Role of the Protein Quality Control System in basal and stress conditions in fission yeast:

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A mi madre, Nieves

“Do or do not. There is no try.”

Master Yoda

The Empire strikes back (1980)

Agradecimientos

En primer lugar, quiero agradecerle a Elena que me diera la oportunidad de realizar el doctorado en su laboratorio y su dirección durante estos años. A todos mis compañeros que han sido amigos fuera y dentro del 383. Por supuesto, a los amigos que Barcelona me ha traído que la han convertido en mi casa. Y por último, y por eso la más importante, dar las gracias a mi madre que con su esfuerzo ha permitido que llegara hasta aquí y si algo nunca me ha faltado ha sido su apoyo.

Abstract

Proteostasis is the proper homeostasis of the concentration and the molecular structure of the proteins, which preserves the integrity of the proteome. Proteostasis is constantly challenged by intrinsic and extrinsic factors which must be buffered by the Protein Quality Control (PQC) system that is composed by a large network of the Ubiquitin Proteasome System machinery (UPS) and the molecular chaperones. Indeed, many diseases are associated with the PQC system failure such as neurovegetative diseases, aging and cancer.

During this thesis project we were developed Schizosaccharomyces pombe as model to study the relation between the PQC system and the stress response. First, we study how the maintaining of the proper turnover of the proteins in basal conditions is important for the regulation of transduction sig-nal cascades. So that, we have developed the transcription factor Pap1 as model to characterize the mechanisms and identify the UPS machinery in-volved in its degradation.

Second, the accumulation of non-native proteins can lead to toxic effect in the cell. We are interested in studying the cellular response upon this kind of proteotoxic stress. In this way, we characterized the expression of the hu-man non-native protein reports HttNTD.nQ and TDP-43 in fission yeast.

Resum

La proteostasi és l'homeòstasi adequada de la concentració i l'estructura molecular de les proteïnes, que preserva la integritat del proteoma. La proteostasi és veu alterada constantment per factors intrínsecs i extrínsecs que han de ser reequilibrats pel sistema de control de qualitat de les proteïnes (PQC) que està format per una gran xarxa de maquinària del sistema Ubiquitina Proteasoma (UPS) i de les xaperones moleculars. De fet, moltes malalties estan associades a la fallada del sistema PQC com ara malalties neurodegeneratives, envelli-ment i càncer.

Durant aquest projecte de tesi, es va desenvolupar Schizosaccharomyces pombe com a model per estudiar la relació entre el sistema PQC i la resposta a l'estrès. Primer, estudiem com d'important és el manteniment dels nivells adequats de les proteïnes en condicions basals per a la regulació de les cascades de transducció de senyal. Per tant, hem escollit el factor de transcripció Pap1 com a model per caracteritzar els mecanismes i identificar la maquinària d'UPS implicada en la seva degradació.

En segon lloc, l'acumulació de proteïnes no natives pot provocar efectes tòxics a la cèl·lula. Ens interessa estudiar la resposta cel·lular davant d'aquest tipus d'estrès proteotòxic. D'aquesta manera, es va caracteritzar l'expressió de proteïnes humanes no natives HttNTD.nQ i TDP-43 en el llevat de fissió.

Contents

Introduction

Introduction

1. Basis of a healthy proteome: the proteostasis network

Proteins come in many sizes and shapes, and display diverse chemical properties. That is why they can perform an amazing array of diverse functions inside and outside cells, allowing them to live and function properly. Proteins can be classified in diverse functional groups, such as structural proteins, scaffold proteins, enzymes, transporters, signaling, molecular machines… The term proteome was created to refer to the entire protein complement of a given cell type. The process of protein synthesis itself, or translation, and the post-translational regulation of stability, modification or localization of proteins, is crucial for protein function and, therefore, for cell survival. Each one of these steps is subject of intense investigation.

During protein synthesis, protein folding into the native state needs to occur in a timely fashion to reach proteins' correct shapes and functions. The cell makes a set of proteins called chaperones to facilitate the proper folding of nascent proteins. As will be widely explained below, the inappropriate covalent modifications in the protein's structure during synthesis can lead to misfolding protein intermediates, which should be targeted for proteolytic degradation. Therefore, chaperones and degradation machineries are required by all cell types to protect the synthesis of their proteomes even in the absence of additional stressors (Fig. 1).

After synthesis, the structure of proteins is continuously challenged by intrinsic and extrinsic cues. The functional or native protein can give rise to non-native intermediates, with concomitant loss of function and aggregation propensity. Therefore, chaperones of different families are involved in the recognition of damaged protein structures and their refolding (Fig. 1).

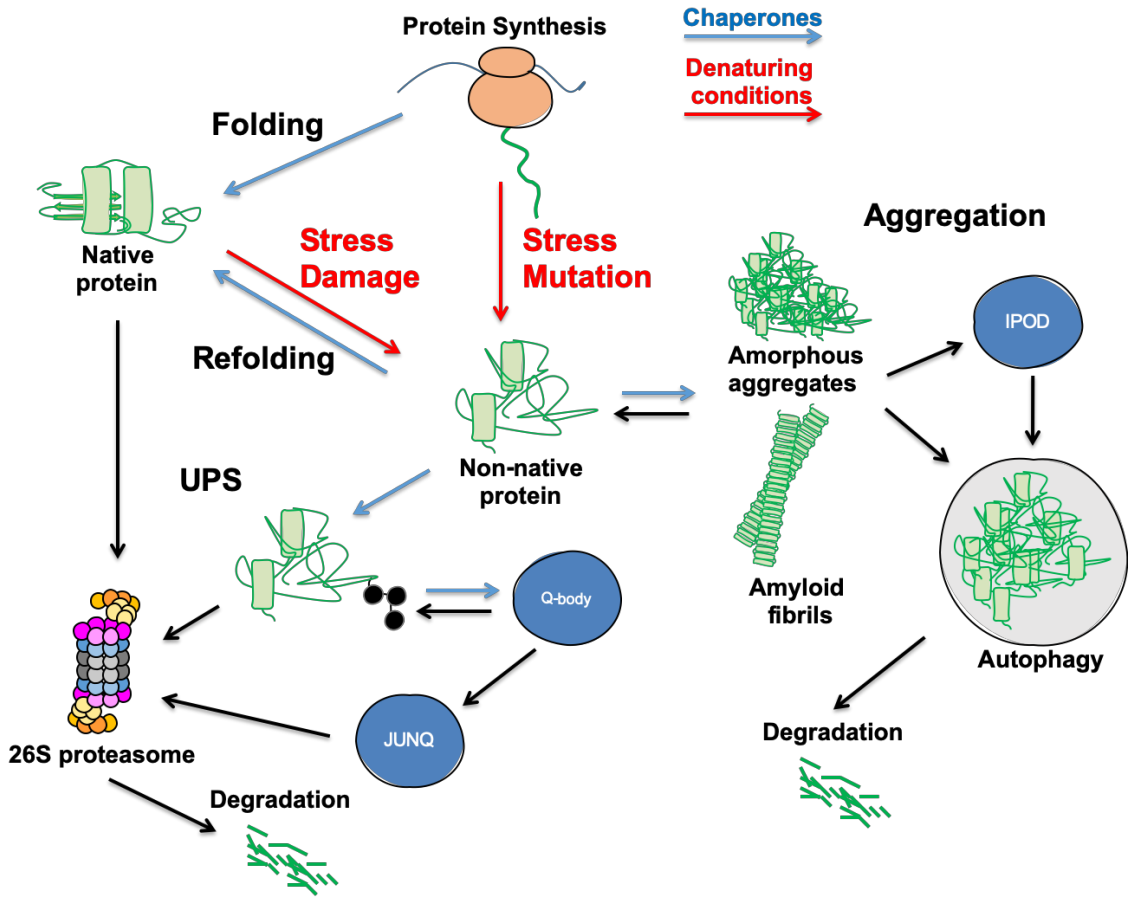

Figure 1. Proteostasis map. Proteostasis is multicompartmental system that coordinates protein synthesis, (re)folding, aggregation and degradation. Red arrows indicate denaturing events, whereas blue arrows indicate processes where chaperones are involved (modified from (Hartl, Bracher et al. 2011).

Another important aspect of post-translational control is the regulation of protein stability. Proteins have to be maintained at certain concentrations to accomplish their functions. The rate of synthesis through transcription and translation is important; however, the life span of intracellular proteins also modulates their steady-state concentrations. Protein stability is controlled primarily by regulated protein degradation, again. Therefore, there are two especially important roles for protein degradation (Fig. 2):

I. The removal of proteins that are toxic, damaged or improperly folded;
II. The controlled degradation of otherwise native healthy proteins; this provides a powerful mechanism for reaching the proper steady-state levels of a given protein, and for promoting rapid changes in protein levels in response to changing conditions.

In both cases, the controlled degradation of proteins is performed in eukaryotes at the level or the proteasome (see 2.2.2.1.2).

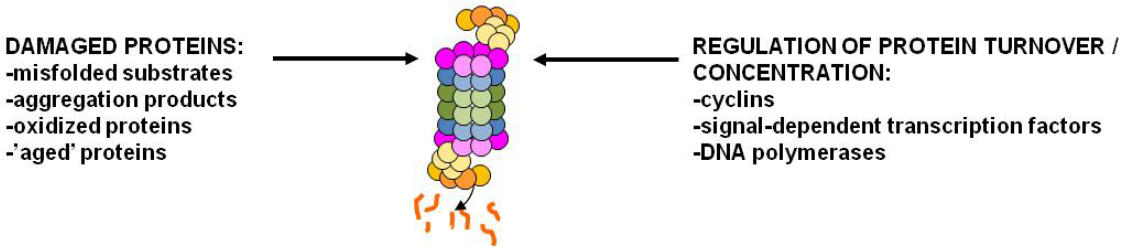

Figure 2. Regulation of protein stability by the proteasome. Dual function of the protein degradation.

In summary, during my thesis project I have studied **proteostasis** using the unicellular eukaryote *Schizosaccharomyces pombe* as a model system. Proteostasis, or correct homeostasis of the cellular proteome, is maintained through numerous biological pathways within the cell, including chaperones and degradation machineries, to control the concentration, folding and degradation of normal or damaged proteins. I have studied the control of protein concentration of a specific transcription factor, Pap1, and have also tested the effect of protein aggregation on cellular fitness.

2. Protein Quality Control (PQC) system: the guardian of proteostasis

As explained above, the concentration and structure of native proteins is not rigid, and the whole proteome is exposed to many intrinsic and extrinsic factors that can affect it. For instance, a massive increase in protein synthesis in response to stress, or temperature up-shifts which promote general protein unfolding, can challenge the network of basal proteostasis machineries, generating proteotoxicity. These effects are associated with several diseases, particularly with neurodegenerative diseases, such as Huntington's Disease (HD), Alzheimer's disease or amyotrophic lateral sclerosis (ALS), where it is usually found accumulated non-native proteins (Muchowski and Wacker 2005). In addition, proteostasis failure is also related to aging and cancer (Chen, Retzlaff et al. 2011).

