing PMSF (17.4 mg/ml in EtOH). Cell organelles and membranes were removed by centrifugation at 5'000 g for 20 minutes, and the total protein concentration of each sample was measured using the Coomassie (Bradford) Protein Assay Kit (Thermo Scientific). Insoluble aggregated proteins were obtained by centrifugation at 16'000 g for 20 minutes, membrane proteins were removed washing twice with Nonidet P-40 (2% in Lysis Buffer w/o EDTA), and aggregates were resuspended in Buffer A (5.0 mM MgCl2, 10 mM Tris–HCl, pH 4.0). Insoluble aggregates were digested with RNase A (4 mg/mL in Buffer A, Roche Diagnostic) and DNase I (0.5 mg/mL in Buffer A, Roche Diagnostic), incubating in ice for 10 minutes, to digest and remove nucleic acids, and solubilized in Urea Buffer (50 mM Tris–HCl, 6.0 M urea, 5% SDS, pH 7.5).

All experiments were done at least in triplicate.

Insoluble Aggregates Analysis

Equal amounts of proteins (30 µg) of each extract were resolved by SDS-PAGE on 8% or 10% polyacrylamide gels. Pre-stained protein markers (range 12-225 kDa, Amersham ECL Rainbow Marker – Full Range) were also loaded.

Silver staining. After electrophoresis, 10% polyacrylamide gel slabs were fixed in 50% methanol, 10% acetic acid for 30 minutes and for an additional 30 minutes with 5% methanol, 7% acetic acid. Gels were washed overnight in water and rinsed in the

morning with fresh water for 30 minutes. Gels were incubated with 5 µg/ml dithiothreitol (DTT) for 30 minutes, then with 0.1% silver nitrate in the dark for 30 minutes. After a wash with water and two washes with the development solution, the gels were submerged in the development solution (3% Na_2CO_3, 0.05% CH2O in water). As the protein bands reached the desired intensity, the development was terminated by adding 2.3 M citric acid and leaving the gels in agitation for 30 minutes. The gels were then washed for 30 minutes in fresh water and stocked in 2% glycerol. Silver staining was used to visualize total protein patterns.

Western Blot analysis. Gels at 8% polyacrylamide were blotted onto nitrocellulose blotting membranes (Amersham Protran 0.45 mm), a constant current of 150 mA for 2 hours. Filters were washed in TBS (0.01 M Tris, 0.9% NaCl, pH 7.4) followed by 1 hour 30 minutes saturation in TBS containing 0.1% Tween 20 and 10% skimmed milk. Immunodetection was performed overnight using anti-ubiquitin monoclonal antibody (IgG mouse clone P4D1, Enzo Life Sciences) at 1:1'000 dilution in TBS containing 0.1% Tween 20 and 5% skimmed milk. After 3 washes of 30 minutes each in TBS and 0.2% Tween 20, membranes were incubated with secondary anti-mouse antibody (ECL anti-mouse IgG Horseradish Peroxidase-Linked Species-Specific Whole Antibody, Amersham), diluted 1:20'000 in TBS, 0.1% Tween 20, 2% skimmed, milk for 2 hours. After 3 washes of 15 minutes each in TBS and 0.3% Tween 20, binding was visualized using ECL Western Blotting Detection Reagent (Amersham), followed by acquisition with the ChemiDoc Imaging System (BioRad).

All experiments were done at least in triplicate.

Fluorescence Microscopy

Cells were pre-fixed adding 3.7% formaldehyde in the growth medium and incubating for 10 minutes at room temperature. After elimination of the medium, cells were fixed with 3.7% formaldehyde in 100 mM PBS, pH 6.5 for 30 minutes at room temperature, followed by 3 washes with 100 mM PBS and incubation with Zymolyase 20T for 90 minutes in Digestion Buffer (1.2 M sorbitol, 0.12 M K2HPO4, pH 5.9) to digest the glucans of the cell wall. After 2 washes with 100 mM PBS to discard the enzyme, cells were incubated for 3 hours with anti-ubiquitin monoclonal antibody (IgG mouse clone FK2, Calbiochem) at 1:500 dilution in 100 mM PBS, 0.025% Tween 20, 1 mg/ml BSA, pH 6.5. After 3 washes with 100 mM PBS, cells were incubated for 2 hours with secondary antibody Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) at 1:500 dilution in 100 mM PBS, 0.025% Tween 20, 1 mg/ml BSA, pH 6.5. Cells were visualized using a Nikon Eclipse E600 fluorescence microscope equipped with a Nikon Digital Sight DS Qi1 camera and FITC filter (wavelength 467 – 556 nm) for the visualization of ubiquitinated proteins. Digital images were acquired and processed using Nikon software NIS-Elements.

All experiments were done at least in triplicate.

RESULTS

Ethanol Stimulates the Formation of Ubiquitin-Rich Aggregates in S. cerevisiae

Ethanol treatment at sublethal doses (4-10% (v/v)) is known to cause the denaturation of pre-existing proteins, and the generation of misfolded protein and protein aggregates (Piper, 1995; Escusa-Toret *et al*., 2013), however, no further analysis of this aggregates has been conducted. In yeast cells, many different protein inclusions generate following a wide range of stress stimuli (Saarinkangas and Barral, 2016), therefore, it is important to understand under which category ethanol-induced aggregates should be allocated. One of the most conserved responses carried out by yeast cells to many stress stimuli is the formation of ribonucleoprotein (RNP) aggregates, composed by mRNAs and RNA-binding proteins, such as stress granules and processing bodies, or P-bodies (Saarikangas and Barral, 2016). To evaluate the presence of inclusions other than P-bodies and stress granules under conditions of ethanol-induced stress, we initially looked for nucleic acid-free aggregates. Therefore, protein extracts were digested with RNase A and DNase I, to the purpose of eliminating the nucleic acid, which we suggest can dispose the disaggregation of RNP bodies. As shown in Fig. 1, the treatment with 10% ethanol (v/v) clearly induced the accumulation of insoluble proteins. The total protein stain (Silver stain) allowed to appreciate the differences in the patterns, showing a clear reduction in detectable proteins following the digestion with nucleases. This decrease is consistent with the expected elimination of the RNP bodies. We then observed the presence of ubiquitinated proteins, through Western Blot analysis (Fig. 1c), using the anti-ubiquitin monoclonal antibody P4D1 (Enzo Life Sciences). Importantly, while the control,

represented by untreated cells, displays no ubiquitin, in the samples obtained from ethanol-stress cells we detected a ubiquitinated pattern, which is revealed even in the fractions treated with the nucleases, suggesting the presence of ubiquitinated aggregates is independent from the nucleic acids. Escusa-Toret *et al.* (2013) revealed that under ethanol stress misfolded proteins coalescence into large inclusion bodies, reported by the research group as Q-bodies, which appear to function as a storage for different misfolded and stress-denatured proteins *en route* to degradation, as they demonstrated the colocalization with Ubc9ts (Escusa-Toret *et al.*, 2013). Our results support the data of Escusa-Toret *et al.*, demonstrating that under severe ethanol stress ubiquitin plays an important role in the guidance of misfolded proteins. However, no evidence suggests that the ubiquitinated aggregates we identified can be categorized as Q-bodies, as Q-bodies are known to accumulate following heat shock temperatures as well (Escusa-Toret *et al.*, 2013), and to be associated with the endoplasmic reticulum membrane (Fang *et al.*, 2014); such characteristics contrast with what we observed for the aggregates we identified (see below).

In 2019, Kato and collaborators described how ubiquitinated insoluble aggregates accumulate under severe ethanol stress in a manner similar to the ones that aggregate following heat shock temperatures. Our data are consistent with what they obtained following the treatment with 10% ethanol (v/v), however, we reported different results regarding heat shock temperatures (see paragraph “Heat Shock Temperatures Stimulate the Formation of Protein Aggregates Associated to Nucleic Acids”). The digestion with nucleases that we performed on the extracted insoluble aggregates describes different characteristics between the ethanol- and heat shock-induced aggregates, highlighting how the presence of nucleic acids can affect the accumulation of different species of aggregates.

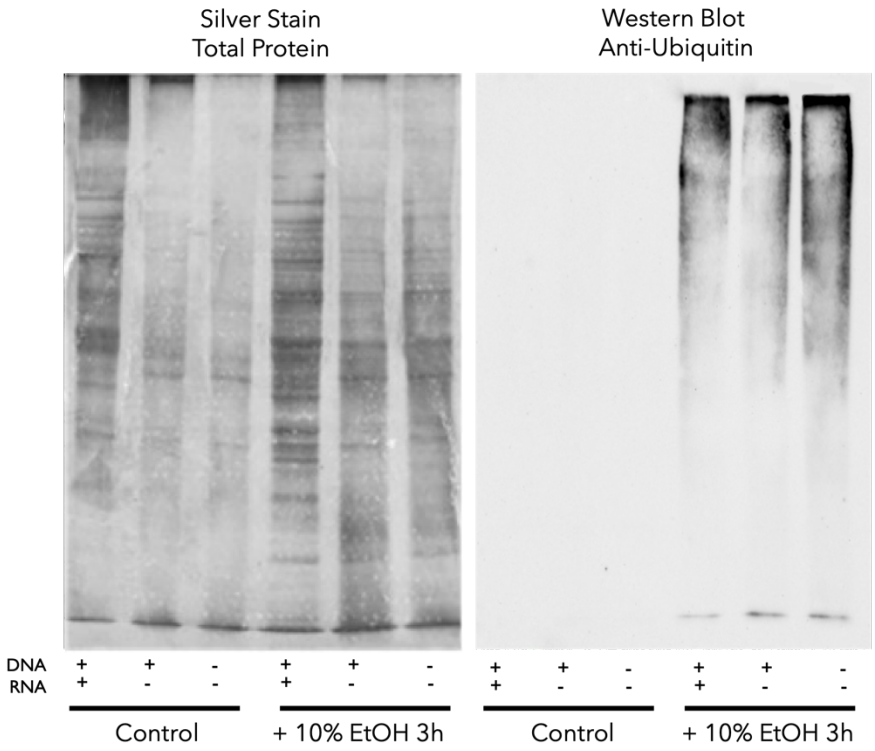

Figure 1. BY4741 cells were treated ethanol 10% (v/v) for 3 hours in YPD medium. Insoluble aggregated protein in yeast cells were extracted before (control) and after (+ 10% EtOH 3h) the ethanol stress treatment. The accumulation of ubiquitinated protein is observable after the addition of 10% ethanol for 3 hours, and it is persistent even after the digestion of nucleic acids with nucleases, suggesting nucleic acids are not a component of the aggregates. Samples were separated using 10% polyacrylamide gel electrophoresis and visualized by Silver staining for total protein levels. Ubiquitinated proteins were detected by Western Blot analysis using the anti-ubiquitin monoclonal antibody P4D1 (dilution 1:1'000) on 8% polyacrylamide gel. For all the gels, loaded 30 µg of each sample.

Heat Shock Temperatures Stimulate the Formation of Protein Aggregates Associated to Nucleic Acids

Heat shock causes similar changes in the plasma membrane to the ones induced by ethanol stress, modifying the protein composition and increasing its permeability

(Piper, 1995). Likewise, heat shock at 42°C causes the generation of misfolded protein, protein aggregation (Grousl *et al.*, 2013), and the synthesis of heat shock proteins, which were shown to concentrate in Q-bodies together with Ubc9ts (Escusa-Toret *et al.*, 2013). For this reason, we exposed *S. cerevisiae* cells to heat shock at 42°C and we applied the extraction protocol for insoluble protein. As described for ethanol stress, the incubation at 42°C induced the accumulation of insoluble proteins (Fig. 2) and Silver staining for total proteins highlighted the difference between the total protein fraction and the aggregates fractions, indicating that the digestion carried out by the nucleases led to the disaggregation of the RNP bodies. The Western Blot analysis, however, marked the presence of ubiquitinated protein only prior to the digestion of RNA and DNA. This result contrasts with what Kato and collaborators observed regarding the accumulation of ubiquitinated insoluble protein in yeast following heat shock at 42°C for 1 hour (Kato *et al.*, 2019). We suggest that the aggregates formed under this stress condition also include RNA and, therefore, are dismembered after the treatment with the nucleases. Indeed, it is known that heat shock temperatures increase the levels of phospho-eIF2α and shut down the translational machinery, leading to the generation of stress granules, a type of RNP bodies, which do not include ubiquitinated proteins, but rather RNA and RNA-binding proteins (Grousl *et al.*, 2021). Moreover, Escusa-Toret and collaborators reported that, following heat shock temperatures, misfolded proteins coalescence into Q-bodies, depositions sites for misfolded and stress-denatured proteins *en route* to degradation, as they colocalize with Ubc9ts. As for stress granules, such aggregates probably include RNA and RNA-binding proteins and, therefore, are dismembered after the digestion of nucleic acids with nucleases.

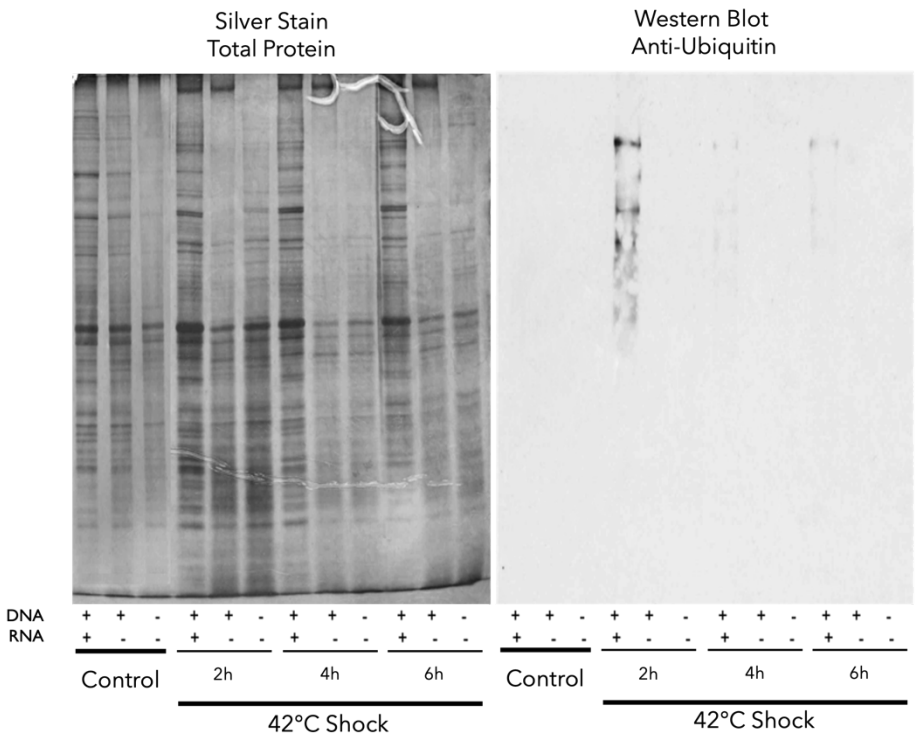

Figure 2. BY4741 cells were exposed to heat shock at 42°C for 6 hours in YPD medium. Insoluble aggregated protein in yeast cells were extracted before (control) and after the exposure (42°C Shock), at different time points every 2 hours (2h, 4h and 6h). Ubiquitin is present only prior to the digestion of the nucleic acids, suggesting that this stress condition leads to the formation of aggregates associated to nucleic acids, which are therefore dismembered after the treatment with nucleases. Samples were separated using 10% polyacrylamide gel electrophoresis and visualized by Silver staining for total protein levels. Ubiquitinated proteins were detected by Western Blot analysis using the anti-ubiquitin monoclonal antibody P4D1 (dilution 1:1'000) on 8% polyacrylamide gel. For all the gels, loaded 30 µg of each sample.