Therefore, cells have developed the Protein Quality Control (PQC) system with the goal of preserving the proteome integrity. This system is constituted by a network of molecular chaperones and by degradation machineries, namely the Ubiquitin Proteasome System (UPS) and autophagy. Thus, these PQC components will recognize proteins that can alter the proteome integrity and will perform three alternative tasks (refolding, degradation or sequestration) to promote cellular fitness. We will first summarize the different families of chaperones (see 2.1), to then describe the three fates of chaperone-bound protein complexes (see 2.2):

I. Refolding towards the native conformation (see 2.2.1)
II. Degradation by the UPS (involved not only in degrading damaged proteins but also regulating protein stability (Fig. 2) (see 2.2.2)
III. Aggregation of terminally-misfolded proteins and sequestration in sub-cellular compartments (see 2.2.3).

2.1. Chaperone families

Chaperones are defined as proteins that interact with, stabilize or help other proteins to acquire their functionally active conformations, but without being present in their final structure. Thus, chaperones perform many essential cellular functions such as folding of newly synthesized proteins, assembly and disassembly of macromolecular protein structures, or selecting the fate of misfolded proteins towards refolding, degradation or aggregation. Often, chaperones are referred as Heat Shock Proteins (Hsp) as their expression increases after heat stress through the activation of the transcription factor Hsf1. There are several conserved chaperone families that are mostly classified according to their molecular weight (Hsp40, Hsp70, Hsp90, Hsp100, and the small Hsp) (reviewed in (Balchin, Hayer-Hartl et al. 2016).

Ribosome associated chaperones

During synthesis, the chain of amino acids is folded into the native structure while it is at the ribosome. For this reason, there are chaperone complexes that are

associated directly to the ribosome. They are providing support for a proper folding, as well as prevent the aberrant association between non-native new proteins and other misfolded proteins. These complexes are the Trigger Factor in bacteria, and the Ribosome associated complex (RAC) and the Nascent chain Associated Complex (NAC) in Eukarya (Balchin, Hayer-Hartl et al. 2016).

HSP70/40 System

The Heat Shock Protein 70 KDa (Hsp70) family of chaperones is a large ubiquitous ATP-dependent family of proteins that binds to substrates which usually expose a sequence of 5 to 7 amino acid sequence that are enriched in hydrophobic residues and are typically exposed by non-native proteins. Hsp70s participate in many biological processes such as folding, refolding, protein assembly and disassembly, translocation, aggregation prevention or facilitated degradation (Walsh, Bursac et al. 2004, Mandal, Nillegoda et al. 2008, Kampinga and Craig 2010, Craig and Marszalek 2017). However, they have just one biochemical activity: an ATP-dependent client binding and releasing cycle of non-native substrates. This activity allows the transfer of non-native proteins to other PQC components (Kampinga and Craig 2010, Balchin, Hayer-Hartl et al. 2016).

Hsp70s have a simple structure. They have two domains: a 40 kDa N-terminal nucleotide-binding domain (NBD) and a 30 kDa C-terminal substrate-binding domain (SBD) with and inner hydrophobic linker. At the same time, the SBD consists of a ß-sandwich domain, that has the peptide-binding site, and an α-helical lid segment. Peptide substrate binds in an extended conformation in a groove in the b-sandwich domain.

Hsp70 canonical function cycle is dependent on nucleotide binding, and at the same time, this interaction depends on the regulation of co-chaperones Hsp40s and nucleotide exchange factors (NEFs). First, the co-chaperones Hsp40 recognize and transfer substrates to Hsp70 in the ATP state where SBD is open. Interaction of Hsp40 with Hsp70 accelerates the ATP hydrolysis resulting in an Hsp70 ADP-bound state where SBD and NBD are loosely associated, and the SBD is in the closed conformation trapping the substrate. Now, NEF

binding to NBD facilitates ADP-ATP exchange turning the SBD to open, and the substrate is released to a downstream proteostasis network machinery (Fig. 3).

Recently it has been described that the NEF protein in *S. cerevisiae* Fes1 is involved in proteotoxic misfolded protein degradation by the UPS (Gowda, Kaimal et al. 2016). There is just one NEF protein (Fes1) in *S. pombe* that is essential, which confirms the biological relevance of the Hsp70 cycle.

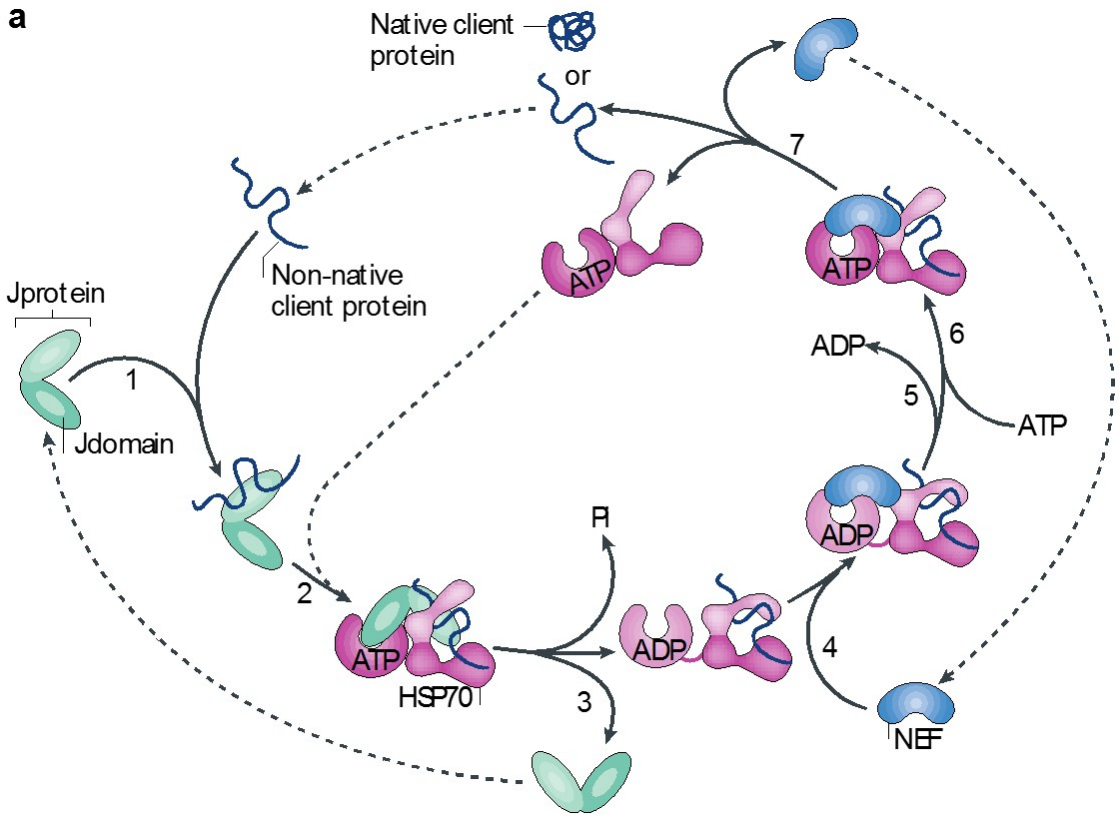

Figure 3. Functional cycle of Hsp70/40 chaperones system. Non-native proteins are recognized by Hsp40 and then transfer to Hsp70. Hsp40 promotes ATP hydrolysis and Hsp70 encage the substrate. NEFs promote the exchange of ADP for ATP and the substrate release ((Kampinga and Craig 2010).

Now, how do Hsp70s have a huge diversity of cellular functions with only one biochemical activity? Thanks to the Hsp40 co-chaperone family. Hsp40s bind to Hsp70s and localize them in specific sub-cellular locations or provide specific substrates. Hsp40 enzymes are also known as J-proteins because their structure is characterized by a conserved J domain that directs their interaction with a partner Hsp70 and that controls the rate of ATP hydrolysis by the Hsp70 protein. Hsp40 has just one simple function: it accelerates the ATPase function

of Hsp70. However, the different classes of J-proteins may have many different sequences and structures that give them different locations and recognition of substrates, explaining the multi-functionality of the Hsp70 system (Kampinga and Craig 2010, Balchin, Hayer-Hartl et al. 2016, Craig and Marszalek 2017). Moreover, there is a limited number of Hsp70 types per cell even thought they are very abundant, while each cell has many different Hsp40 family members. This implies that each Hsp70 has more than a J-protein partner.

The first characterized J-protein in yeast was the *S. cerevisiae* protein Ydj1 (Yeast dnaJ) (Caplan and Douglas 1991). Ydj1 is related to several cellular process such as stabilization of a huge number of Ssa1 substrates, particularly kinases (Mandal, Nillegoda et al. 2008); interaction between Hsp90 and its clients (Flom, Lemieszek et al. 2008); DNA damage response (Sluder, Nitika et al. 2018), and the clearance of misfolded proteins via UPS (see 2.2.2.1.4) (Park, Bolender et al. 2007).

Another important Hsp40 in *S. cerevisiae*, which is homologue of the human DnaJB1, is Sis1. Sis1 recognizes cytosolic misfolded proteins and transfers them to the UPS for degradation (Park, Kukushkin et al. 2013, Samant, Livingston et al. 2018). Sis1 also is associated with the prevention of prions-associated toxicity. Moreover, Sis1 is also related to the compartmentalization of aggregates (Sontag, Samant et al. 2017). Both Sis1 and Ydj1 regulate the activity of the Hsp70 Ssa1.

In *S. pombe*, the Ydj1 homologue Mas5 and the Hsp70 Ssa2 have been described to repress the transcription factor Hsf1 (see 4.2.3) in the absence of heat stress. Cells lacking Mas5 display constitutive activation of the Hsf1-dependent expression program (Vjestica, Zhang et al. 2013).

Hsp100

In the 1990s the group of Susan Lindquist demonstrated that the *S. cerevisiae* Hsp104 chaperone is able to dissolve protein aggregates (Parsell, Kowal et al. 1994, Parsell, Kowal et al. 1994, Glover and Lindquist 1998). This was a big change in proteostasis research because until that moment it was believed that protein aggregates were an irreversible state.

Hsp104 belongs to the ClpB/Hsp100 family of protein-remodelling machines or AAA+ proteins (ATPases associated with various cellular activities), that use ATP to rescue proteins from an aggregated state (Doyle and Wickner 2009). These proteins are the main disaggregases in yeast (Hsp104), plants (Hsp101) and eubacteria (ClpB), but they are absent in mammalian cells. However, the exogenous expression of Hsp104 in human cells improves their chaperone capacity (Mosser, Ho et al. 2004).

Hsp104 is a 102 kDa large protein that forms a tunnel and it is defined by a N-terminal Domain (ND), a Nucleotide Binding Domain 1 (NBD1), a Middle Domain (MD), another NBD2 and a C-terminal Domain (CD). ND is not essential for thermotolerance, but its deletion leads to some problems in recognition of specific substrates. NBDs have the canonical AAA+ sequence where the ATP hydrolysis occurs. The MD is necessary for Hsp70/40 interaction. Finally, the CD is related to hexamer assembly (Doyle and Wickner 2009).

Hsp104 does not have an important role in normal growth, but it is involved in many aggregation-related processes such as prions homeostasis and it is essential for a correct fitness recovering after stress (Parsell, Kowal et al. 1994). Hsp104 alone can recognize specific substrates like prions. Indeed, a high concentration of Hsp104 *in vitro* prevents the formation of large fibrils of [PSI+] yeast prion by elimination of oligomeric intermediates that nucleate fibril formation (Shorter and Lindquist 2004). Furthermore, it was demonstrated that Hsp104 remodels other non-prion substrates such as soluble aggregates (Doyle, Shorter et al. 2007). However, for rescuing native proteins from aggregates Hsp104 requires the additional presence of the Hsp70/40 couple (Glover and Lindquist 1998), (Fig. 4).

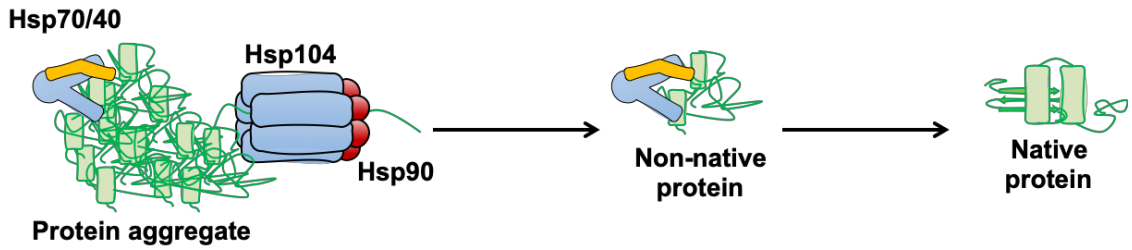

Figure 4. Hsp104 cooperates with other chaperones during disaggregation. Hsp70/40 and Hsp90 make easier the disaggregation function of Hsp104. When one misfolded protein is token from the aggregate, chaperones bind it to ensure its soluble state and refolding (based in (Bosl, Grimminger et al. 2006)).

Chaperonines

This family of chaperones includes huge complexes which provide an appropriate environment for protein folding. Chaperonins form a 1 MDa cylindrical complex consisting in two rings of seven to nine 60 KDa subunits. Its function is enclosing non-native single proteins in order to avoid aggregation and give them a perfect environment for folding by smoothing their folding energy landscape using ATP. Chaperonins may act downstream of the Hsp70 system. Among them, we highlight the following:

I. GroEL (bacterial cytosol), Hsp60 (mitochondria) and Cpn60 (chloroplasts).
II. Thermosome (archeas) and TRiC (eukaryotic cytosol).

Chaperonines of each one of these two groups display important differences regarding client recognition and folding system mechanism, and therefore clients of one group cannot be clients of the other (Balchin, Hayer-Hartl et al. 2016).

HSP90

Hsp90 is a very conserved family of chaperones and it is the most abundant in the cytosol. Hsp90 family members support not only protein folding but also conformational maturation and maintenance of its substrates which are important proteins such as transcription factors, steroid hormone receptors and proto-oncogenic kinases (Oppermann, Levinson et al. 1981, Joab, Radanyi et al. 1984, Wilhelmsson, Cuthill et al. 1990, Aligue, Akhavan-Niak et al. 1994, Cutforth and Rubin 1994). It has been described that Hsp90s are not only involved in non-native protein folding but also in degradation. Clients of Hsp90s can be identified because in absence of Hsp90 activity, their function is impaired because they are degraded by the proteasome or aggregated (Taipale, Jarosz et al. 2010). They cooperate with Hsp70 promoting ubiquitination (McClellan, Scott et al. 2005, Samant, Livingston et al. 2018).

Hsp90 is a homodimer, where each subunit consists of an N-terminal nucleotide-binding domain, a middle domain and a C-terminal dimerization domain.

Hsp90 have different conformations that depend on the nucleotide, substrate or co-factors binding.

Small Hsp

Small Hsps (sHsps) are chaperones of low molecular weight (12-43 kDa) that associate with non-native proteins in order to prevent irreversible aggregates in an ATP-independent manner. Thus, they allow the subsequent substrate solubilisation and refolding by ATP-dependent chaperones (Hsp70, Hsp100). Recently, in *E. coli*, it has been demonstrated that in the sHsp-substrate complex the oligomers of sHsp are displaced by Hsp70 and then Hsp70 recruits Hsp100 to resolubilize substrates (Zwirowski, Klosowska et al. 2017) (Fig. 5). sHsps are present from bacteria to mammalian cells, but with low conservation. In bacteria they are termed IbpAB, in *S. cerevisiae,* Hsp26 or Hsp42 and in *S. pombe,* Hsp16 or Hsp9.

sHsp structure is very important for their function and they are able to assemble into oligomers in basal conditions in a dynamic continuous exchange of monomers. Upon stress conditions, usually sHsps shift from oligomeric to smaller forms, often dimers, which bind to misfolded proteins (reviewed in (Haslbeck and Vierling 2015).

Furthermore, sHsp are involved in the maturation of insoluble aggregates. They collaborate in the fusion of small aggregates in bigger inclusion (Coelho, Lade et al. 2014).

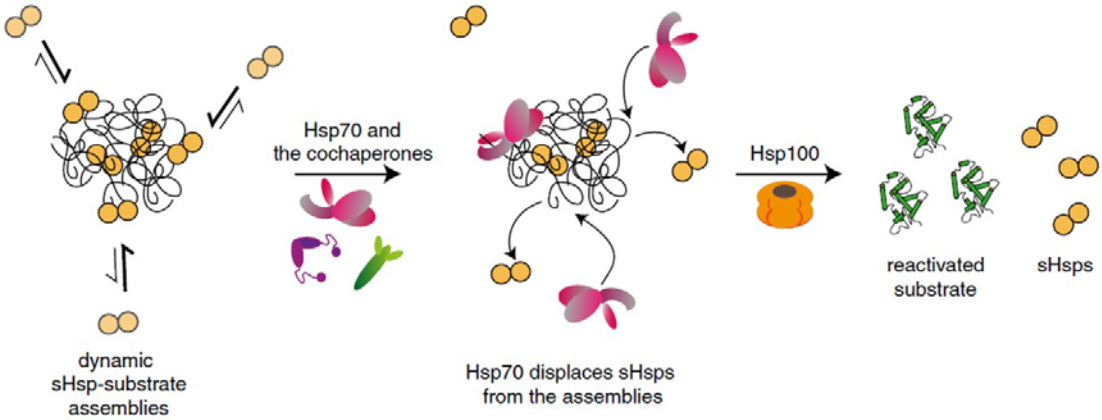

Figure 5. Action model of sHsp. sHsps form dynamic sHsp-substrate assemblies waiting for Hsp70 action that displaces sHsps and consequently Hsp100 cooperate with Hsp70-substate for disaggregation (Zwirowski, Klosowska et al. 2017).

2.2. Non-native proteins fates

Now I will explain the three different fates of non-native proteins, which are orchestrated by the PQC system: refolding, degradation and sequestration of aggregates.

2.2.1. Folding and refolding

As I mentioned before, the structure of a protein is crucial for its function. The protein folding is the physical process by which an amino acids sequence acquires a proper functional native 3D conformation. However, once synthesized, the native structure of mature proteins is vulnerable and can be disturbed by many insults. Then, non-native proteins are recognized by PQC chaperones network and they can be refolded to the proper conformation again.

It was in the 1950s in the Anfinsen's laboratory where it was demonstrated that small proteins refold spontaneously *in vitro* on removal of denaturalization conditions (Anfinsen 1973). Nevertheless, during 1980s it has described that larger proteins need the assistance from chaperones for folding (Hartl 1996). High energy unfolded proteins have to go through substantial kinetic energy barriers and different favourable fold intermediate states to reach a native conformation. Thus, chaperones provide assistance to protein folding in two ways:

I. Lowering free-energies barriers promoting the correct intramolecular conformation.
II. Preventing intermolecular associations avoiding thermodynamically stable protein aggregates (Chiti and Dobson 2006, Balchin, Hayer-Hartl et al. 2016) (Fig. 6).

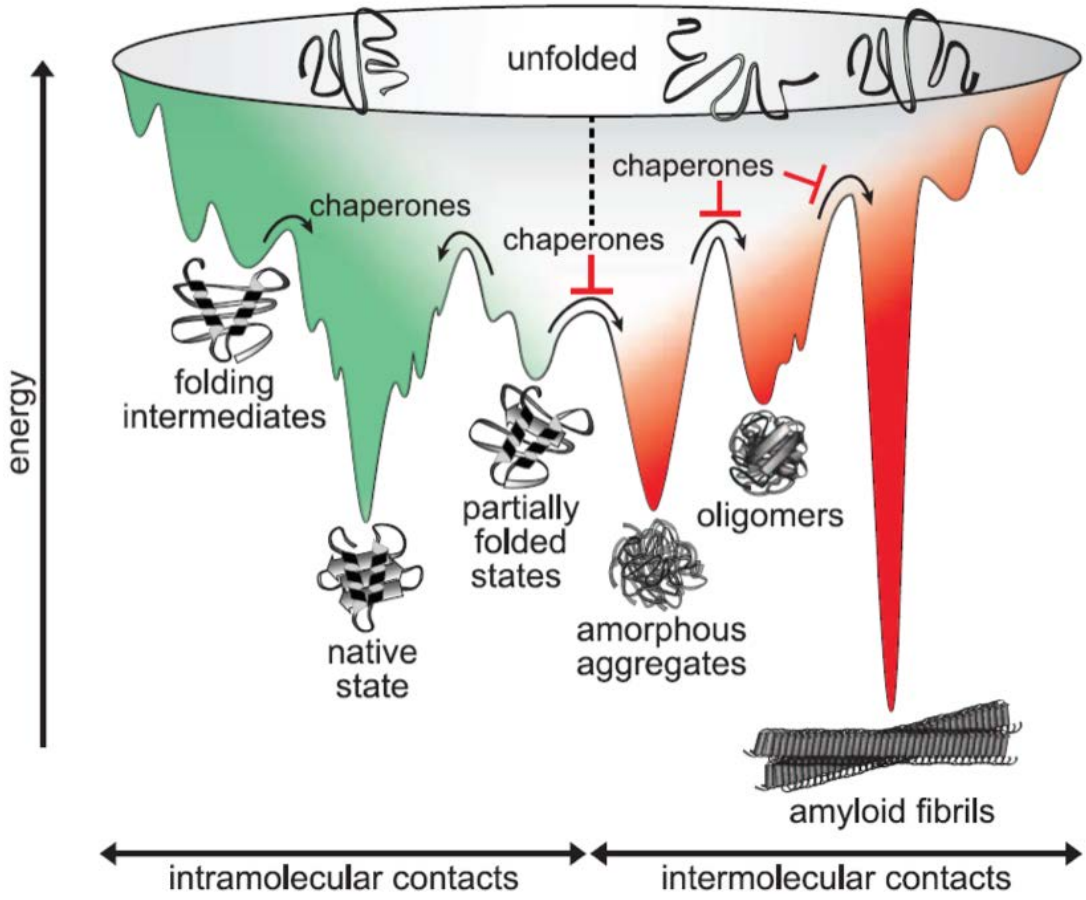

Figure 6. Thermodynamic landscape of protein folding. Unfolded proteins are in a high energy state and they must travel downhill on a more thermodynamically favourable state. Green map points to intramolecular contacts of different folding intermediates. Whereas, red mad points to intermolecular interactions. Molecular chaperones assist the proper folding and prevent the aberrant intermolecular contacts (Balchin, Hayer-Hartl et al. 2016).