Nitrogen Starvation Leads to the Formation of Ubiquitinated Protein Aggregates

It is known that nitrogen depletion causes the reversible formation of protein assemblies. The lack of nitrogen sources causes the activation of GCN2 (General Control non-derepressible 2), a kinase involved in the phosphorylation of eIF2a. This phosphorylation leads to the stalling of translation initiation, allowing the normal process of elongation, which results in polysome disassembly and in the aggregation of stress granules and P-bodies (Kedersha and Anderson, 2009). However, no investigation was performed to evaluate the presence of ubiquitin under this stress condition. Hence, we questioned if ubiquitinated protein assemblies could accumulate following the elimination of all nitrogen sources. To do so, *S. cerevisiae* cells were deprived of amino acids and ammonium sulphate for 24 hours and samples were taken at different time-points (3h, 6h, 24h). Fig. 3 shows the presence of insoluble proteins and the difference between the three fractions of aggregates in the Silver-stained slab gels (Fig. 3). We observed the presence of ubiquitinated proteins through Western Blot analysis. The results display that the treated samples present a ubiquitinated pattern in the samples extracted from cells after 24 hours of nitrogen starvation, which is revealed even in the fractions treated with the nucleases, suggesting the presence of ubiquitinated aggregates is independent from the nucleic acids. Control and previous time-points (3h and 6h) shows no ubiquitin presence.

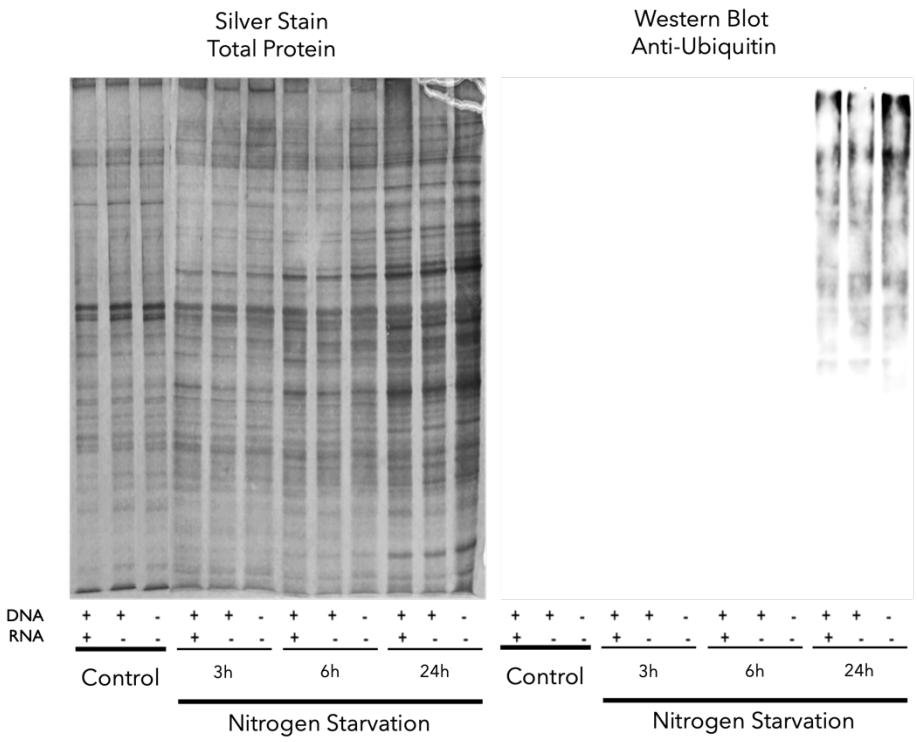

Figure 3. BY4741 were grown in medium depleted of nitrogen for 24 hours. Insoluble aggregated protein in yeast cells were extracted before (control) and after the depletion of nitrogen (nitrogen starvation), at different time points (3h, 6h and 24h). The accumulation of ubiquitinated protein is observable after 24 hours of nitrogen starvation, and it is persistent even after the digestion of nucleic acids with nucleases, suggesting nucleic acids are not a component of the aggregates. Samples were separated using 10% polyacrylamide gel electrophoresis and visualized by Silver staining for total protein levels. Ubiquitinated proteins were detected by Western Blot analysis using the anti-ubiquitin monoclonal antibody P4D1 (dilution 1:1'000) on 8% polyacrylamide gel. For all the gels, loaded 30 µg of each sample.

Glucose Depletion does not Induce the Formation of Ubiquitin-Rich Aggregates

Glucose depletion stress induces the accumulation of proteasome-storage granules (PSGs), that act as storage of proteasome subunits (Karmon and Ben Aroya, 2020). PSGs are transient structures, as they are dismembered upon glucose restoration, and their formation is regulated by free monoubiquitin, moreover, they colocalize with GFP-tagged Ubi4 and Ubp6 (Gu *et al.*, 2017). Hence, we decided to investigate if ubiquitinated proteins accumulate under this stress condition. To do so, *S. cerevisiae* cells were deprived of glucose for 24 hours and samples were taken at different time-points (3h, 6h, 24h). As shown in Fig. 4, we can assess the presence of insoluble proteins and the difference between the three fractions of aggregates, however, Western Blot displays no presence of ubiquitinated proteins. To understand if the lack of ubiquitin depends on the concentration of the sample, a second analysis was done loading twice the amount of protein extract (60 µg), but even in this case, there was no trace of ubiquitin (Fig. 4B). Glucose starvation is known to cause a severe inhibition of translation initiation which leads to the redistribution of components of the protein synthesis machinery and mRNAs into reversible membraneless cytoplasmic foci, such as P-bodies, with the function of storage or degradation of mRNA (Hoyle *et al.*, 2007; Janapala *et al.*, 2019). Therefore, we conclude that, most likely, the structures that form upon glucose removal are protein-nucleic acid inclusions, rather than ubiquitin-rich aggregates.

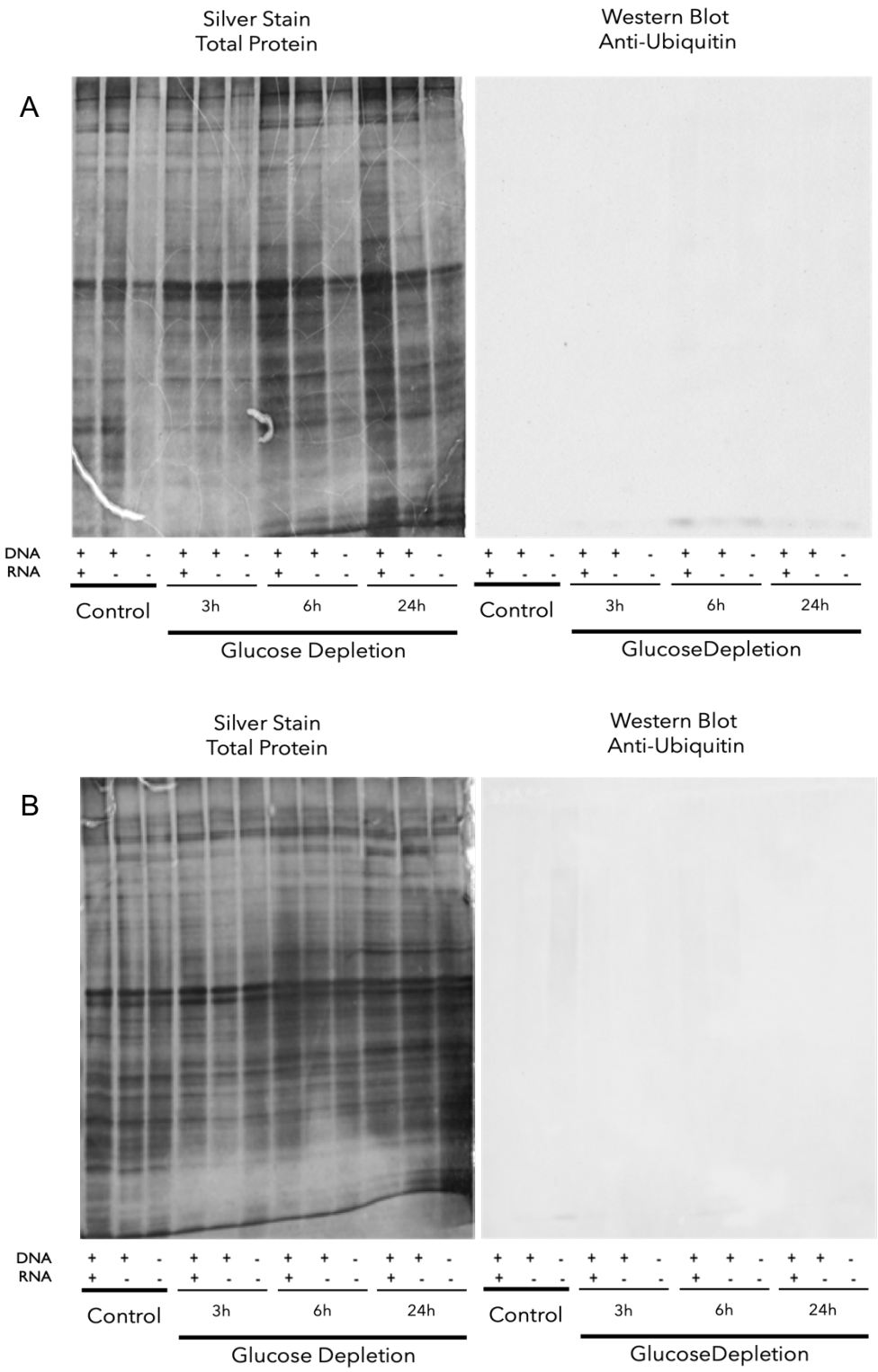

Figure 4. BY4741 were grown in medium lacking glucose for 24 hours. Insoluble aggregated protein in yeast cells were extracted before (control) and after the depletion of glucose (glucose depletion), at different time points (3h, 6h and 24h). (A) As shown in the Western Blot analysis, there is no presence of ubiquitinated proteins both for the control and for glucose

depletion. For both gels, loaded 30 µg of proteins. (B) Western Blot was repeated, loading twice the amount of protein extract (60 µg), but even in this case, there was no trace of ubiquitin for all the different time-points. These results suggest that under this stress condition the only aggregates formed are the ones associated to nucleic acids, which are much likely dismembered after the treatment with nucleases. Samples were separated using 10% polyacrylamide gel electrophoresis and visualized by Silver staining for total protein levels. Ubiquitinated proteins were detected by Western Blot analysis using the anti-ubiquitin monoclonal antibody P4D1 (dilution 1:1'000) on 8% polyacrylamide gel.

Puromycin Leads to the Aggregation of Ubiquitinated Protein in Puromycin-Sensitive S. cerevisiae Strain

Dendritic cell Aggresome-Like Induced Structure (DALIS) are membraneless protein aggregates which function as transient deposits for ubiquitinated defective polypeptides in mammalian dendritic cells during their maturation in response to several stresses, including fever-like temperatures, nutritional stress, and puromycin treatment (Lelouard *et al.*, 2004). Puromycin is an antibiotic which is erroneously incorporated during protein synthesis at the place of the correct amino acid, resulting in a premature termination of translation and truncated nascent proteins (Aviner, 2020). Therefore, we decided to expose yeast cells to puromycin treatment. Since yeasts are naturally resistant to puromycin because they metabolize it to inactive derivatives (Melcher, 1971), a puromycin-sensitive triple mutant EPP (*erg6Δpdr1Δpdr3Δ*), gently given by Cary *et al.*, 2014, was tested both *in vitro* and *in vivo* for the resistance to different puromycin concentrations (Fig. 6). Fig. 6A shows the difference in the growth of 5-fold serial dilutions of wild type (WT), EPP, and single or double deletion (*erg6Δ*, *pdr1Δ*, *pdr3Δ*, *pdr1Δpdr3Δ*) mutant cells on YPD agar

containing crescent concentrations of puromycin, demonstrating the inhibiting effect of puromycin on the triple mutant at concentration as high as 500 µM. As puromycin 500 µM showed a partial inhibition in the growth of EPP on agar plate, we analyzed its effect in liquid media containing a lower concentration of the drug (Fig. 6B). As expected, while 200 µM puromycin had no effect on the growth of WT cells, the treatment with the same concentration on the EPP triple mutant induced a slow growth rate, confirming that the deletion of these genes led to the sensibilization of *S. cerevisiae* to the drug.