Very recently, it has been shown that the rate of synthesis that can be regulated in order to optimize folding. A slower ratio of translation prevents misfolding of the nascent polypeptides (Spencer, Siller et al. 2012, O'Brien, Vendruscolo et al. 2014).

2.2.2. Degradation: UPS and Autophagy

In the 1940s, Rudolph Schoenheimer demonstrated that cell's proteins turnover (Schoenheimer, Ratner et al. 1939). The life span of proteins can be very variable, from few minutes until as long as the life of an organism.

There are two cellular processes for degradation of proteins: the Ubiquitin Proteasome System (UPS) and autophagy. The UPS is a selective proteolytic system where degradation of substrates is conjugated with the ubiquitin protein and then they are cleaved by the proteasome complex. On the other hand, autophagy degrades cytosolic spaces through the lysosome. The UPS is responsible of 80-90% of cellular proteolysis, whereas autophagy does the other 10-20% (reviewed (Kwon and Ciechanover 2017).

2.2.2.1. Ubiquitin Proteome System (UPS)

In the late 1990s, Aaron Ciechanover defined the UPS as the cellular system to eliminate proteins in a specific manner (Hershko and Ciechanover 1998). Thereby, I will explain the UPS function that is divided in two steps:

I. The substrate recognition and the concomitant covalent ubiquitin attaching.
II. Degradation through 26S proteasome complex.

Then, I will talk about the two kind of substrates of the UPS:

I. Non-native proteins.
II. Functionally proteins whose elimination is programmed.

2.2.2.1.1. <u>Ubiquitination</u>

The specificity of UPS is achieved by the previous conjugation of substrates with ubiquitin. However, ubiquitination is more than a degradation signal, is a post-translational modification that can mark different protein fates following a ubiquitin code.

<u>Ubiquitin Machinery</u>

Ubiquitination is mediated by the cooperative action of three enzymes known as ubiquitin machinery: the ubiquitin-activating enzyme (E1), the ubiquitin-transferring enzyme (E2), and the ubiquitin ligase (E3) (Fig. 7).

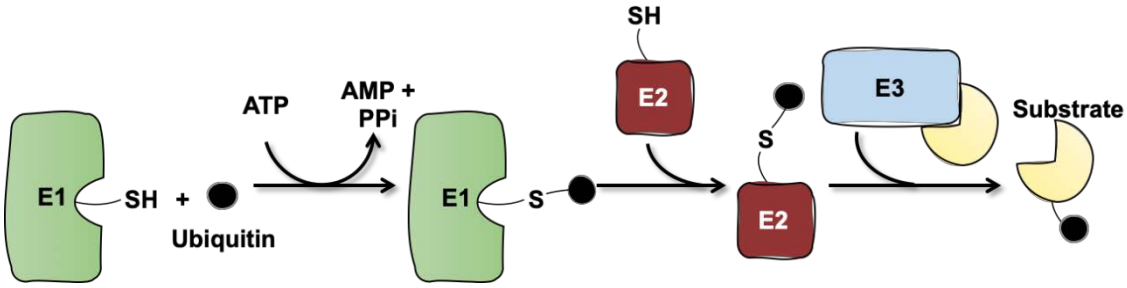

Figure 7. Ubiquitination substrate steps. Ubiquitin molecule is activated by E1 using ATP. Active ubiquitin is transferred to the E2-conjugating enzyme, and E3 facilitates the transfer of the active ubiquitin to a specific substrate (Bhogaraju and Dikic 2016).

The E1 enzymes activate ubiquitin molecules using ATP and they form a ubiquitin adenylate. Eukaryotic organisms often code only for a single E1. For example, *S. cerevisiae* only express a single and essential E1, Uba1 (McGrath, Jentsch et al. 1991).

The active ubiquitin is transferred to the E2-conjugating enzyme through a thioester bond. The E2s determine the kind of the conjugated ubiquitin chain: mono or poly-ubiquitinated.

E3 ligases bind to specific substrates, promoting the transfer of ubiquitin from E2s to the substrates. The E3 ligases generate an isopeptide bond between the C-terminal glycine (Gly) of ubiquitin and a lysine (Lys) residue on the substrate (Hershko and Ciechanover 1998). In rare cases, ubiquitin is conjugated to non-Lys residues of substrates such as serine (Ser), threonine (Thr) or cysteine (Cys). These atypical linkages mediate various non-proteolytic processes as will be explained in the next section (the ubiquitin code) (Kwon and Ciechanover 2017).

E3 ligases can be classified in big groups depending on their ubiquitination domains: the RING finger proteins, the HECT (homologous to E6-AP C terminus) proteins, and the U-box domain proteins. Most of the E3s have a RING domain that is conserved from yeast to humans (Deshaies and Joazeiro 2009).

An important example of RING E3 is Ubr1**.** Ubr1 is a protein conserved from yeast to human cells (Tran 2019). In *S. cerevisiae*, it has been described as an important element of the cytosolic PQC, as it promotes the degradation of misfolded proteins (see 2.2.2.1.4) (Samant, Livingston et al. 2018). In addition, Ubr1 in *S. cerevisiae* is the main E3 involved in the N-terminal degradation rule

(see 2.2.2.1.3) (Hwang, Shemorry et al. 2010), whereas in *S. pombe* it is not (Fujiwara, Tanaka et al. 2013). In fission yeast, Ubr1 has been related to different nuclear regulation pathways. For instance, it participates in the degradation of selected nuclear substrates such as the transcription factor Pap1 (Kitamura, Taki et al. 2011, Penney, Samejima et al. 2012) and the RNA-binding protein involved in meiosis Mei2 (Kitamura, Katayama et al. 2001).

The U-box domain E3 ligases protein group is also known as E4 ligase which serves as scaffold to help in the transfer of ubiquitin to a previously conjugated ubiquitin moiety, resulting in elongation of poly-ubiquitin chains (Koegl, Hoppe et al. 1999).

Yeast Hul5 is a HECT E3 ligase that in *S. cerevisiae* is required for ubiquitination of cytosolic misfolded proteins upon stress and it is necessary for fitness recovery after HS. The Hul5 localization under stress conditions is cytosolic, whereas in basal conditions is nuclear. Moreover, in basal conditions Hul5 is involved in the degradation of short-lived misfolded proteins. Thereby, Hul5 shows a dual functionality depending on cell conditions (Fang, Ng et al. 2011).

Ubiquitin code

Nowadays, we know that ubiquitination of proteins is not only a destruction signal but it is also a post-translational modification that can affect function, fate and localization of proteins. Thus, there is a ubiquitin code that manages these processes.

Mono-ubiquitination is the first ubiquitination that may happen in multiple lysine (K) residues of the substrate (multi-mono-ubiquitination). Actually, it has been shown recently that mono-ubiquitination of one or several substrate residues is sufficient to induce proteolysis in a large number of yeast and human proteins (Braten, Livneh et al. 2016). Then, the E2 ligase can add more ubiquitin molecules to this first one to any of its seven lysine residues, causing poly-ubiquitination. Which lysine is conjugated depends on the ubiquitin code that marks the substrate fate. Lys48 and Lys11 linkages mediate proteasomal degradation of normally folded short-lived proteins, whereas Lys48, Lys11 and Lys63 linkages

are involved in the degradation of misfolded proteins through the UPS or autophagy. Lys63 linkage facilitates the autophagic degradation of substrates in a 'cis'-mode as well as their associated cellular materials, such as damaged mitochondria and invading pathogens, in a 'trans'-mode. Lys63 linkages can also modulate various non-degradative processes such as DNA repair and the activation of protein kinases. Compared with Lys48 and Lys63, relatively little is known about the functions and mechanisms of atypical linkages such Lys6, Lys27, Lys29, Lys33, and Met1. Proteins with Ubiquitin-Binding Domains (UBDs) 'read' the ubiquitin code and link the ubiquitinated substrates to the downstream processes (reviewed in (Kwon and Ciechanover 2017)) (Figure 8).

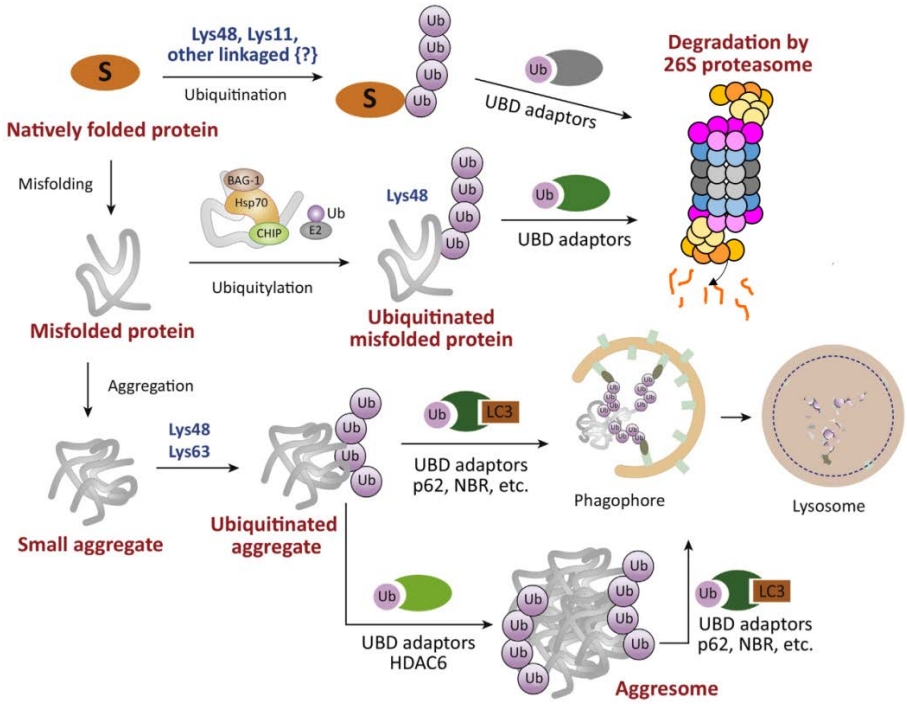

Figure 8. Roles of ubiquitin linkages in PQC. The ubiquitin linkage of substrate specifies the posterior degradation way (Kwon and Ciechanover 2017).