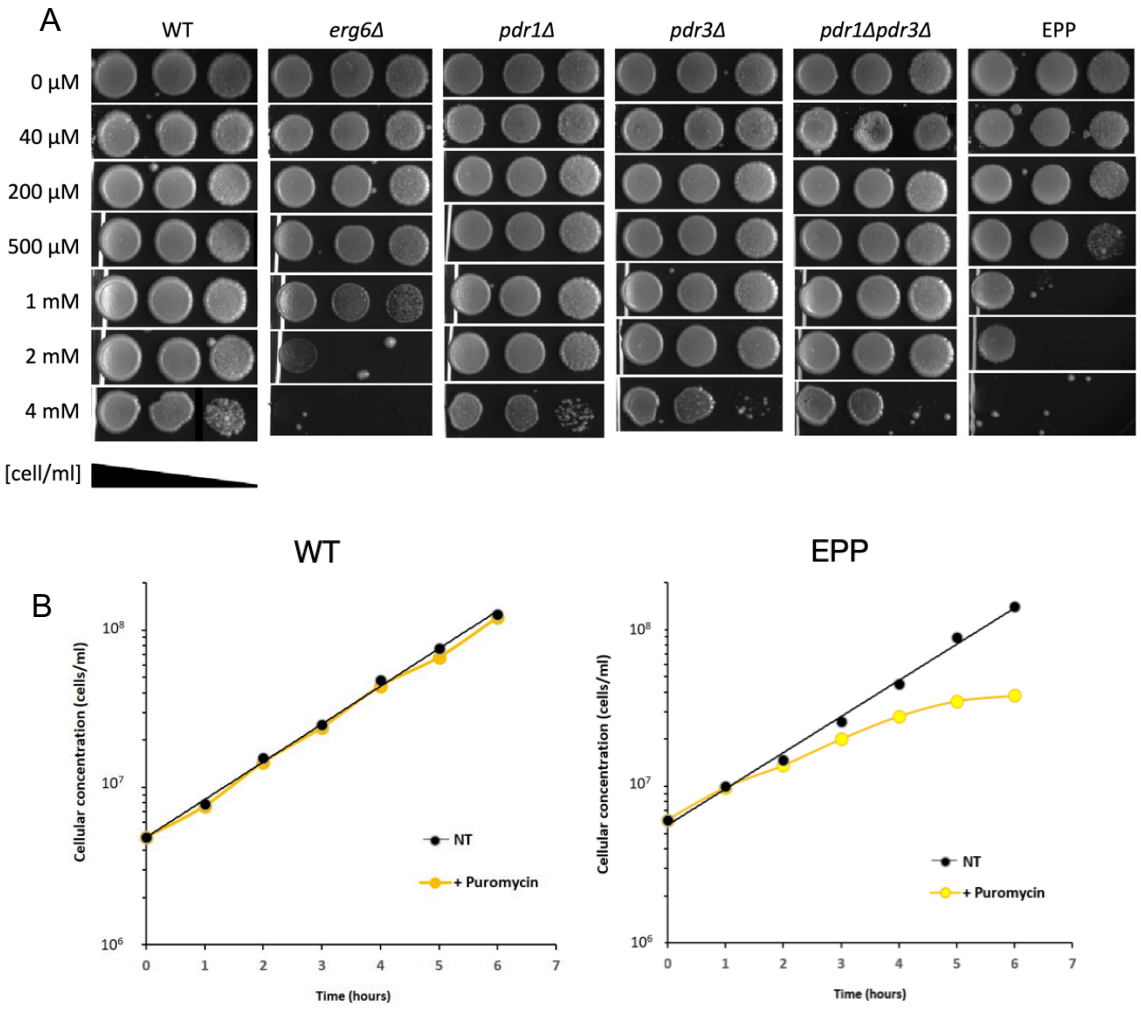

Figure 6. Characterization of the puromycin-sensitive triple mutant *erg6Δpdr1Δpdr3Δ* (EPP) of the BY4741 strain. (A) Growth of 5-fold serial dilutions of wild type (WT), EPP, *erg6Δ*, *pdr1Δ*, *pdr3Δ*, and *pdr1Δpdr3Δ* cells on YPD agar containing 0 µM, 40 µM, 200 µM, 500 µM, 1 mM,

2 mM and 4 mM puromycin. The inhibiting effect of puromycin is demonstrated on the triple mutant EPP at concentration as high as 500 μM. (B) Growth curves for WT and EPP cells in liquid media containing 0 μM (black) and 200 μM (yellow) puromycin. 200 μM puromycin was shown to slow, without inhibiting, the growth of the EPP mutant strain, whereas it had no effect on the growth of WT cells.

At this point, we decided to investigate whether the treatment with puromycin causes the aggregation of ubiquitinated proteins in yeast. First, the protein analysis of insoluble aggregates extracted from non-treated single, double, and triple mutants revealed that the deletion of the *ERG6*, *PDR1*, and *PDR3* genes did not generate changes in the patterns of insoluble proteins, which result to be similar to the WT one (Fig. 7).

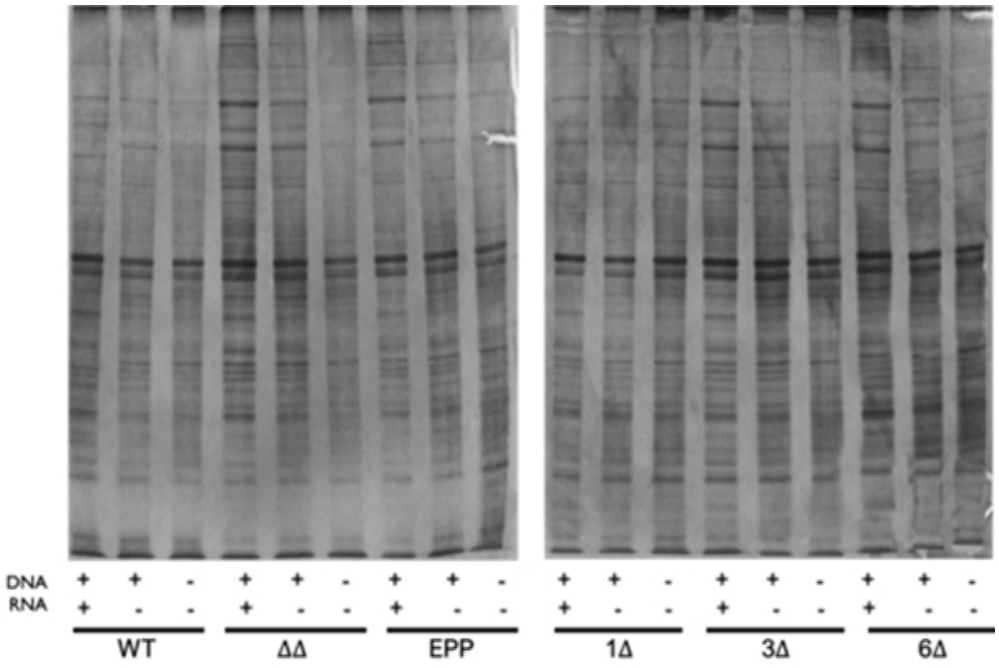

Figure 7. Analysis of insoluble aggregates extracted from non-treated wild type (WT), *pdr1Δpdr3Δ* (ΔΔ), *erg6Δpdr1Δpdr3Δ* (EPP), *pdr1Δ* (1Δ), *pdr3Δ* (3Δ), and *erg6Δ* (6Δ) in YPD medium. See Figure 1 for caption. For all the gels, loaded 30 μg of each sample. As expected,

the deletion of the *ERG6*, *PDR1*, and *PDR3* genes did not generate changes in the patterns of insoluble proteins, which was shown to be similar to the WT one. Samples were separated using 10% polyacrylamide gel electrophoresis and visualized by Silver staining for total protein levels. For all the gels, loaded 30 µg of each sample.

Then, we performed a Western Blot analysis (Fig. 8) against ubiquitin for the WT and the EPP cells treated with 200 µM puromycin for 24 h. The analysis demonstrated the presence of ubiquitinated proteins in the EPP triple mutant 24 h after the treatment with the drug, whereas the WT displays no ubiquitin pattern, coherently with the fact that puromycin has no effect on *S. cerevisiae* (Melcher, 1971).

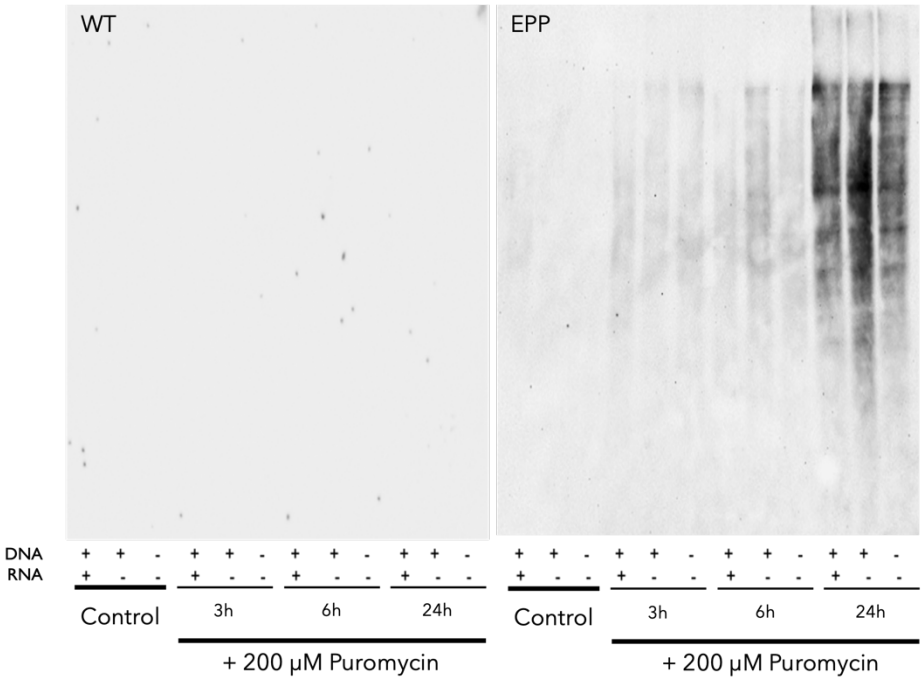

Figure 8. Insoluble aggregates analysis of WT and EPP cells treated with 200 µM puromycin for 24 hours. Insoluble aggregated protein in yeast cells were extracted before (control) and after the treatment with puromycin (+ 200 µM puromycin), at different time points (3h, 6h and 24h). Western Blot analysis demonstrated the presence of ubiquitinated proteins only in the puromycin-sensitive EPP triple mutant 24 hours after the treatment with the drug, revealing

the persistence even after the digestion of nucleic acids with nucleases. This suggests nucleic acids are not a component of the aggregates. Ubiquitinated proteins were detected by Western Blot analysis using the anti-ubiquitin monoclonal antibody P4D1 (dilution 1:1'000) on 8% polyacrylamide gel. For all the gels, loaded 30 µg of each sample.

Ubiquitin-rich Aggregates are Round-Shaped, Disseminated in the Cytosol and not Colocalized with any other Cellular Structure

We decided to study the cellular localization of ubiquitinated aggregates, using fluorescence microscopy. As the protein analysis revealed that ubiquitinated aggregates form in yeast following the exposure to ethanol treatment and nitrogen starvation, we decided to investigate these two conditions. To do so, cells were grown to exponential phase, then were treated with 10% ethanol for 3 hours or depleted of nitrogen for 24 hours. After the exposure to the stress, cells were fixed and immune decorated for mono- and polyubiquitinated proteins using the FK2 monoclonal antibody (Calbiochem). As expected, ubiquitin-rich aggregates formed in yeast cells under the two stress conditions (Fig. 9). Ethanol treatment induced the accumulation of small, round ubiquitinated protein aggregates, which seemed not to colocalize with any cellular structure, but rather are disseminated in the cytosol (Fig. 9A). Also, nitrogen starvation induced the formation of ubiquitinated aggregates, but these appeared to be larger in size and less numerous in the cell, however, they still seemed not to be colocalized with any cellular compartment (Fig. 9B). These results show that ubiquitin-rich aggregates can accumulate in *S. cerevisiae* cells under different stress conditions.

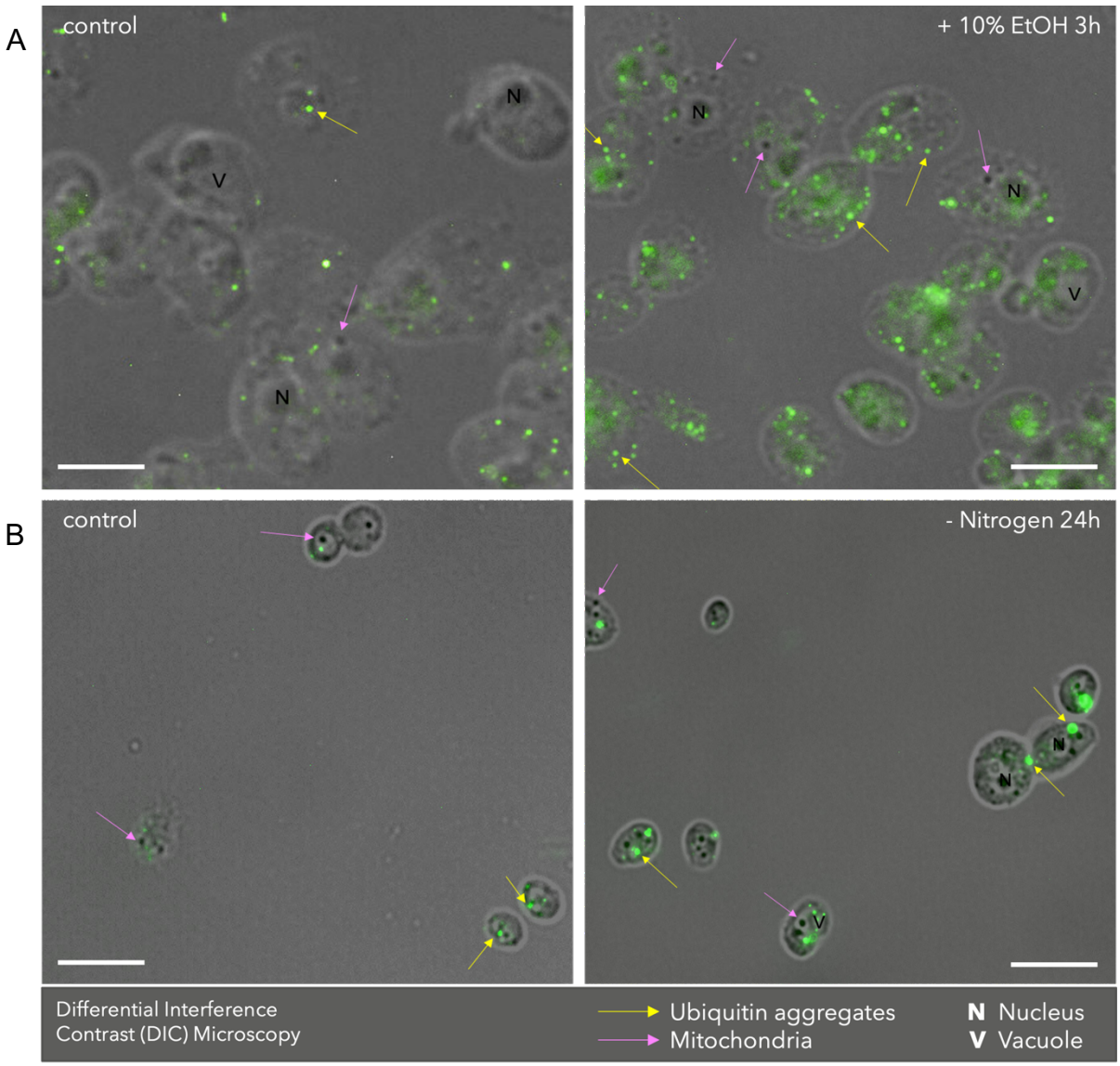

Figure 9. Ubiquitin-rich aggregates are visualized in *S. cerevisiae* under different stress conditions. (A) cells grown in YPD medium, under no stress (control), or treated with 10% ethanol for 3 hours (+ 10% EtOH 3h). (A) It is observable the presence of a few ubiquitinated spots in non-treated cells, in contrast to the ethanol-treated cells, where it is noticeable the higher frequency of small cytoplasmic round-shaped ubiquitinated spots in the cytoplasm, apparently not colocalized with any other cellular component. (B) cells grown in minimal medium, under no stress (control), or under nitrogen starvation for 24 hours (- Nitrogen 24h). It is observable the presence of a few small ubiquitinated spots in non-treated cells, in contrast to nitrogen-depleted cells, where it is noticeable the presence of round-shaped ubiquitinated spots in the cytoplasm, which seem to appear larger and in a lower frequency compared to

the ethanol-exposed cells, but still not colocalized with any other cellular component. Ubiquitin is analyzed with FK2 anti-ubiquitin monoclonal antibody (1:500) and detected with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500). Size bar 5 μm.

DISCUSSION

By prevailing opinion, protein aggregation represents a dangerous phenomenon, being associated to age-related neurodegenerative disorders, such as Alzheimer's or Parkinson's Diseases, which involve incorrect protein folding processes and protein aggregation (Hipp *et al.*, 2019). However, this event not always is a synonym of cellular failure or cell death, instead, the coalescence of proteins into large multimeric intracellular bodies, such as mRNP bodies, is a conserved cellular response adopted by eukaryotic cells to deal with an unfamiliar situation. This mechanism is also employed by healthy cells to overcome the presence of large amounts of misfolded polypeptides, generated in response to external stresses, which cannot properly be rescued by the overloaded proteolytic machinery (Gallardo *et al.*, 2021; Muscolino *et al.*, 2021). Such aggregates are used as temporary storages for potentially toxic proteins, which will either be rescued or addressed for clearance by the ubiquitin proteasome system, chaperone-mediated autophagy, or aggrephagy (Lamark and Johansen, 2012).

In the present study, the accumulation of insoluble proteins was confirmed in yeast cells following exposure to the previously described stress conditions. Heat shock temperature at 42°C and glucose starvation caused the accumulation of protein assemblies, however, the treatment with nucleases suggested that the inclusions formed under these conditions are predominantly messenger ribonucleoprotein (mRNP) bodies, composed of RNA and RNA-binding proteins. These structures are transient, and have distinct composition, but are spatially, compositionally, and functionally linked. Indeed, they are mainly implied in the storage and decay of RNA, playing a crucial role in the control of gene expression (Kedersha and Anderson, 2009).

On the other hand, nitrogen starvation, ethanol treatment and puromycin addition caused the accumulation of different cytoplasmic aggregates. The inclusions formed under these conditions are ubiquitin-positive, even following the elimination of the nucleic acids, suggesting they should be allocated under a different category. A species of ubiquitin-positive aggregates can be found in mammalian dendritic cells. Dendritic cell Aggresome-Like Induced Structures (DALISs) are membraneless protein aggregates composed by defective ribosomal products (DRiPs), which function as transient deposits for ubiquitinated defective proteins in dendritic cells (Pierre, 2005). They were originally found in mammalian dendritic cells, but additional studies assessed their presence also in macrophages and in some non-immune cells (Szeto *et al.*, 2006). In antigen presenting cells, they act as antigen storage and ubiquitination sites for DRiPs (Wenger *et al.*, 2012), whereas in other cell types their function is still shrouded in mystery. We suggest that yeast ubiquitin-rich aggregates, like the ones identified during the present study, are more closely related to the mammalian DALISs, rather than stress granules or P-bodies. Supporting this hypothesis, is the evidence that DALISs form in response to several stresses, including the treatment with the protein synthesis inhibitor puromycin (Lelouard *et al.*, 2004). Likewise, they are characterized by a round-shaped morphology, and they appear as independent structures, not colocalized with any other cellular compartment, in the cytosol of mammalian cells (Lelouard *et al.*, 2004).

Ubiquitin-rich structures equivalent to those found in mammalian cells had never been detected in yeast, however, the conservation degree between mammalian and yeast cells encourages the idea of yeast as an extremely suitable model to conduct a preliminary study on such cytoplasmic protein aggregates.

The present study is a preliminary investigation aimed at determining whether and under which conditions ubiquitin-rich protein aggregates form in the yeast *Saccharomyces cerevisiae*. It is premature to classify these ubiquitinated inclusions under a specific category, as further studies are needed to shed light on their function, composition, and clearance. However, the similarities with mammalian DALIS suggest that they might be a form of conserved response to stress conditions, and that they might help the cell dealing with the consequently generated misfolded proteins.

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The role of Atg1 and the ubiquitin- and Atg8-binding protein Cue5 in ethanol-induced ubiquitin-rich aggregates clearance and ubiquitination

INTRODUCTION

The formation of protein aggregates is a conserved process engaged by eukaryotic cells in response to external stimuli that alter the physiological cellular environment (Tyedmers *et al.*, 2010). Among the plethora of environmental stresses that stimulate protein aggregates, severe ethanol stress is known to induce protein dysfunction, as it induces the denaturation of polypeptides, leading to the accumulation of insoluble misfolded proteins (Piper, 1995; Ma and Liu, 2010; Escusa-Toret *et al.*, 2013).

In previous studies, the exposure to severe ethanol stress conditions and the analysis of protein extracts, allowed us to isolate ubiquitin-rich protein aggregates in *Saccharomyces cerevisiae*. In mammalian cells, ubiquitinated aggregates can be detected in dendritic cells, where they form in response to different stress conditions (Pierre, 2005). These inclusions, called Dendritic cells Aggresome-Like Induced Structures (DALISs), represent transient deposits for ubiquitinated defective proteins and they have recently been identified in macrophages and non-immune cells as well, where their biological role is still not fully understood (Szeto *et al.*, 2006). These aggregates have never been identified in other cellular models, however, the ubiquitin-rich aggregated proteins we observed in yeast share some analogies DALISs. For

instance, they both form after the induction of a stress, they are composed by proteins and ubiquitin, they are round in shape and not colocalized with any other cellular structure (Lelouard *et al.*, 2004). Nonetheless, additional investigations are needed to corroborate this hypothesis, such as the evaluation of their clearance.

Hence, we decided to investigate how ethanol-induced ubiquitin-rich protein aggregates are cleared inside the cell. Whereas the nitrogen starvation condition is known to stimulate the formation of protein aggregates that are cleared by aggrephagy, the selective autophagy of protein aggregates (Lamark and Johansen, 2012), it is not certain what mechanisms are involved in the clearance of protein aggregates under severe ethanol stress. Aggrephagy, the selective ubiquitin-dependent autophagy involved in the elimination of protein aggregates (Lamark and Johansen, 2012), may represent a key mechanism. The yeast protein Cue5 is a ubiquitin-binding protein (Lu *et al.*, 2014), which is known to be recruited to ubiquitinated lipid droplets through its ubiquitin-binding domain, co-degrading together with lipid droplets through lipophagy (Kumar *et al.*, 2022). Cue5 represents an appealing candidate as aggrephagy receptor for the ubiquitin-rich protein aggregates under ethanol stress. Therefore, the aim of the present project is to investigate if and what role Cue5 might have in the ubiquitin-rich aggregates, unravelling its potential function in their clearance.

Strains and Media

The *Saccharomyces cerevisiae* strains used in this study are shown in Table 1.

Mutant	Strain	Background	Genotype	Source
WT	BY4742	BY4742	MAT**a** *his3Δ 1leu2Δ0 leu2Δ0 ura3Δ0*	Brachman *et al.*, 1998
WT	SMR31	BY4742	MAT**a** *his3Δ 1leu2Δ0 leu2Δ0 ura3Δ0 ERG6-GFP::HIS3*	Nazarko Lab
atg1Δ	SMR35	BY4742	MAT**a** *his3Δ 1leu2Δ0 leu2Δ0 ura3Δ0 atg1::KanMX ERG6-GFP::HIS3*	Nazarko Lab
cue5Δ	SMR91	BY4742	MAT**a** *his3Δ 1leu2Δ0 leu2Δ0 ura3Δ0 cue5::KanMX ERG6-GFP::HIS3*	Nazarko Lab

Table 1. *Saccharomyces cerevisiae* strains used in this study.

Cells were grown in batches in Yeast Extract Peptone Dextrose (YPD) medium (2% dextrose, 1% yeast extract, 2% peptone), SD+CSM-His medium (1.7 g/L Yeast Nitrogen Base (YNB) without amino acids and ammonium sulfate, 20 g/L dextrose, 5 g/L ammonium sulfate, 0.78 g/L CSM-His), or in minimal medium (1.7 g/L YNB (added

with 5 g/L ammonium sulfate, 10 mg/L Methionine, 10 mg/L Histidine and 10 mg/L Tryptophan), 2% dextrose, 200 mg/L Histidine, 200 mg/L Uracil, 400 mg/L Leucin, 400 mg/L Lysin, 400 mg/L Methionine), at 30°C at 250 rpm.

Equal biomass (100 OD_{600}) of mid-log phase cells in YPD or SD+CSM-Ura media were supplemented with 10% ethanol (v/v) for 3 hours and 24 hours. At designated time-points, aliquots of the yeast cultures were harvested and centrifuged to remove medium, cell pellets were frozen at −20°C until protein extraction was performed.

All experiments were done at least in triplicate.

Insoluble Aggregates and Protein Extraction

Insoluble aggregated proteins were analyzed adapting the protocol of Kato *et al.* (2019). Briefly, cells were incubated in Lysis Buffer (50 mM PBS, 1.0 mM EDTA, 5% glycerol, pH 7.0), added of Zymolyase 20T (2.5 mg/mL) and PMSF (17.4 mg/ml in EtOH) at room temperature for 20 minutes and disrupted by vortexing with glass. Cell organelles and membranes were removed by centrifugation at 5'000 g for 20 minutes at 4°C, total protein concentration of each sample was measured using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) and protein concentration were normalized accordingly. After normalization, 10% of each total protein sample was collected and temporarily stored at +4°C. The total protein samples were then centrifuged at 16'000 g for 20 minutes at +4°C, to obtain a pellet fraction, containing the insoluble aggregated proteins, and a supernatant fraction. 10% of the supernatant fraction was collected and temporarily stored at +4°C, the remaining supernatant was discarded. 100% of the pellet fraction was collected and washed twice with Lysis Buffer + 2% Nonidet P-40 (492018 | NP-40 Alternative, PROTEIN GRADE, EMD Millipore) to

remove membrane proteins, and then solubilized in Urea Buffer (50 mM Tris–HCl, 6.0 M urea, 5% SDS, pH 7.5) and stored at -20°C.

Total protein and supernatant fractions were processed using trichloroacetic acid (TCA) protein precipitation assay. Briefly, 12.5% of 100% TCA was added to each sample, which were soon after centrifuged at 14'000 g for 5 minutes at room temperature to allow protein to pellet. Pellets were washed twice with 80% acetone, and let dry completely, then were resuspended in Urea Buffer (50 mM Tris–HCl, 6.0 M urea, 5% SDS, pH 7.5) and stored at -20°C.

All experiments were done at least in triplicate.

Insoluble Aggregates and Protein Analysis

30 µl of pellets, 10 µl of total protein and 10 µl of supernatants were resolved by SDS-PAGE on 8% or 10% polyacrylamide gels. 5 µl of pre-stained protein markers (BioRad Precision Protein Plus Kaleidoscope Ladder, range 10-250 kDa) were also loaded.

Western Blot analysis. Polyacrylamide gels were blotted onto 0.2 µm nitrocellulose blotting membranes (Bio-Rad), a constant current of 90 V was applied for 1 hour. Total proteins were stained with REVERT™ Total Protein Stain Kits (LI-COR®) and imaged with Odyssey® DLx (LI-COR®). Filters were then incubated at room temperature for 1 hour in 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad) + TBST 1X for saturation. Immunodetection was performed overnight using anti-ubiquitin monoclonal antibody (IgG_1 mouse clone P4D1, sc-8017 Santa Cruz Biotechnology) at 1:1'000 dilution in 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad) + TBST 1X, or anti-GFP antibody (IgG_1 mouse clones 7.1 and 13.1, Roche) at 1:1'000 dilution in 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad) + TBST 1X. After 3 washes of 5

minutes each in TBST 1X, membranes were incubated with IRDye® 800CW Goat anti-Mouse IgG Secondary Antibody (LI-COR®) diluted 1:15'000 in TBST 1X for 1 hour. After 2 washes of 5 minutes each in TBST 1X and 1 wash in TBS 1X, binding was visualized using Odyssey® DLx (LI-COR®).
All experiments were done at least in triplicate.