Upon non-native proteins accumulation, ubiquitin is not only important for marking misfolded proteins for degradation but it has been demonstrated that ubiquitin keeps soluble state of misfolded proteins in order to avoid insoluble aggregate formation (Miller, Mogk et al. 2015) as it will be discuss (see 3.1.2).

DUBs

The ubiquitin code is edited by the deubiquitinases (DUBs). There are many types of DUBs, but all of them are able to remove ubiquitin moieties from targeted proteins. DUBs hydrolyse after the C-terminal Gly76 of ubiquitin. DUBs not only recycle ubiquitin from ubiquitinated substrates, but also can modulate the degradation rate of the substrates (Leznicki and Kulathu 2017, Mevissen and Komander 2017). In addition, DUBs are necessary for correct proteasome function. In *S. pombe* the DUB Ubp3 is associated to the proteasome and it removes the ubiquitin of misfolded target proteins in order to be degraded by it. In cells lacking proteasomal Ubp3, misfolded substrates accumulate (Kriegenburg, Jakopec et al. 2014).

2.2.2.1.2. Proteasome

The proteasome is a large protein complex that works as a molecular machine to degrade proteins that have been previously ubiquitinated. It is constituted by 60 protein subunits. It can be divided into different parts: 20S proteasome and two 19S caps.

The 20S proteasome is a cylindrical, barrel-like catalytic core. There is a 19S cap complex bound to two ends of this core that regulates the activity of the catalytic core. The 20S and two 19S together constitute the 26S complex.

Six of the nineteen proteins of 19S complex have ATPase activity to unfold substrates and transfer them to the inner chamber of the 20S. The 20S complex is formed by two inner rings of seven β subunits each, with three proteolytic active sites per ring. These active sites can cleave peptide bonds of substrates at hydrophobic, acidic or basic residues (Fig. 9).

The products of this catalytic process are short peptides that are rapidly degraded by cytosolic peptidases to single amino acids.

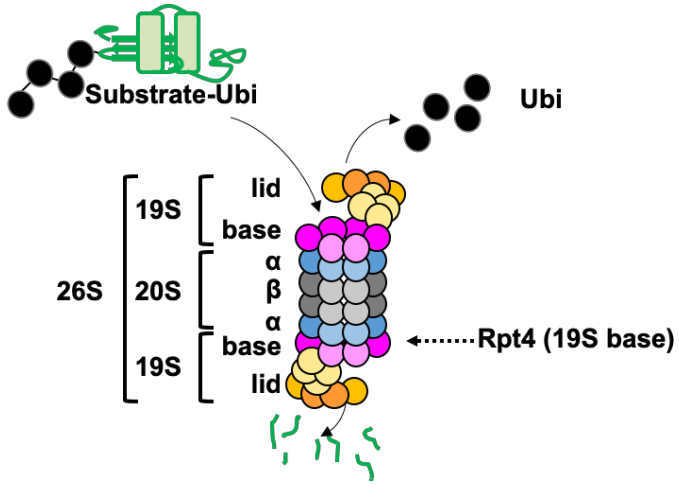

Figure 9. Schematic 26S proteasome complex. 26S proteasome degrade ubiquitinated proteins. Is structured in three parts: a central 20S catalytic core subunit and two 19S cap co plexes in the tips of the 20S.

In *S. cerevisiae* one of the important chaperones participating in the 26S assembly is Nas6, providing a dual mechanism to control affinity interactions between the lid, the base and the core particle of the proteasome complex (Li, Tian et al. 2017). Although little is now about the specific role of *S. pombe* homologue Nas6, cells lacking Nas6 show dysfunctional proteasome degradation (Kriegenburg, Jakopec et al. 2014).

Other interesting subunit of the proteasome is one of the very few non-essential proteasome subunits, Rpt4. This protein belongs to the 19S, specifically to the base (Bohn, Beck et al. 2010) (Fig. 9). Recently, Rpt4 has been linked to a non-proteasomal function: it avoids heterochromatin spreading (Seo, Kwon et al. 2018).

In fission yeast the 26S proteasome is located in the inner side of the nuclear envelope, as demonstrated by electron microscopy (Wilkinson, Wallace et al. 1998) and fluorescent microscopy (Takeda, Yoshida et al. 2010). However, the proteasome is dislocated in the cytosol during nitrogen starvation (Takeda, Yoshida et al. 2010).

2.2.2.1.3. Controlled degradation

Proteins can be degraded even if they have not been damaged. That can occur in short lived proteins, and also in proteins which degradation is signal-dependent. This system is a powerful tool to regulate protein activity and levels. Thereby, it is easy for the cell to respond to changing conditions, for instance, controlling the abundance of transcription factors.

Which are the cellular mechanisms for detecting when short lived proteins must be degraded? These proteins have degrons, or sequences containing the minimal elements that mediate the interaction of proteins with degradation machineries to promote proteolysis, namely E3 ligases. The interaction of degrons with E3 ligases is a regulated process. I will explain three different mechanisms of interaction: (i) the N-terminal rule, (ii) the post-ransductional modification of proteins or (iii) the localization.

N-terminal rule

One of those minimal elements is the N-terminal rule. The only feature of this rule is that the very N-terminal amino acid of a protein sequence determines its stability. The first peptide characterized as substrate of the N-terminal rule was e^k of *E. coli* in Alexander Varshavsky's lab (Bachmair, Finley et al. 1986, Bachmair and Varshavsky 1989). In eukaryotes, there are two types of destabilizing amino acids in the N-terminal: basic (Arg, Lys, His) and bulky hydrophobic amino acids (Ile, Leu, Phe, Tyr, Trp) (Bachmair, Finley et al. 1986, Bachmair and Varshavsky 1989, Varshavsky 2011).

In eukaryotic cells, the UBR family interacts with the N-degron (Bartel, Wunning et al. 1990). Particularly, in *S. pombe* there are two Ubr proteins: Ubr1 and Ubr11. As explained above, Ubr11, but not Ubr1, is the N-terminal rule ubiquitin ligase (Fujiwara, Tanaka et al. 2013, Kitamura and Fujiwara 2013), whereas, in *S. cerevisiae* Ubr1 is essential for the N-terminal rule degradation pathway (Rao, Uhlmann et al. 2001).

For instance, the N-terminal rule participates in the proteolysis of the subunit Scc1 of the cohesin complex in yeast. The caspase-like protease Esp1 generates a N-terminal destabilizing amino acid (Arg) in Scc1 which will be degraded by Ubr1. This process is essential for chromosome segregation (Rao, Uhlmann et al. 2001).

Post-translational modification

Cyclins are the proteins that allow the progression to the different cell cycle stages. A cyclin of a particular cell cycle phase has to be degraded in a specific moment in order to go through the cycle. Cyclins are phosphorylated and then suffer a conformational change that leads to the exposure of a degron sequence. Thereby, the E3 ligases APC will ubiquitinate them and they will be processed by the 26S proteasome (Glotzer, Murray et al. 1991).

Localization

This mechanism usually is related to the regulation of signalling cascades. I am going to focus on the regulation of stress response signalling pathways since it is a pivotal topic of this thesis.

In mammalian cells the transcription factor Nrf2 regulates Antioxidant-Response Element (ARE)-mediated expression genes. The abundance of Nrf2 is regulated by UPS. In basal conditions Nrf2 is localized in the cytosol and it is constitutively ubiquitinated by Keap1/Cul3 complex. Keap1 is an E3 ligase while Cul3 is an adaptor between Keap1 and Nrf2. Upon oxidative stress, Nrf2 dissociates from Cul3 and translocates to the nucleus, leading to expression of the ARE-containing genes (Chapple, Siow et al. 2012).

Similarly, in *S. cerevisiae*, the activity of the transcription factor Yap1 is regulated by the UPS. Yap1 is constitutively cytosolic and upon oxidative stress it translocates to the nucleus where DNA-bound Yap1 is marked for proteasomal degradation by the E3 ligase Not4. This points to a regulated way of limiting Yap1-dependent transcriptional activation (Gulshan, Thommandru et al. 2012). In addition, the amount of *S. pombe* transcription factor Pap1 (homologue of Yap1) is regulated by the UPS as well. Cells lacking the Ubc2 E2 conjugating

enzyme or the Ubr1 E3 ligase display resistance to caffeine or oxidative stress treatment in Pap1-dependent manner (Fig. 15) (Kitamura, Taki et al. 2011, Penney, Samejima et al. 2012).

2.2.2.1.4. Clearance of misfolded proteins through UPS

The PQC system can limit toxic effect of misfolded proteins accumulation by degradation through UPS. The UPS machinery cooperates with molecular chaperones to promote soluble misfolded proteins clearance. This process has two consecutive steps: the molecular chaperones are responsible of the recognition of the substrates and the UPS machinery labels and eliminates the clients.

Depending on the characteristic and the localization of the substrates we can define different chaperones and ubiquitination circuits that eliminate the misfolded proteins in different localizations.

Cytosol

In the cytosol, the new synthesized proteins can be damaged by many insults such as mutations or stress. So, already here the yeast ribosomal E3 ligases Ltn1 and Rnq1 recognize those non-native proteins and ubiquitinate them avoiding the spreading (Mathiassen, Larsen et al. 2015).

If those proteins skip this first ribosomal control, they must be eliminated from de the cellular milieu. If they are not recognized by chaperones for degradation, they aggregate waiting for degradation. The cytosolic aggregates will be solubilized and degraded in a Hsp104- and UPS-dependent manner (Mathiassen, Larsen et al. 2015).

For misfolded soluble proteins, Judith Frydman's laboratory has defined in detail the cytosolic circuit for degradation using different kind of misfolded protein reporters. Misfolded proteins must be bound to Hsp70 proteins to be maintained soluble prior to degradation. Hsp40 bring the substrates to Hsp70 and are responsible of the next steps for degradation. *S. cerevisiae* Hsp40 Sis1 and Ydj1 are involved in this chaperone-mediated degradation. However, they have different pathways. Ydj1 promotes that cytosolic E3 ligase Ubr1 labels the target

with Lys48-linked ubiquitin. Sis1 mediates the alternative proteasomal-targeting signal, Lys11-linked ubiquitin by ER E3 ligases Hrd1 and Doa10. Both, Lys11 and Lys48-linked ubiquitin are necessary for the cytosolic degradation of the substrates, both chains make easier that the proteasome recognize the substrate (Samant, Livingston et al. 2018).