Fluorescence Microscopy

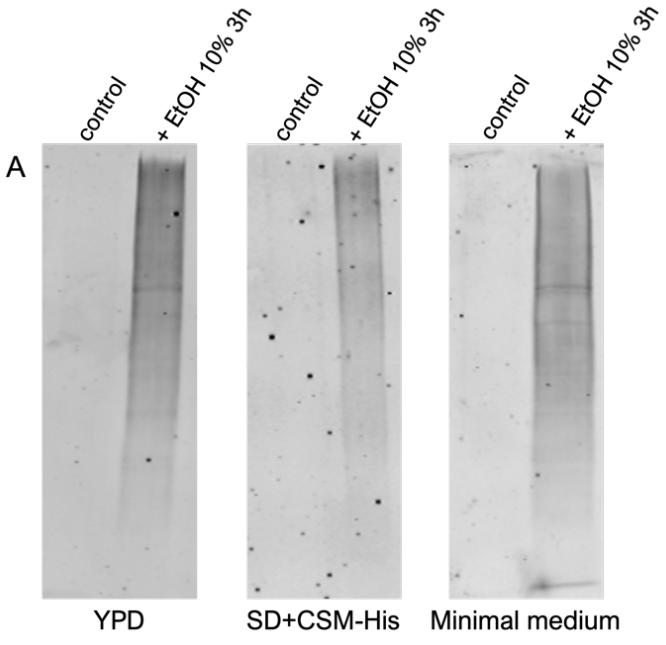
2 ml of cells were collected at each time point: mid-log phase untreated cells, cells treated for 3 hours with 10% ethanol, and cells treated for 24 hours with 10% ethanol. Cells were centrifuged to eliminate medium and resuspended in 1 ml of 100% methanol for fixation for 10 minutes at room temperature. Cells were then centrifuged to eliminate methanol and washed 3 times with Phosphate Buffer (100 mM K_2HPO_4, 100 mM KH_2PO_4), followed by 1 wash with Digestion buffer (1.2 M sorbitol, 0.12 M K2HPO4, pH 5.9). An incubation with Zymolyase 20T (5 mg/ml) for 55 minutes in Digestion Buffer allowed to digest the glucans of the cell wall. Cells were centrifuged and washed once with 100 mM Digestion Buffer w/o enzyme to discard the enzyme, and then stored in Digestion Buffer at +4°C for a maximum of 2 days. For immune decoration, cells were placed on fresh poly-lysine-coated slides and washed at least 10 times with 100 mM Phosphate Buffer, 1.2 M sorbitol, 0.05% Tween 20. Then, they were incubated for 3 hours with anti-ubiquitin monoclonal antibody (IgG_1 mouse clone P4D1, sc-8017 Santa Cruz Biotechnology) at 1:200 dilution in 100 mM Phosphate Buffer, 0.025% Tween 20, 1 mg/ml BSA, pH 6.5, at +4°C in moist chamber. After 3 hours, cells were washed at least 10 times with 100 mM Phosphate Buffer, 1.2 M sorbitol, and subsequently incubated for 2 hours in a dark moist chamber with

secondary antibody Alexa Fluor® 488 AffiniPure Donkey Anti-Mouse IgG (H+L) (Jackson ImmunoResearch) at 1:800 dilution in 100 mM Phosphate Buffer, 0.025% Tween 20, 1 mg/ml BSA, pH 6.5. After 10 washes with 100 mM Phosphate Buffer, 1.2 M sorbitol, cells were incubated with AUTOdot Autophagy Visualization Dye Monodansylpentane (MDH) (SM1'000a; Abcepta, San Diego, CA, USA) at 1:10'000 dilution in 100 mM Phosphate Buffer, 1.2 M sorbitol for 30 minutes in a dark moist chamber. Then cells were visualized using a Nikon Eclipse Ti2, equipped with a Photometrics Prime 95B camera. The filters FITC (wavelength 467 – 556 nm) and DAPI (wavelength 352 – 477 nm) were used for the visualization of ubiquitinated proteins and lipid droplets respectively. Digital images were acquired and processed using Nikon software NIS-Elements.

All experiments were done at least in triplicate.

Ethanol Stress Causes the Aggregation of Ubiquitin-Rich Bodies Including Erg6-GFP

Based on what we observed in previous experiments, we decided to evaluate the presence of ubiquitinated aggregates in the *S. cereviasiae* strain SMR31, henceforth indicated as WT, under 10% ethanol (v/v) for 3 hours. In this strain, Erg6 is tagged with the green fluorescent protein (GFP), which is an established marker of lipid droplets (Kumar *et al.*, 2020). We chose this strain to further investigate Cue5, a ubiquitin-binding protein that in known to be recruited to lipid droplets through its ubiquitin-binding domain. Interestingly, we observed the accumulation of ubiquitin-rich protein aggregates in the strain after 3 hours of ethanol stress in YPD. The same result was observed also in SD+CSM-His and minimal medium, confirming that under 3 hours of severe ethanol stress there is presence of ubiquitinated aggregates in WT cells (Fig. 1A). At the same time, we assessed the presence of Erg6-GFP in the aggregates, which was observed equally for the three different media (Fig. 1B).



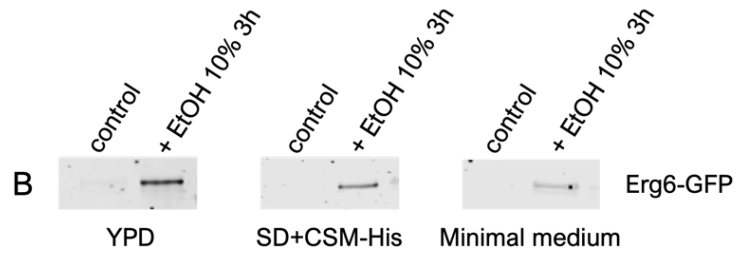


Figure 1. Western Blot analysis of ubiquitin and GFP in the insoluble aggregates of the strain SMR31 under ethanol stress in different media. (A) The presence of ubiquitin is consistent in the WT cells following the addition of 10% ethanol for 3 hours throughout the different media. Samples were separated using 8% polyacrylamide gel electrophoresis and ubiquitinated proteins were detected by Western Blot analysis using the anti-ubiquitin monoclonal antibody P4D1 (dilution 1:1'000). For all the gels, loaded 30 µl of each sample. (B) Erg6-GFP is present in WT cells under ethanol stress for 3 hours. Samples were separated using 10% polyacrylamide gel electrophoresis and GFP was detected by Western Blot analysis using the anti-GFP antibody (dilution 1:1'000). For all the gels, loaded 30 µl of each sample.

ATG1 and CUE5 are Implied in the Clearance and Formation of Ubiquitin-Rich Aggregates in S. cerevisiae

We decided to deeply investigate the protein aggregation upon prolonged severe ethanol stress, which was extended to 24 hours. For this study, we evaluated the effects of 10% ethanol on the mutant strains *atg1Δ*, deleted of the *ATG1* gene, and *cue5Δ*, deleted of the *CUE5* gene. Preliminary studies showed an accumulation of ubiquitinated aggregates in the *atg1Δ* strain 3 hours after the addition of 10% ethanol, which persisted through the 24 hours of stress. On the other hand, for *cue5Δ*, such accumulation was not as sharp as for the *atg1Δ* mutant cells, on the contrary, the presence of ubiquitin remained similar to the ubiquitin level of the control, which was obtained before the treatment with ethanol (Fig. 2).

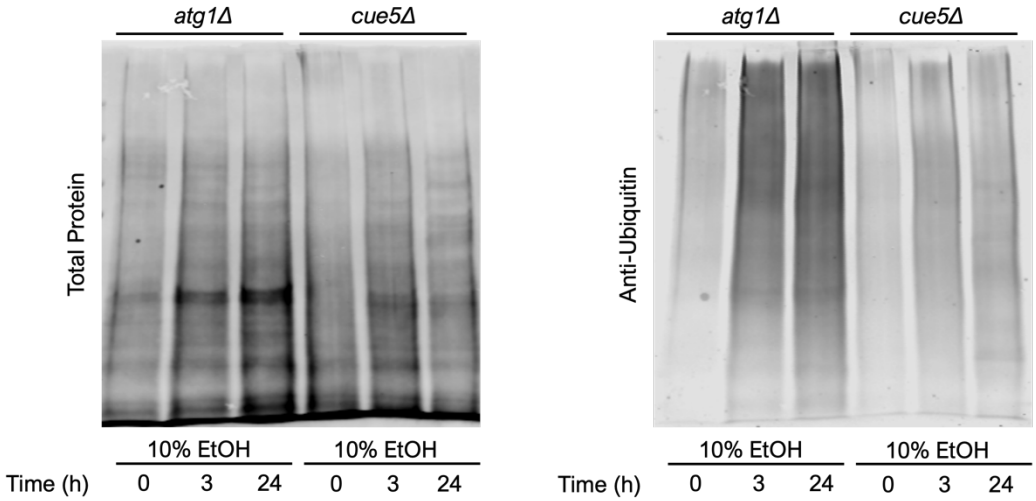


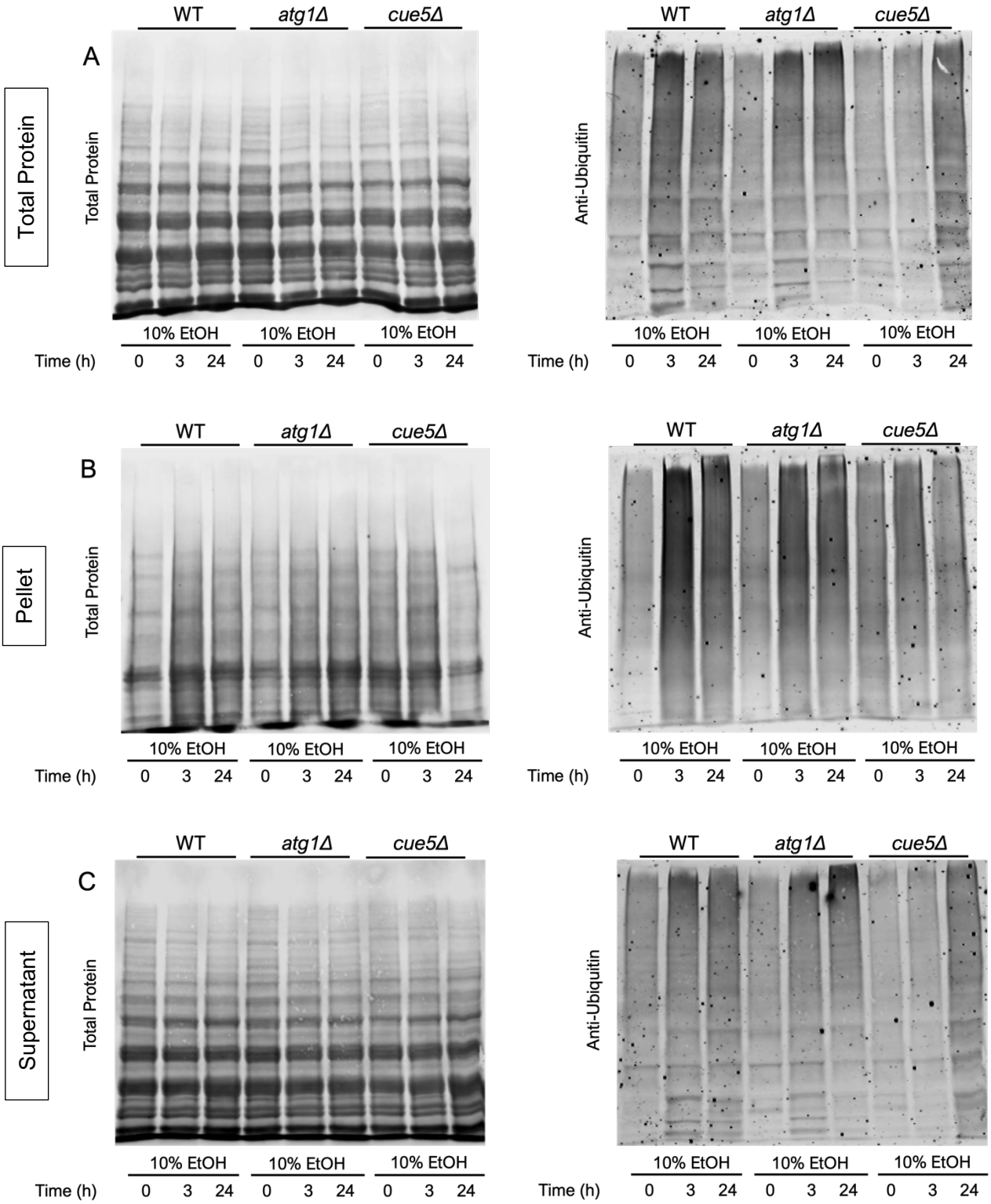
Figure 2. Preliminary Western Blot analysis of ubiquitin in the insoluble aggregates of *atg1Δ* and *cue5Δ* mutant cells after 3 and 24 hours of ethanol stress. An accumulation of ubiquitinated aggregates is observable in the *atg1Δ* strain after the addition of 10% ethanol, which persisted through the 24 hours of stress. For *cue5Δ*, ubiquitin level was similar to that of the control even after the induction of the stress. Samples were separated using 8% polyacrylamide gel electrophoresis and ubiquitin was detected by Western Blot analysis using the anti-ubiquitin monoclonal antibody P4D1 (dilution 1:1'000). Loaded 30 µl of each sample.

These results suggested that *ATG1* might be implied in the clearance of ubiquitin-rich aggregates, which explains the accumulation when the gene is depleted, and that *CUE5* might instead stimulate their formation, hence the lower presence of ubiquitin when the gene is deleted.

We decided to investigate deeper this hypothesis. To do so, we further collected (i) the supernatant fraction, complementary to the insoluble aggregates (or pellet) fraction and therefore containing the soluble proteins, and (ii) the total protein fraction, inclusive of both the soluble (supernatant) and insoluble (pellet) ones, to exhaustively comprehend how ubiquitinated proteins are distributed in the different fractions.

Western Blot analysis of the three fractions (Fig. 3), confirmed the previous hypothesis. The total protein fraction displayed an enrichment in ubiquitin after 3 hours of 10% ethanol stress for both WT and *atg1Δ*. On the other hand, the level of ubiquitin after 3 hours of ethanol stress remained unchanged for *cue5Δ*. After 24 hours of ethanol stress, the ubiquitin level of WT slightly decreased, whereas, for *atg1Δ* and *cue5Δ* it increased. For *atg1Δ*, the level of ubiquitinated proteins after 24 hours of stress was even higher than after 3 hours (Fig. 3A). For the pellet, or insoluble aggregates fraction, we could observe a strong increase in the ubiquitinated proteins level of WT after 3 hours of the stress, which decreased after 24 hours. For *atg1Δ*, results showed an increase after 3 hours of stress, which remained stable even after 24 hours of stress. Regarding *cue5Δ*, we noticed a light increase after 3 hours of ethanol stress, which returned to the initial level after 24 hours (Fig. 3B). Such results are consistent with those obtained in the preliminary study for the mutant strains. The supernatant fraction confirmed, as expected, to be complementary to the pellet fraction. Interestingly, we could notice that the abundance of ubiquitinated proteins observed in the total protein fraction was caused by the supernatant fraction, meaning that the majority of ubiquitinated proteins present in *cue5Δ* after 24 hours of ethanol stress are soluble and not aggregated (Fig. 3C).

BY4742 was included in the study as a control strain, to remark that Erg6-GFP did not introduce any difference in the protein patterns of the other strains. As expected, the overall ubiquitin profile of BY4742 was the same as the WT. Indeed, we could observe an accumulation of ubiquitin after 3 hours of 10% ethanol stress, which decreased after 24 hours (Fig. 3D).


A
Total Protein
Total Protein
Anti-Ubiquitin
WT
atg1Δ
cue5Δ
10% EtOH
Time (h)
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B
Pellet
C
Supernatant

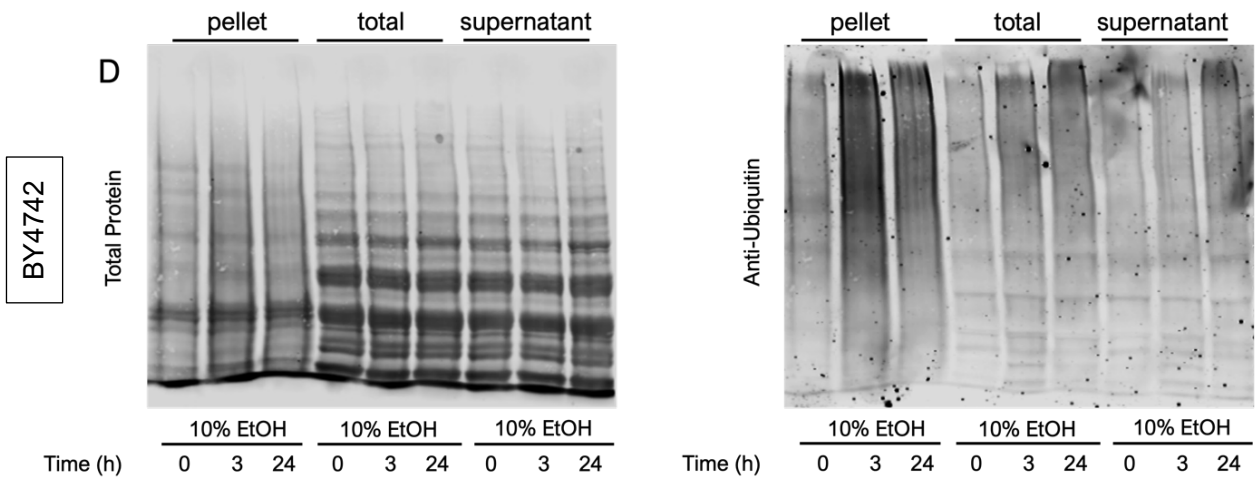
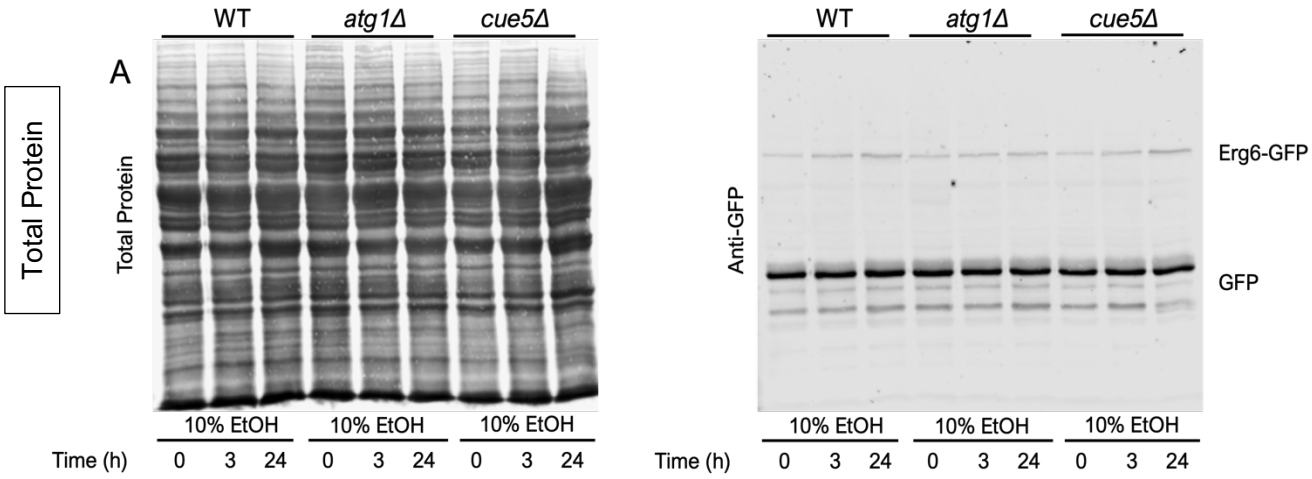


Figure 3. Western Blot analysis of ubiquitin in the WT, *atg1Δ* and *cue5Δ* strains after 3 and 24 hours of 10% ethanol stress. (A) The total protein fraction displays the presence of ubiquitin in the cells. (B) The pellet shows the presence of ubiquitin in the insoluble protein fraction. (C) The supernatant highlights the presence of ubiquitin in the soluble protein fraction, confirming, as expected, to be complementary to the pellet fraction. (D) The ubiquitin profile of BY4742 highlights the similarities with the WT strain, displaying that Erg6-GFP caused no difference in the protein pattern. In the overall, the results are consistent with the ones obtained in the preliminary experiment. Samples were separated using 8% polyacrylamide gel electrophoresis and ubiquitin was detected by Western Blot analysis using the anti-ubiquitin monoclonal antibody P4D1 (dilution 1:1'000). Loaded 10 µl for the total protein and supernatant fractions, and 30 µl for the pellet fraction.

Erg6-GFP is Present in the Ubiquitin-Rich Aggregates Formed Following Severe Ethanol Stress

Since we observed the presence of Erg6-GFP in the insoluble aggregates formed following the stress with 10% ethanol for 3 hours in the preliminary experiment, we decided to investigate whether the same happens in the mutant strains. To do so, we analyzed through Western Blot against GFP the three protein fractions, total, insoluble,

and soluble proteins. Western Blot analysis confirmed the presence of Erg6-GFP in the aggregates. As shown in Fig. 4, we could observe the comprehensive presence of GFP in the total protein fraction, mainly as processed GFP, and, at a lower rate, as Erg6-GFP (Fig. 4A). Interesting is the subdivision of GFP in the other two fractions. While in the supernatant, or soluble fraction, it seems to locate the majority of processed GFP (Fig. 4C), almost all Erg6-GFP seems to be located in the pellet, hence insoluble, fraction (Fig, 4B). While the concentration of free GFP remains constant throughout the different time points for all the three strains, the overall Erg6-GFP seems to progressively increase from the control to the 24 hours stress condition in the total protein fraction. Notable is the accumulation of Erg6-GFP in the pellet of the three strains after 3 hours of ethanol stress, which seems to decrease after 24 hours. The decrease at 24 hours might be explainable with the supernatant fraction. Indeed, this shows an increment of Erg6-GFP at the same time point, suggesting a portion of the Erg6-GFP does not remain colocalized with the insoluble aggregates, but instead becomes soluble at some point between the 3 and the 24 hours. BY4742 was included in the study as a negative control (Fig. 4D).



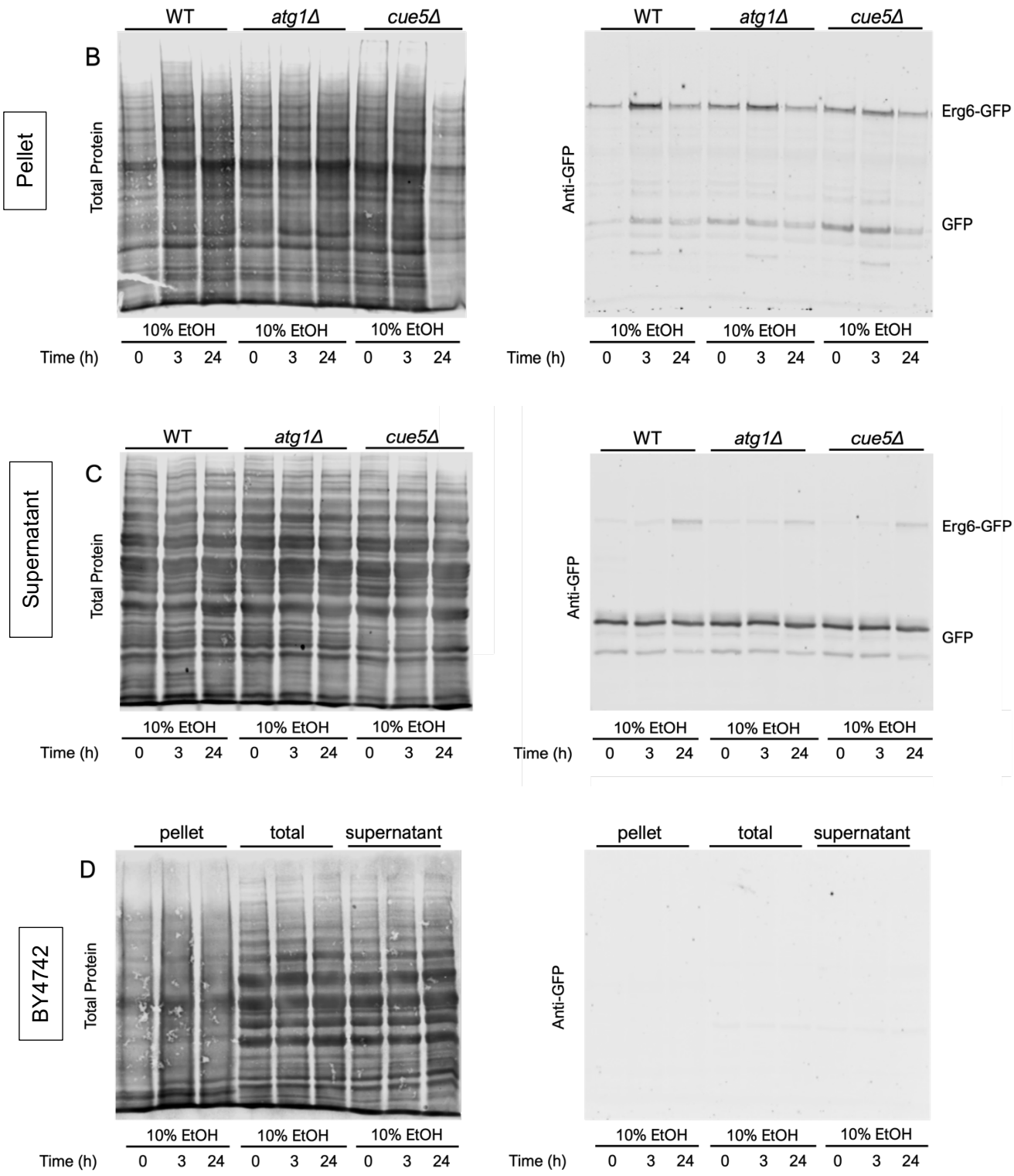


Figure 4. Western Blot analysis of Erg6-GFP in the WT, *atg1Δ* and *cue5Δ* mutants after 3 and 24 hours of 10% ethanol stress. (A) The total protein fraction displays the overall presence of GFP in the cells. (B) The pellet shows the majority of Erg6-GFP in the insoluble protein fraction. (C) The supernatant displays, instead, that processed GFP resides in the soluble

fraction, being, as expected, complementary to the pellet fraction. (D) The GFP profile of BY4742 was included as negative control. In the overall, the results are consistent with the ones obtained in the preliminary experiment. Samples were separated using 10% polyacrylamide gel electrophoresis and GFP was detected by Western Blot analysis using the anti-GFP antibody (dilution 1:1'000). Loaded 10 µl for the total protein and supernatant fractions, and 30 µl for the pellet fraction.

Ethanol-Induced Ubiquitin-Rich Aggregates are Independent Structures Located in a Peripheral Position of the Cytoplasm

Erg6 is a protein associated with lipid droplets in the yeast *Saccharomyces cerevisiae*, hence, we hypothesized that ubiquitin-rich aggregates might colocalize with these structures. Since we observed the presence of Erg6-GFP in the ubiquitinated protein aggregates of all the strains through Western Blot analysis, we decided to further investigate the result by fluorescence microscopy. For this purpose, WT, *atg1Δ*, *cue5Δ* and BY4742 cells were grown to exponential phase, then were treated with 10% ethanol (v/v) for 24 hours and aliquots were taken before the addition of ethanol (control), after 3 hours of treatment and after 24 hours. After the exposure to the stress, cells were immune decorated, ubiquitin was visualized using an anti-ubiquitin antibody (P4D1, monoclonal) and lipid droplets were stained with a specific dye (MDH).
GFP fluorescence in Erg6-GFP cells was demonstrated to fade away after the digestion with Zymolyase 20T during the preparation of the cells for the immune decoration (Fig. 5), therefore, they were processed as BY4742 cells.

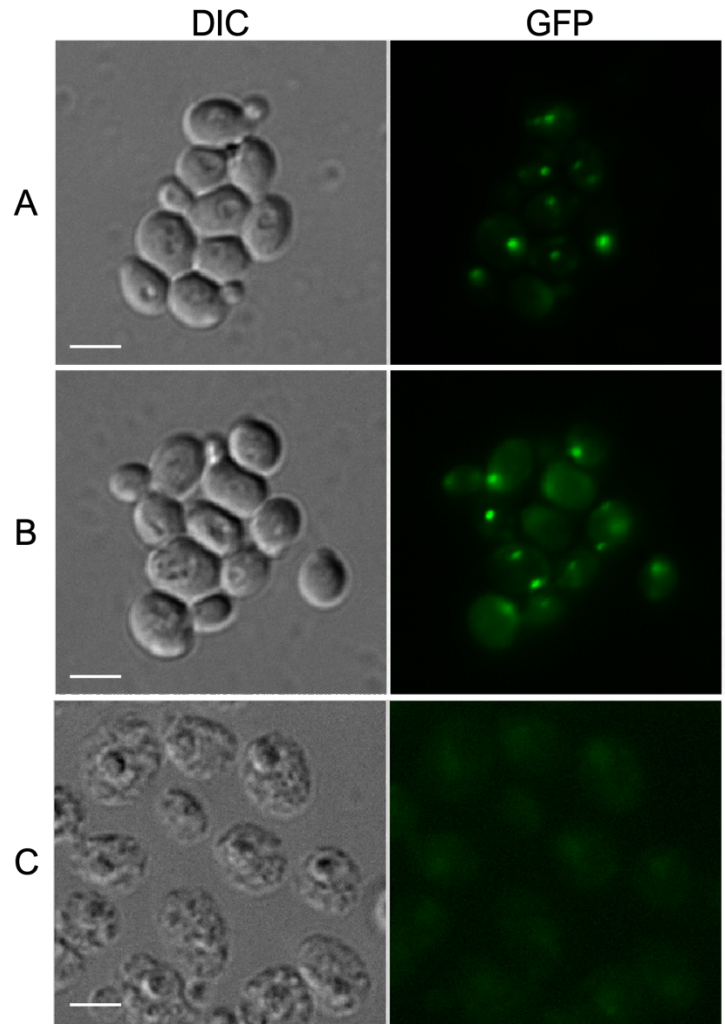
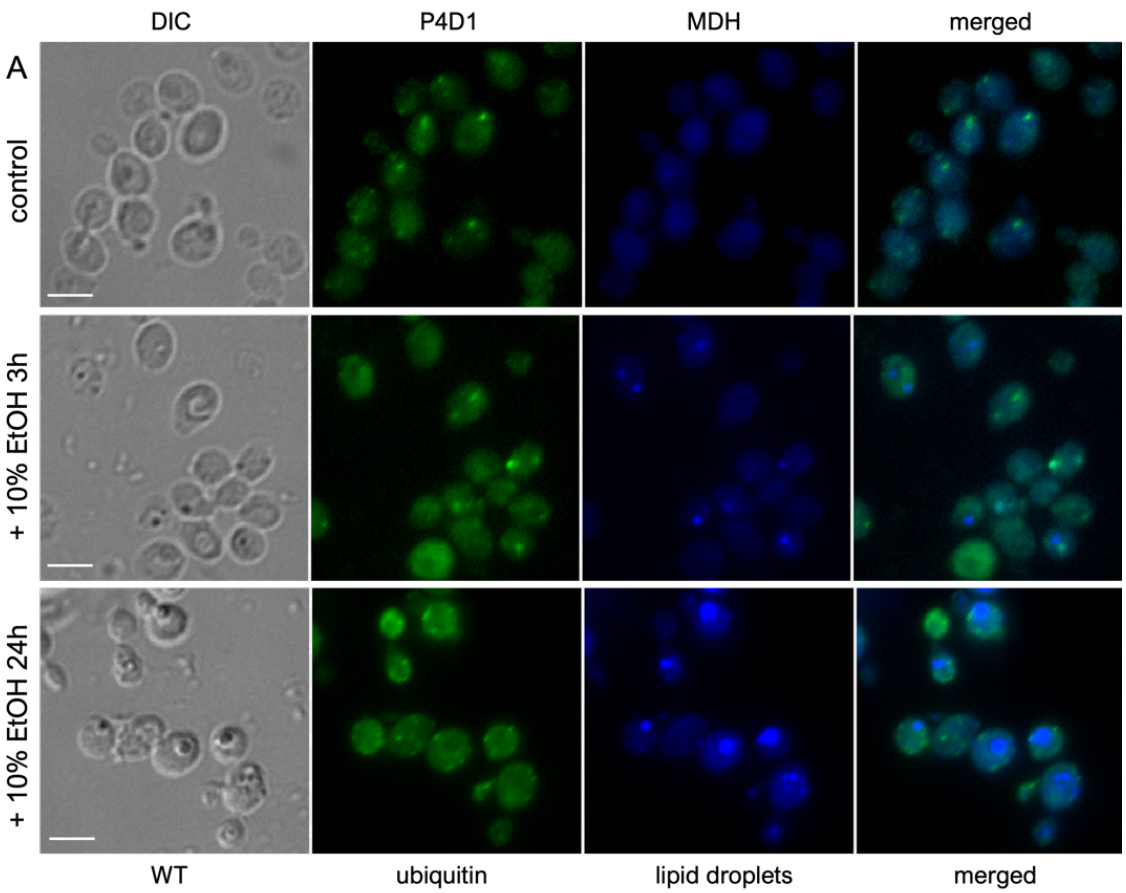