Mitochondria

Recently, it has been characterized in yeast a new circuit for degradation of cytosolic substrates through mitochondria. Cytosolic aggregation-prone proteins are set close to the mitochondria and imported to intermembrane space for degradation. Although Hsp104 is necessary for the entrance into the mitochondria, Hsp70 are not necessary for this degradation pathway. Thus, mitochondria participate in the clearance of cytosolic misfolded proteins.(Ruan, Zhou et al. 2017).

Nucleus

The nucleus also has a misfolded degradation pathway for nuclear proteins. In addition, many misfolded cytosolic proteins are actively translocated to the nucleus or just move passively through nuclear pores to be degraded in the nucleus. Several publications suggest that San1 is the main nuclear E3 ligase in yeast (Gardner, Nelson et al. 2005, Kriegenburg, Jakopec et al. 2014, Samant, Livingston et al. 2018). San1 target by K48-linked ubiquitin, so that the K11-linked ubiquitin is not necessary for nuclear degradation. This may be facilitated by the nuclear protein Dsk2, which binds to K48 chains and carry them to proteasome (Samant, Livingston et al. 2018). In *S. pombe* there are more E3 ligases involved in ubiquitination of nuclear targets: Ubr11 (Kriegenburg, Jakopec et al. 2014) or Pib1 and Pub1 (Kampmeyer, Karakostova et al. 2017). Hsp40 participation in nuclear degradation is unknown.

Besides Hsp40 there is more co-chaperones that interact with Hsp70 for degradation. *S. pombe* proteins Bag101 and 102 of family of UBL/BAG-domain proteins are co-chaperones of Hsp70 that associate with the proteasome. That way misfolded substrate is set close to the proteasome for degradation (Kriegenburg, Jakopec et al. 2014, Poulsen, Kampmeyer et al. 2017).

Endoplasmic Reticulum

The endoplasmic reticulum has a degradation circuit as well, the Endoplasmic Reticulum-Associated Degradation (ERAD). Misfolded reticulum proteins are recognized at the lumen of the reticulum and when they are secreted are ubiquitinated by Doa10 or Hrd1 (Vembar and Brodsky 2008).

2.2.2.2. Autophagy

Autophagy is a lysosomal organelle process that was discovered by Christian de Duve in the 1960s. This catabolic process is designed to break down damaged organelles and cytosolic misfolded protein aggregates. Also, autophagy is induced under stress conditions, such as nutrient starvation, in order to recycle cytosolic organelles to obtain nutrients.

The process of autophagy consists of three basic steps. First, during the nucleation of the phagophore, an isolated membrane sequesters a small portion of the cytoplasm, including soluble materials and organelles. Second, the phagophore elongates to form the autophagosome. Third, the autophagosome fuses with the lysosome (or the vacuole in yeasts and plants) to become an autolysosome and degrades the materials contained within it (Levine and Kroemer 2008) (Fig. 10).

Although the proteasome is the main route for protein degradation, it cannot handle big misfolded protein inclusions or insoluble aggregates. In addition, some protein aggregates associated to different neurodegenerative diseases can inhibit proteasomal activity. Then, these aggregates are efficiently degraded by autophagy in the lysosome.

Autophagy receptors such us optineurin, NBR1 and P62 bound to lipidated Atg8 (LC3 in mammals) are decorating the inner side of the active phagophore. Poly-ubiquitination of protein aggregates via K63-linked as well as K48-linked ubiquitin can be recognized by the autophagy receptors. Thus, ubiquitinated aggregates are includes in the autophagosome and then it traffics and fuses with

the lysosome and the contents of the resultant autolysosome are degraded by lysosomal hydrolases (Galves, Rathi et al. 2019) (Fig. 10).

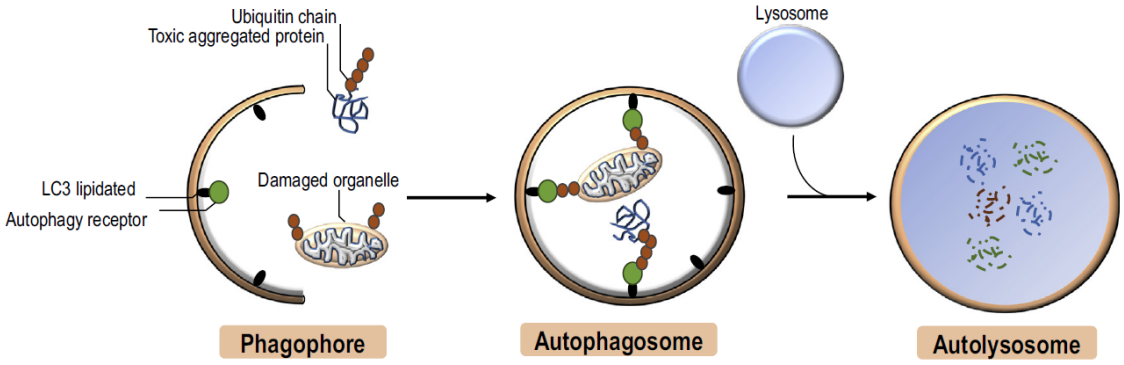

Figure 10. Schematic steps of autophagy mechanism. Recognition of aggregate-prone proteins by the phagophore membranes. Next autophagosome formation and fusion with the lysosome (autolysosome) to degrade ubiquitylated cargo proteins and damaged organelles (Galves, Rathi et al. 2019).

2.2.3. Aggregation

Finally, non-native proteins can form aggregates instead of being refolded or degraded. Non-native proteins may exhibit hydrophobic patches that are normally hidden in the native conformation. These regions besides be recognized by the UPS and chaperones, can cause the association with similar molecules forming favourable thermodynamically protein aggregates (Fig. 6).

Often these non-native protein aggregates are related to diseases, and originally they were just described as toxic by-products due to a PQC failure. Nowadays, we know that protein aggregation is a very complex and regulated process where aggregates can be toxic, but they can also become beneficial and even protect the cell from proteotoxic stress.

Furthermore, molecular chaperones and UPS machinery are actively involved in the regulation of aggregate deposition, besides the cytoskeleton and sorting factors (Miller, Mogk et al. 2015). Of course, the cellular state deeply influences all these processes (Sontag, Samant et al. 2017).

There are different classes of non-native protein aggregates which are distinguished based on their physical properties and spatial sequestration.

2.2.3.1. Solubility of aggregates

When we talk about physic properties of the aggregates, that usually refers to the solubility of the aggregates and we can divide them in two groups: soluble and insoluble aggregates.

The soluble aggregates have an amorphous conformation and they are more dynamic than the other aggregates (Bolognesi, Faure et al. 2019).

On the other hand, different molecules of the same misfolded protein may associate in an orderly way forming insoluble aggregates that are termed amyloids. Amyloid is a filamentous homopolymer with well-ordered structural motifs. The monomers of this structure are the same misfolded protein that stacks linearly to form ß-sheet structures. At the same time ß-sheet conformation nuclei into oligomers that get mature to reach very stable fibrils. Very recently, it has been explained at the molecular level why these structures are so stable. Sequence of poly-glutamines (polyQ) spell folds into a helical structure that is stabilized by the formation of unusual hydrogen bonds between glutamines and carbonyl groups of the main chain, and their helicity directly correlates with tract length (Escobedo, Topal et al. 2019). Indeed, those fewer stable pre-fibril structures are more toxic than more stable bigger ß-sheet conformations.

This kind of structure was studied in the association of prion proteins of *S. cerevisiae*. Prions are defined as proteins that easily acquire an unfolded non-functional conformation and which promote that similar proteins adopt the same conformation. Then, they associate with each other in amyloid fibrils. That is facilitated by a domain termed prion domain, with low complexity, which contains predominantly hydrophobic amino acids. Likewise, in many organisms as humans, there are many proteins that have prion-like domains that are similar to yeast's prion domains. In general, prion-like domains facilitate the safe-assembly into large complexes or aggregates. There are several prion-like proteins that form amyloid aggregates and they are associated to many neurodegenerative

diseases such as Alzheimer's disease, Huntington's disease, Parkinson's disease or amyotrophic lateral sclerosis (reviewed in ((Monahan, Rhoads et al. 2018)).

2.2.3.2. PQC compartments

It has been described that integral part of PQC is the spatial sequestration of non-native proteins in different PQC compartments: the Insoluble Proteins Deposits (IPODs), the Q-bodies, the Juxtanuclear Quality Control Compartments (JUNQs) and Age-dependent Protein Deposits (APODs).

IPODs

Firstly, IPODs are formed by insoluble aggregates. Those compartments are formed in normal growth conditions and contain amyloid like proteins. IPODs are localized in the periphery of vacuoles in yeast and it seems that they are immobile. Furthermore, it is thought that their localization is not casual because IPOD clearance is associated to autophagy (Kaganovich, Kopito et al. 2008).

Q-bodies and JUNQ

Upon non-extreme stress conditions, soluble misfolded proteins firstly deposit in dynamic aggregates in an early quality control structure called Q-bodies (Escusa-Toret, Vonk et al. 2013). The dynamic of those structures depends on the energy of chaperones, but they are cytoskeleton independent. Normally, Q-bodies are anchored to the ER and are rapidly cleared through the UPS (Escusa-Toret, Vonk et al. 2013). However, if the proteasome is impaired and Q-bodies cannot be removed, they may collapse in bigger structures: JUNQs. These compartments are localized in the vicinity of the nucleus and they are more dynamic than IPODs. Thereby, JUNQs can act as a reservoir of soluble misfolded protein for subsequent refolding or degradation. Furthermore, it is known that proteasomes are translocated close to the JUNQ under high stress conditions (Kaganovich, Kopito et al. 2008). However, under prolonged or severe stress or blocked UPS, non-

amyloidogenic compartments can form insoluble aggregates and be localized in IPODs (Kaganovich, Kopito et al. 2008).

It has been reported that proteins at JUNQs are ubiquitinated, which suggests that ubiquitin may help maintaining the solubility of coalescent bodies. Furthermore, the DUB Ubp3 in *S. cerevisiae* is involved in disassembly of JUNQs but not other coalescent bodies (Oling, Eisele et al. 2014, Nostramo, Varia et al. 2016). Thus, ubiquitination could be essential to the sorting of misfolded proteins in different compartments (Fig. 11).