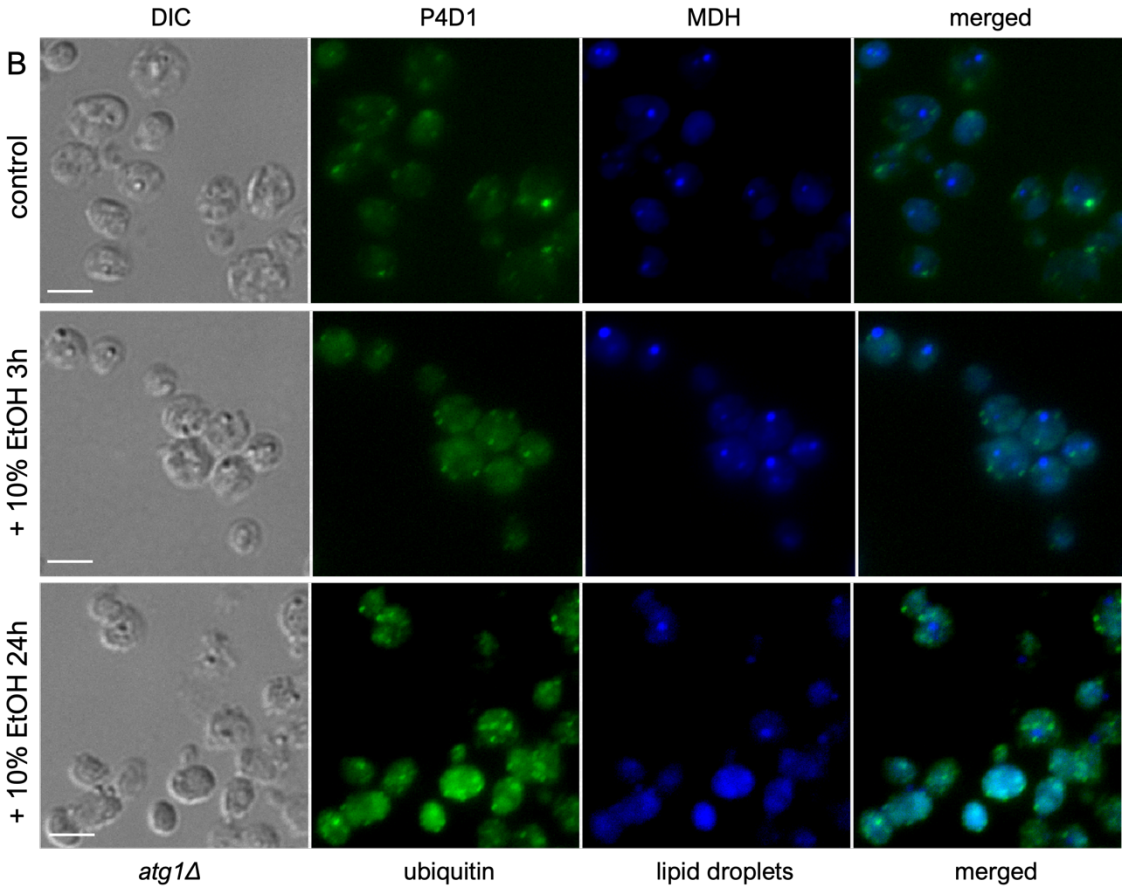
Figure 5. GFP fluorescence in Erg6-GFP cells fades away after the digestion with Zymolyase 20T. (A) Erg6-GFP cells grown in YPD medium under no stress, not processed. (B) Erg6-GFP cells grown in YPD medium under no stress, fixed for 10 minutes in 100% methanol. (C) Erg6-GFP cells grown in YPD medium under no stress, fixed for 10 minutes in 100% methanol, and digested for 55 minutes with Zymolyase 20T (5 mg/ml). Size bar 5 μm.

The formation of ubiquitin-rich aggregates in yeast cells after the treatment with 10% ethanol is shown in Figure 6. For all the strains, it is observable the presence of a few round spots before the treatment with 10% ethanol. In WT cells (Fig. 6A), the accumulation of ubiquitin after 3 hours of 10% ethanol stress is not different from the control. However, after 24 hours of stress, we can notice a greater number of ubiquitin-

rich aggregates. They appear to be smaller in size and do not colocalize with lipid droplets, instead they seem to reside as independent structures in a peripheral position of the cytoplasm. Similarly, in *atg1Δ* cells (Fig. 6B), it is appreciable the accumulation of small, round-shaped ubiquitinated protein aggregates. They appear soon after 3 hours of ethanol stress, and they seem not to colocalize with any cellular structure, but rather to be disseminated in a peripheral location in the cytosol. Interestingly, after 24 hours of stress the accumulation of ubiquitin-positive dots seems to be slightly greater than the one of the other strains, confirming what was previously observed in the Western Blot analysis. For *cue5Δ* cells (Fig. 6C), the presence of ubiquitin-rich aggregates seems to be inferior after the induction of ethanol stress compared to other strains, which is consistent to what was observed in the Western Blot analysis. Even in this strain, ubiquitinated spots are small and round-shaped, and do not colocalized with lipid droplets or other cellular structures, but appear to be free in the cytoplasm.

BY4742 cells (Fig. 6D) were used as a control, to highlight the differences between the initial strain and the Erg6-GFP strains. As shown in Fig. 6D, no major differences are detected within the strains. In BY4742 cells it is observable the accumulation of ubiquitin-rich aggregates after the treatment with 10% ethanol for 3 hours. For this strain as well, the dots appear to be small and round-shaped, in a peripheral location in the cytosol and not colocalized with any other cellular structure.


DIC
P4D1
MDH
merged
A
control
+ 10% EtOH 3h
+ 10% EtOH 24h
WT
ubiquitin
lipid droplets
merged


DIC
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MDH
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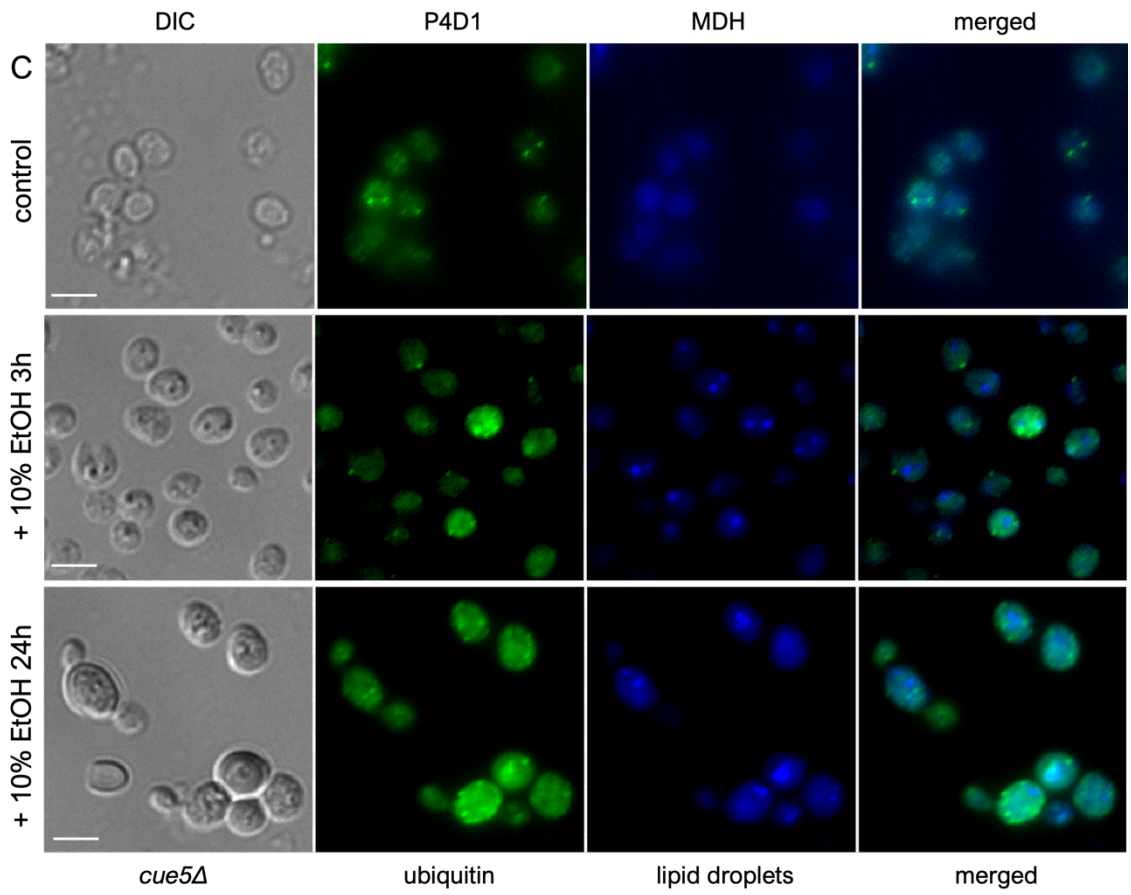
B
control
+ 10% EtOH 3h
+ 10% EtOH 24h
atg1Δ
ubiquitin
lipid droplets
merged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lipid droplets
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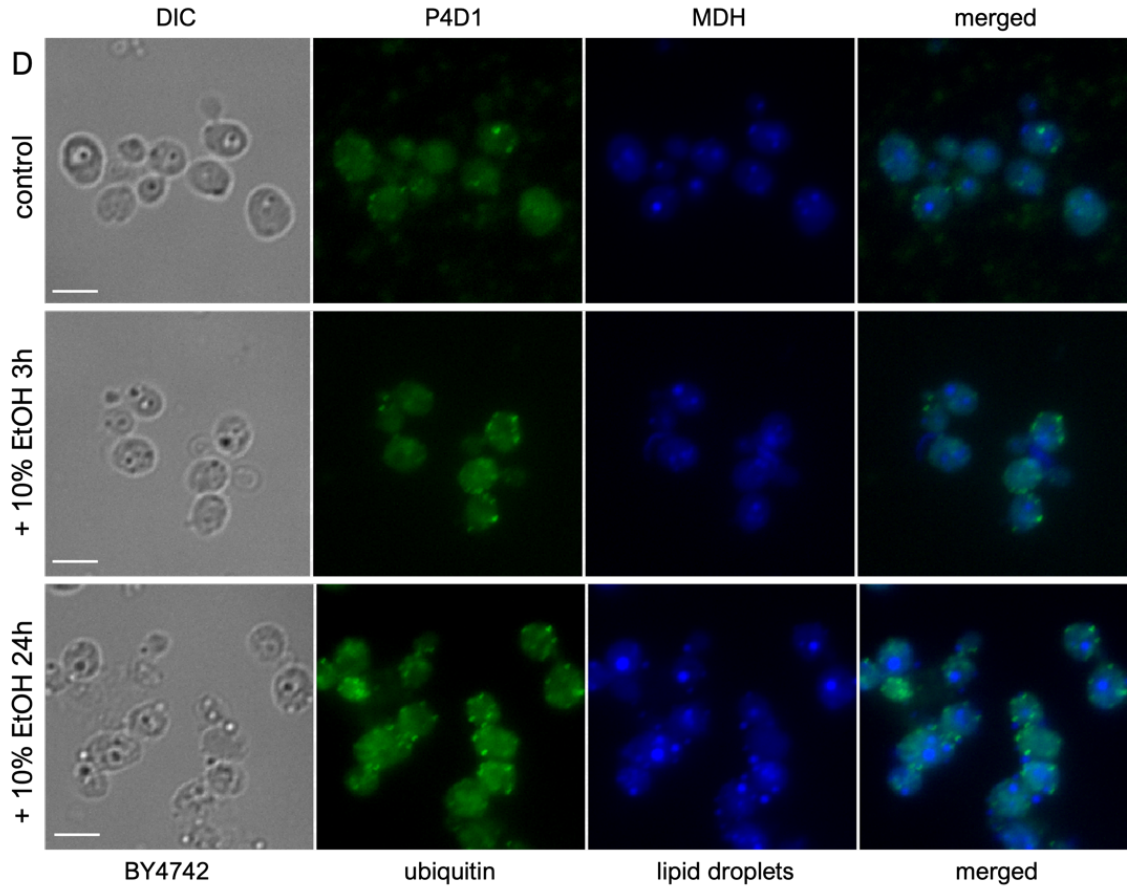
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BY4742
ubiquitin
lipid droplets
merged

Figure 6. Ubiquitin-rich aggregates visualization in Erg6-GFP strains. For all the strains, it is observable the presence of a few round spots before the treatment with 10% ethanol (v/v). (A) WT cells display accumulation of small round-shaped ubiquitin dots after 24 hours of 10% ethanol stress, which do not colocalize with lipid droplets. (B) *atg1Δ* cells show the accumulation of small, round-shaped ubiquitinated protein aggregates soon after 3 hours of stress. They are disseminated in a peripheral location in the cytosol and do not colocalize with other cellular structures. (C) in *cue5Δ* cells, the presence of ubiquitin-rich aggregates is inferior compared to other strains. Ubiquitinated spots are small and round-shaped, and do not colocalized with lipid droplets or other cellular structures but are free in the cytoplasm. (D) BY4742 is the control strain. It exhibits the accumulation of ubiquitin-rich aggregates soon after 3 hours of 10% ethanol treatment. The dots are small and round-shaped, in a peripheral location in the cytosol and do not colocalize with any other cellular structure. Ubiquitin is analyzed with P4D1 anti-ubiquitin monoclonal antibody (1:200) and detected with Alexa Fluor® 488 AffiniPure Donkey Anti-Mouse IgG (1:800). Lipid droplets are labeled with AUTOdot Autophagy Visualization Dye Monodansylpentane (MDH) (1:10’000). Size bar 5 µm.