APODs

Upon replicative and chronological aging two protein deposits, labelled with Hsp104, have been termed APOD. Those deposits are formed by irreversibly damaged proteins and insoluble proteins aggregates resulting from an overflowed PQC. The formation of these compartments is promoted by Hsp42 and counteracted by Hsp104 and Hsp70 in *S. cerevisiae*. Those structures correlate with aging: Hsp104 or Hsp70 deletion, which cause enhanced APOD formation, life span is shorter; whereas if the assembly of APODs is impaired for example by Hsp42 deletion mutant life expand is extended. Furthermore, cells with APODs can form Q-bodies upon heat shock and have an enhanced proteasomal activity. So, APODs are not consequence of an aged or impaired proteostasis. APODs are not dynamic structures unlike Q-bodies, they are really stable in time as it has been described that APODs contain prion-like proteins in their amyloid state that points to these structures are amyloid-like (Saarikangas and Barral 2016).

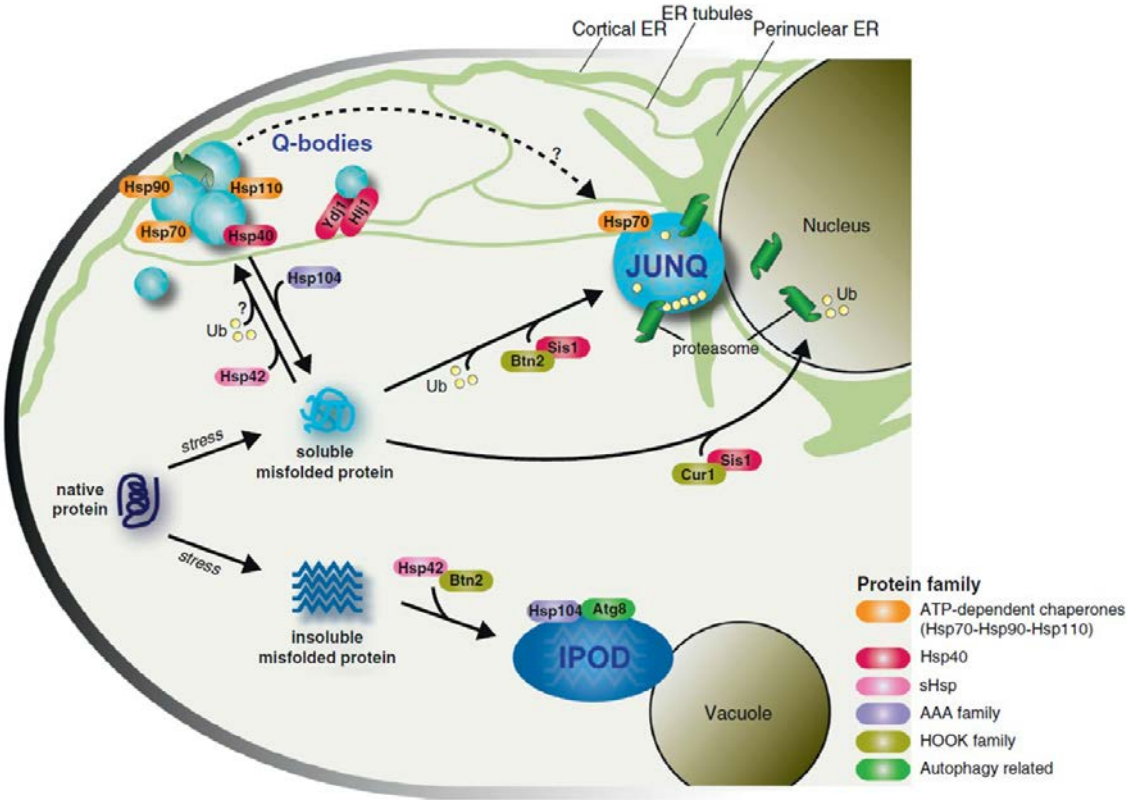

Figure 11. Schematic distribution of the different PQC compartments. Non-native proteins are spatial distributed by different chaperones, sorting factors and their regulation pathways into the different PQC compartments, leading to refolding, degradation or isolating (Sontag, Vonk et al. 2014).

2.2.3.3. Chaperones and aggregation

All of those compartments are actively assembled by different chaperones. For example, Hsp40 Ydj1 has been linked to the formation and localization of Q-bodies (Escusa-Toret, Vonk et al. 2013), Sis1 has a potential role in maturation of Q-bodies to JUNQs (Sontag, Samant et al. 2017) and Hsp104 is necessary for amylogenic aggregates formation such as mutated huntingtin aggregates and for their disaggregation (Glover and Lindquist 1998, Krobitsch and Lindquist 2000). Actually, Hsp104 is often used as a marker of protein aggregates. Stress-inducible proteins Btn2 and Cur1 in *S. cerevisiae* act as protein-sorting factors and are essential for PQC compartmentalization of misfolded proteins. For this sorting, Btn2 and Cur1 interact with chaperones as Sis1 and Hsp42 (Malinovska, Kroschwald et al. 2012). Moreover, the small chaperone Hsp42 has been described to be necessary for IPOD maturation. Hsp42 acts adding misfolded proteins to the immature IPOD (Specht, Miller et al. 2011). Similar to *S. cerevisiae's*

Hsp42, Hsp16 from fission yeast allows the fusion of age- and dead-associated aggregates promoting maturation of those aggregates. This fusion is necessary for maintaining those aggregates localized in the mother cell upon division, promoting their asymmetric distribution to preserve the fitness of one descendant (Coelho, Dereli et al. 2013, Coelho, Lade et al. 2014).

2.2.3.4. UPS and aggregation

UPS has an important role in the sorting of misfolded proteins into different compartments. Misfolded proteins can be ubiquitinated for clearance, but when this is impaired, Q-bodies cease to accumulate into JUNQs and instead they accumulate in IPODs. Thus, ubiquitination is essential to keep solubility of misfolded proteins (Escusa-Toret, Vonk et al. 2013). Also, if chaperones that promote ubiquitination are deleted, some misfolded proteins are localized in IPODs, whereas other misfolded proteins remain in JUNQs. Furthermore, if ubiquitination of an amyloidogenic protein is enhanced, it is directly localized in JUNQs instead of IPODs (Sontag, Samant et al. 2017). Thus, ubiquitination acts as an essential signal for misfolded proteins in soluble PQC compartments in a chaperone network-dependent manner (Kaganovich, Kopito et al. 2008). On the other hand, IPODs are not removed by the UPS but by autophagy (Kaganovich, Kopito et al. 2008).

2.2.3.5. PQC compartment functions

Thus, some functions are proposed for these compartments: (i) enhancing aggregated protein clearance by the degradation machineries, (ii) keeping the cell safe from toxic prion-like molecules or (iii) avoiding degradation of misfolded protein that will be useful after stress again. Nevertheless, all of them point to a protective role of aggregation upon proteotoxic stress conditions.

Aggregated proteins fate will depend on the PQC compartments where they are. In JUNQs, misfolded proteins maintain a soluble state in order to be removed by proteasome or be refolded by the chaperones. It has shown that

after aggregation formation and recovery in permissive conditions, misfolded proteins that was in soluble compartments (but not in IPODs) recover native structure in a Hsp104 dependent- manner (Kaganovich, Kopito et al. 2008).

Supporting the idea of that PQC may actively promote aggregation, the formation of those PQC compartments have been related to cellular fitness upon stress conditions. Cells with depletion of chaperones such as Hsp104 or Hsp42 are deficient in PQC compartment sequestration pathways. Those mutant strains display fitness problems upon stress (Escusa-Toret, Vonk et al. 2013). Sequestration of misfolded proteins could avoid deleterious effects of free misfolded proteins in the cellular milieu.

Furthermore, aggregates may have protective role for proteins as well. It has been proposed that during stress some aggregated proteins maintained in a reversible state. And after stress cessation, these proteins come back to the soluble fraction and normal activity (Wallace, Kear-Scott et al. 2015).

Those compartments can facilitate the clearance of the misfolded proteins. PQC compartments are close to different clearance systems (UPS and autophagy). So, it easier for cells to have delimitated locations for misfolded protein removal or refolding.

Regarding on-off switches, some examples rely on the formation of aggregate-like centers. For instance, during cycle progression, Whi3 and Rim4 are prion-like proteins that sequester mRNA of cyclins in *S. cerevisiae* (Cln3 and Clb3 respectively) avoiding cell cycle progression (Caudron and Barral 2013). Also, upon nutrient starvation yeast cells enter in quiescence, concomitant with the formation of aggregation centers containing metabolic enzymes, proteasomal subunits, protein kinases and phosphatases and cytoskeletal proteins (Saad, Cereghetti et al. 2017). Although the function of those aggregates remains unclear, some of those proteins maintain their activity in the coalescence state; it has been suggested that this could spatially control of enzymatic activity or could protect proteome from damage and catabolic processes that are enhanced during quiescence (reviewed in (Saarikangas and Barral 2016)).

We can conclude that even though it was believed that aggregation of misfolded proteins was a passive event causing toxicity, the view has changed. Currently, we know that aggregation is a more complex process. Apparently, aggregation is an active and regulated cellular process and it is necessary for cell fitness. All of it points to a beneficial effect of coalescence of misfolded proteins into aggregates, to either facilitate their clearance or to avoid its toxic effects. In addition, protein aggregates could also preserve those misfolded proteins from degradation, or they may even have a regulation function during specific cellular processes.

3. Challenging the PQC: Proteotoxic Stress

The accumulation of non-native proteins can lead to cell toxicity due to the following:

I. Loss of the physiological function of the non-native protein.
II. Gain-of-function of the misfolded protein which interferes with the normal activity of the cell.

Both types of events have been related to many diseases such as neurodegenerative diseases, aging or cancer. The PQC system is involved in maintaining the integrity of the proteome in the absence or presence of perturbing conditions **(**Fig. 1).

Newly synthesized proteins are the most sensitive group to suffer missfolding. Mutations in one gene may impair the correct folding of translated products. Also, some new synthesized proteins need a partner to be stabilized, or they must be transfer to a specific localization. If these processes fail, the newly synthesized proteins become potentially toxic (Sontag, Samant et al. 2017).

Environmental stress is the main trigger of protein miss-function that can affect either mature or new proteins. Furthermore, cellular stress enhances the denaturation of those proteins which are prone to degradation, such us mutated or prion-like proteins. Immediately after proteotoxic stress, cells trigger protective mechanisms to defend the proteome integrity, mainly: (i) the rate of new proteins

synthesis is decreased; and (ii) stress-inducible chaperones are up-regulated (Sontag, Samant et al. 2017). In fact, the PQC system itself has a role in the regulation of stress signalling cascades as well (see 4).

I will describe in more detail the effects of gene mutations (see 3.1) and environmental stress (see 3.2) in proteotoxicity.