DISCUSSION

Ethanol stress is known to induce protein dysfunction, as it induces the denaturation of polypeptides, leading to the accumulation of insoluble misfolded proteins (Piper, 1995; Ma and Liu, 2010; Escusa-Toret *et al.*, 2013). In the present study, we assessed the presence of ubiquitin-rich protein aggregates in the yeast *Saccharomyces cerevisiae* cells after the induction of severe stress with 10% ethanol (v/v) for 3 hours, which persisted for at least 24 hours in the cell. The accumulation of insoluble ubiquitinated proteins in reaction to the addition of ethanol is consistent in *S. cerevisiae* cells, as different strains and media were used during the investigation, and they all showed the same response.

We demonstrated that the accumulation of ubiquitin-rich aggregates is relevant after 3 hours of severe ethanol stress for both the wild type and the *atg1Δ* mutant. Importantly, although the level of ubiquitin decreases in the WT strain after 3 hours of stress, a large amount of ubiquitinated proteins is observable for the *atg1Δ* mutant 24 hours following the induction of the stress. This suggests that WT cells initiate the process of dismantling of ubiquitin-rich aggregates at some point after 3 hours of ethanol addition. On the other hand, *atg1Δ* mutant cells appear not to be able to disassemble ubiquitinated inclusions, which indicates that *ATG1* gene is implied in the process. *ATG1* encodes for the protein Atg1, a serine/threonine kinase which plays a crucial role in the regulation of macroautophagy, henceforth called autophagy. It represents one of the essential components for the formation of autophagosomes, and a null mutation in this gene prevents the induction of autophagy (Kamada *et al.*, 2000; Suzuki and Ohsumi, 2007; Papinski *et al.*, 2014). In our study, in the *atg1Δ* mutant cells, Western Blot analysis revealed that the level of ubiquitin remained stable even after 24 hours of the induction of severe ethanol stress, contrarily to the WT strain. For

this reason, we suggest that autophagy is most likely implied in the clearance of ubiquitin-rich protein aggregates in the yeast *Saccharomyces cerevisiae*. Further studies are necessary to confirm the hypothesis, however, this assumption is supported by the well-known process of aggrephagy, the selective degradation of protein aggregates performed by autophagy. Dendritic cells Aggresome-like Induced Structures (DALISs) are a species of ubiquitinated protein aggregates, formed by the accumulation of misfolded polypeptides in mammalian antigen-presenting cells during immune cell maturation (Lelouard *et al.*, 2004). These structures are exceptionally similar to the stress-induced ubiquitin-rich aggregates we observed in *S. cerevisiae*. Indeed, they accumulate upon induction of several stress conditions, such as puromycin addition, oxidative stress, starvation, and transfection, they function as ubiquitination sites for Defective Ribosomal Products (DRiPs) and their only components are proteins, ubiquitin and ubiquitin enzymes (Pierre, 2005). Interestingly, these mammalian inclusions were demonstrated to be cleared by the Chaperone-Assisted Selective Autophagy (CASA), a type of aggrephagy, which represents the selective autophagy of misfolded proteins following a chaperone-mediated formation of protein aggregates that are targeted to form autophagosomes (Lamark and Johansen, 2012).

On the other hand, *cue5Δ* mutant cells displayed a minor accumulation of ubiquitin after severe ethanol stress induction, compared to wild type cells. The yeast protein Cue5 is a ubiquitin-binding protein, which possesses a ubiquitin-binding domain (CUE) and an Atg8-interacting motif (AIM). Atg8 is structurally similar to ubiquitin, but it is required for the formation of the autophagosome and functions as a dock for substrates on their way to lysosomal degradation through the autophagy pathway (Mizushima *et al.*, 2011). Cue5 operates as a ubiquitin-Atg8 adaptor, binding both

ubiquitin and Atg8, hence, mediating the autophagic degradation by directly recognizing the ubiquitin conjugates in the ubiquitin-dependent autophagy pathway (Lu *et al.*, 2014). Moreover, when yeast cells are depleted of *CUE5* they are demonstrated to accumulate aggregation-prone proteins (Lu *et al.*, 2014). According to these findings, Cue5 has a role in aggrephagy, which efficiently clears the cell from protein aggregates by the ubiquitin-dependent autophagy pathway. Interestingly, our results show that *cue5Δ* mutant cells do not accumulate ubiquitin-rich aggregates under severe ethanol stress. For this reason, we suggest that Cue5, as ubiquitin-binding adaptor, might have a role in conjugating ubiquitin to the aggregates under severe ethanol stress. Further research is necessary to confirm this hypothesis and to evaluate whether in absence of Cue5 these aggregates do not accumulate at all, or if they form but are not ubiquitinated. Noteworthy, Cue5 is known to collaborate with Ubc4/Ubc5 ubiquitin-conjugating enzymes and the Rsp5 ubiquitin ligase, for the conjugation of ubiquitin to its substrate (Lu *et al.*, 2014). Therefore, it is plausible that under ethanol stress conditions the absence of *CUE5* does not interfere with the accumulation of the aggregates, but inhibits their ubiquitination.

We observed the presence of Erg6-GFP in the protein extracts, for the majority in the pellet fraction, under severe ethanol stress. Erg6 is a well-established marker of lipid droplets (Kumar *et al.*, 2020). In the present study, we demonstrated that Erg6-GFP accumulates in the insoluble fraction for all the three strains, WT, *atg1Δ* and *cue5Δ*, after 3 hours of ethanol stress, whereas it becomes soluble at some time between 3 and 24 hours of stress. This result suggests that the ubiquitin-rich aggregates colocalize or interact at some point with lipid droplets. In previous works, it was observed that proteins that function in ubiquitination pathways or that contain ubiquitin-binding motifs colocalize with lipid droplets, suggesting a LD-associated ubiquitination

complex (Bersuker and Olzmann, 2017). Interestingly, Cue5 is known to be recruited to ubiquitinated lipid droplets thanks to its ubiquitin-binding CUE domain (Kumar *et al.*, 2022). This finding strengthens our hypothesis of Cue5 having a role in the conjugation of ubiquitin to the aggregated substrate under ethanol stress, as in the *cue5Δ* mutant cells we observed no differences in the accumulation of Erg6-GFP, indicating that the aggregates probably accumulate even in absence of Cue5. However, such results have never been observed before, not in yeast as much as in mammals, therefore, further analysis is necessary to explore the functions of Cue5 in the ethanol-induced ubiquitin-rich aggregates.

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Conclusion

By prevailing opinion, protein aggregation represents a dangerous phenomenon, being generally associated to age-related neurodegenerative disorders, such as Alzheimer's or Parkinson's Diseases. However, this mechanism is not always a synonym of cellular failure or cell death. Indeed, evidence suggest that protein aggregation is a well-organized and conserved process employed by healthy eukaryotic cells to overcome the presence of large amounts of misfolded polypeptides, which are generated in response to changes in the physiological cellular environment and cannot promptly be rescued by the overloaded proteolytic machinery.

The yeast *Saccharomyces cerevisiae* represents a simple, yet powerful, model widely used for the research on cellular aggregates. The different dynamic deposition sites formed in this model, such as P-bodies and stress granules, have been demonstrated to portray the equivalent structures found in other eukaryotic species, despite the increased complexity in protein composition and function in multicellular eukaryotes. Indeed, *Saccharomyces cerevisiae* turned out to be a very suitable model for the studies we conducted for the present work.

During the first section of the present thesis, we demonstrated that heat shock temperatures and glucose starvation do not cause the accumulation of ubiquitin-rich aggregates in *S. cerevisiae*. Instead, as suggested in previous works, these stress conditions predominantly cause the accumulation of ribonucleoprotein bodies, mainly composed of RNA and RNA-binding proteins. These structures are transient and are mainly implied in the storage and decay of RNA, henceforth, we propose that the digestion and breakage of nucleic acids lead to the disruption of the aggregates themselves. On the other hand, the formation of ubiquitin-positive protein aggregates

in *S. cerevisiae* was observed in cells following the exposure to severe ethanol stress, under nitrogen starvation conditions, and after the treatment with sublethal doses of the protein synthesis inhibitor puromycin. Evidence showed that this response was persistent aside from the presence of nucleic acids, suggesting they do not belong to the category of RNP bodies. A peculiar species of ubiquitin-positive aggregates can be found in mammalian dendritic cells. Dendritic cell Aggresome-Like Induced Structures (DALISs) are transient deposition sites for ubiquitinated defective proteins in dendritic cells. Such structures are exquisitely similar to the stress-induced ubiquitin-rich aggregates we observed in *S. cerevisiae*, however, further studies should be conducted to shed light on their function, composition, and clearance to confirm the analogies between the two different species of aggregates.

During the second section of this thesis, we focused on investigating the clearance of ethanol-induced ubiquitin-rich protein aggregates in yeast cells. We demonstrated that the accumulation of ubiquitin-rich aggregates is persistent in *atg1Δ* mutant cells, whereas in wild type cells the level of ubiquitinated proteins decreases a few hours after the exposure to the stress. This suggested that autophagy is implied in the clearance of ubiquitinated aggregates, as Atg1 is a kinase which plays a crucial role in the regulation of this process. Further studies are necessary to confirm the hypothesis that autophagy might be implied in the clearance of ubiquitin-positive inclusions, however, this assumption is supported by the well-known process of aggrephagy, the selective degradation of protein aggregates performed by autophagy. DALISs, dendritic cells aggresome-like induced structures, were demonstrated to be cleared by the Chaperone-Assisted Selective Autophagy (CASA), a particular type of aggrephagy.

We demonstrated the presence of Erg6-GFP in the ubiquitin-positive insoluble fraction of the protein extracts of all the strains under severe ethanol stress. Erg6 is a well-known marker of lipid droplet, hence, this result suggests that the ubiquitin-rich aggregates colocalize or interact at some point with lipid droplets. Interestingly, the yeast protein Cue5 is a ubiquitin-binding protein that in known to be recruited to lipid droplets through its ubiquitin-binding domain. We observed that *cue5Δ* mutant cells displayed a minor accumulation of ubiquitin throughout the whole treatment with ethanol, compared to wild type and *atg1Δ* mutant cells. Despite the lack of accumulation of ubiquitin in *cue5Δ* mutant cells, the levels of Erg6-GFP appeared equal to the ones of the other strains. These findings suggest that, under severe ethanol stress, proteins aggregate despite the lack of Cue5, which, on the other hand, might have a role in the conjugation of ubiquitin to the ethanol-induced aggregated proteins. Such hypothesis is supported by the evidence that Cue5 is known to collaborate with the Ubc4/Ubc5 ubiquitin-conjugating enzymes and the Rsp5 ubiquitin ligase for the conjugation of ubiquitin to its substrate. However, these results have never been observed before, not in yeast as much as in mammals, therefore, further analysis is necessary to explore the functions of Cue5 in the ethanol-induced ubiquitin-rich aggregates.

Finally, fluorescence microscopy investigation in *Saccharomyces cerevisiae* cells demonstrated the accumulation of ubiquitin-positive aggregates under nitrogen starvation conditions and severe ethanol stress. In the first case, ubiquitinated aggregates appeared as a few large round-shaped, independent structures localized in a peripheral position in the cytoplasm. In the second case, ubiquitin-positive dots displayed a similar round shape and localization inside the cell, however, they appeared to be smaller in size and more numerous in count. Nonetheless, the

ubiquitin-rich structures we observed in *Saccharomyces cerevisiae* cells through fluorescence microscopy reflect the characteristic morphology reported in dendritic cells for DALIS, encouraging the idea that these two structures, yeast stress-induced ubiquitin-rich aggregates and mammalian DALIS, are similar and should be allocated under the same category.

To summarize, severe ethanol stress is a condition that persistently stimulate the accumulation of ubiquitin-rich protein aggregates in the yeast *Saccharomyces cerevisiae*. Such aggregates appear as many small round-shaped dots, which are not colocalized with any other cellular structure inside the cell, but rather are independent formations localized in a peripheral position in the cytoplasm. Ethanol-induced ubiquitin-positive aggregates are supposedly cleared by macroautophagy and the ubiquitin-binding protein Cue5 seems to be involved in their ubiquitination.

Future Perspectives

The formation of protein aggregates represents a major topic in the scientific research, being widely associated with the pathological onset of several neurodegenerative disorders, such as Alzheimer's, Parkinson's, and Huntington's Disease. Nevertheless, the study of functional protein aggregates formed under stress conditions, other than stress granules and P-bodies, is poor.

The present thesis tries to underline the importance of studying other protein inclusions which might be beneficial to the eukaryotic cell, using *Saccharomyces cerevisiae* as model organism. This preliminary work lays the foundation to unravel the molecular mechanisms involved in the process of aggregates formation and clearance, other than revealing a new type of protein deposition sites in yeast cell. However, further studies are necessary to characterize such aggregates, shedding light on their components and functions in the yeast cells. The identification of marker proteins will help analyzing the ubiquitin-rich aggregates faster, creating an easy reproducible model. Finally, a parallel validation in mammalian systems will elucidate the features of this phenomenon.