3.1 Study of mutated non-native proteins in yeast models

As described above, PQC failures are related to many disorders particularly neurodegenerative diseases, aging or cancer. Thereby, from a medical point of view, PQC components are a logical target for treatments.

Yeast models has been used for decades to study many eukaryotic processes, but in the last years they have also been used to study the origin of some neurodegenerative disorders. Thus, *S. cerevisiae* strains over-expressing the wild type or mutated protein involved in a specific disease have been generated to study aggregate formation. The budding yeast model allows researchers to perform several molecular approaches to understand the molecular causes of toxicity, find out what elements of the PQC are involved, or even find therapeutic targets (Krobitsch and Lindquist 2000, Johnson, McCaffery et al. 2008, Kaganovich, Kopito et al. 2008, Armakola, Hart et al. 2011, Kim, Raphael et al. 2014, McGurk, Lee et al. 2014, Park, Arslan et al. 2018). In the next two sections I will explain what is known about the expression of the mutated protein models TDP-43 (see 3.1.3) and huntingtin (see 3.1.4) in yeast.

3.1.1. TDP-43

The 43-kDa TAR–DNA-binding protein (TDP-43) is an ubiquitously expressed nuclear protein that undergoes a pathological conversion to an aggregated protein with cytoplasmic localization in affected cells (Johnson, McCaffery et al. 2008). This is related to neurodegenerative diseases as amyotrophic lateral sclerosis (ALS) and frontal temporal lobar degeneration with ubiquitin positive inclusions (FTLD-U) (Sontag, Samant et al. 2017). Likewise, TPD-43 aggregates also

appear in skeletal muscle cells of patients with inclusion body myopathy, oculopharyngeal muscular dystrophy and distal myopathies (Vogler, Wheeler et al. 2018).

TDP-43 has been extensively expressed in *S. cerevisiae* as a proteotoxicity model (Johnson, McCaffery et al. 2008, Armakola, Hart et al. 2011, Kim, Raphael et al. 2014, Park, Hong et al. 2017, Park, Arslan et al. 2018)((Bolognesi, Faure et al. 2019). Similar to human affected tissues, TDP-43 is toxic in *S. cerevisiae* when forms cytosolic aggregates. These cytosolic aggregates appear with a high expression of wild-type TDP-43, or upon expression of lower concentrations of the mutated forms. Moreover, it is impossible to detect amyloid formation of TDP-43 in *S. cerevisiae* (Johnson, McCaffery et al. 2008, Park, Arslan et al. 2018) opposite to human inclusions (Vogler, Wheeler et al. 2018). In addition, there are no interactions between TDP-43 and either prion-proteins or chaperones (Hsp104 or Sis1) that usually are associated to amyloid aggregates (Johnson, McCaffery et al. 2008). However, recently, it has been demonstrated that TDP-43 inclusions sequester the Hsp40 Sis1 leading to a decrease of degradation of misfolding proteins. Moreover, Sis1 overexpression rescues this phenotype. Furthermore, when the homologue of Sis1, mammalian DNAJB1, is over-expressed there is a reduction of toxicity in rodent ALS models (Park, Hong et al. 2017). In addition, TDP-43 co-localizes with stress granules, and proteins that modulate stress granule assembly are strong modifiers of TDP-43 toxicity in flies and *S. cerevisiae*. Thereby, it was possible to find new therapeutic strategies for ALS (Kim, Raphael et al. 2014).

Although little is known about the mechanism by which TDP-43 is toxic, we know that the toxic effects of TDP-43 reside in some of the components of its structure. The C-terminal region that is a prion-like domain (PRD), and the RNA recognition motives (RRM), are the domains where mutations leads to cytosolic aggregation (Fig. 12) (Kim, Raphael et al. 2014, Bolognesi, Faure et al. 2019), and they are together necessary for toxicity. Moreover, it was published that aggregation *per se* is not sufficient for toxicity (Johnson, McCaffery et al. 2008).

Figure 12. TDP-43 structure. TDP-43 is a chain of 414 aa that contains 2 RRM domains and prion-like domain at C-terminal.

3.1.2. Huntingtin

The most common repeat sequence in human proteins is the poly-glutamine (polyQ) repeats. When the abnormal expansion of this polyQ stretch exceed a certain threshold, in several proteins is linked to disease. One of them is the Huntington's disease (HD). HD is a progressive hereditary neurodegenerative disease caused by mutation of the exon 1 of *huntingtin* gene. This mutation promotes the abnormal expansion of polyglutamine encoding CAG repeat sequence and in the same way the polyQ stretch of huntingtin protein (Htt). This mutation can lead to toxicity and protein aggregation. HD patients express Htt with polyQ tails is longer than 35 glutamine, and the length of the polyQ sequence is proportional to disease severity (Ha and Fung 2012).

Mutated Htt (mHtt) is used extensively as a model to study polyQ diseases and aggregation. mHtt is a prion-like proteins that aggregate forming compact ß-sheet amyloid aggregates (Sakahira, Breuer et al. 2002). The toxicity is linked to the solubility properties of mHtt, where soluble forms seem to be more toxic than aggregates. Soluble mHtt monomers, but not amyloid aggregates, can impair several basic cellular functions (Behrends, Langer et al. 2006, Caron, Hung et al. 2014, Kim, Hosp et al. 2016). For instance, mHtt monomers are involved in the inhibition of ER degradation promoting ER stress (Leitman, Ulrich Hartl et al. 2013). Also, mHtt increases reactive oxygen species in primary embryonic cortical neurones in mice (Li, Valencia et al. 2010, Sapp, Valencia et al. 2012). Moreover, mHtt is involved in not physiological interaction with transcription factors (Schaffar, Breuer et al. 2004).

Many progresses in understanding proteostasis and proteotoxicity linked to polyQ and other amyloid proteins have been performed expressing the exon 1 of Htt in the budding yeast model. In 1999, the group of Susan Lindquist was the first one in express Htt exon 1 in *S. cerevisiae* fused to GFP. Surprisingly, it was

necessary to express a huge amount of Htt with a very long extension of 103 glutamines to detect cytosolic aggregates (Htt aggregates appear beyond 35 glutamines in human cells). Moreover, mHtt aggregation is dependent on Hsp104, as cells lacking Hsp104 do not display aggregates .(Krobitsch and Lindquist 2000).

Regarding toxicity, in *S. cerevisiae* this is a controversial issue. Although at the very beginning Lindquist's group reported the lack of toxicity of the chimeras, some groups have later described toxic phenotypes upon expression of the same fusion proteins in *S. cerevisiae* (Meriin, Zhang et al. 2002, Johnson, McCaffery et al. 2008, Zurawel, Kabeche et al. 2016, Zhao, Zurawel et al. 2018). There are many publications describing different mechanisms of how mHtt impairs the normal cellular activity. One of these mechanisms is the interaction of the prion protein Rnq1 with mHtt. In *S. cerevisiae* Rnq1 is essential for toxicity and aggregation of mHtt. Thereby, mHtt can be disturbing the normal function of prion-like proteins.

Moreover, it has been published that aggregates are impairing proteasomal degradation, probably by sequestering some essential elements for sorting misfolded proteins to degradation. For instance, and similar to TDP-43 aggregates, mHtt aggregates sequester and inhibit the Hsp40 Sis1 that recognizes cytosolic misfolded proteins and transfer them to nuclear degradation (Park, Kukushkin et al. 2013).

Another research group links mHtt expression with mitochondrial disfunction. mHtt aggregates disturb the mitochondria and the cytoskeleton which promotes the increase of oxidative stress (Solans, Zambrano et al. 2006).

It has also been reported that mHtt aggregates may increase ER stress (Duennwald and Lindquist 2008) leading to defects in endocytosis (Meriin, Zhang et al. 2003).

Recently, the group of Surachai Supattapone reported the expression of Htt chimeras in *S. pombe*. They compared the expression Htt in *S. cerevisiae* and *S. pombe*. Similar to *S. cerevisiae,* only Htt with polyQ 103Q in length forms aggregates in *S. pombe*. However, they demonstrate that at same protein levels of Htt *S. cerevisiae* shows growth defects expressing 103Q, whereas *S. pombe*

do not. They suggested that the formation of aggregates is toxic in cells having a proteome rich in polyQ proteins such as *S. cerevisiae*. They proposed that aggregating mHtt would associate with other endogenous polyQ proteins impairing their activity and generating toxicity (Zurawel, Kabeche et al. 2016). Indeed, they perform a mass spec approach to study the proteins that are associated in a length dependent manner to Htt in both yeast models. They find that there are more proteins associated to Htt that play an important role in nucleoli and mitochondria in *S. cerevisiae* but do not in *S. pombe*. In addition, Htt appears to interact preferentially with polyQ proteins which are few in *S. pombe* (Zhao, Zurawel et al. 2018). Therefore, they conclude that the difference between both yeast models underlies in the amount of aggregation-prone proteins in the respective proteomes.

3.2 Challenging the PCQ with environmental changes

Many kinds of stresses, such as the presence of heavy metals or pH can enhance the amount of misfolded proteins and affect normal proteostasis, but I will focus on oxidative and heat stress.

Oxidative stress

Since our planet changes from a N_2 rich atmosphere to an O_2 atmosphere, cells had to evolve creating powerful antioxidant defences to deal with the oxidative stress created by the new oxidant environment. Reactive Oxygen Species (ROS) are several molecules derived from O_2 such as superoxide anion ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO\cdot$), that have a high reactivity and oxidant power. ROS inside the cells are produced as normal by products of oxygen metabolism and we know that they participate in different biological processes as signalling molecules. In addition, cells express numerous antioxidant barriers that keep ROS at steady-state concentrations. However, the organic molecules are really sensitive to be oxidized by ROS, so uncontrolled increases of ROS mediates oxidized reactions that indiscriminately damage lipids, DNA and specially proteins (reviewed in (Vivancos, Jara et al. 2006)).

ROS-mediated oxidation of proteins leads to amino acid residue modifications, cleavage of peptide bonds, and formation of covalent protein-protein cross-linked derivatives. Those modifications may also reversibly or irreversibly modify amino acid chains. Reversible modifications usually are profited by the cell for signalling process. However, most of ROS modifications lead to irreversible damage, for instance the oxidation of some amino acid residues leads to protein carbonylation. Those irreversible damages promote the loss of native structure of proteins leading to a proteotoxic effect in the cell.

Heat stress

Heat stress has a main role in generating proteotoxic stress, the increase of temperature cause conformational changes that can lead to non-native conformations. Specifically, many noncovalent interactions (hydrogen bonding or hydrophobic interaction) between amino acids residues within a protein, and between protein and solvent, dictate and stabilize the native three-dimensional structure of proteins. These interactions are relatively weak and can be disrupted easily upon modest increases of temperature. This drives to an increase of the exposure of the hydrophobic patches that were sheltered in the core of proteins. So that, heat stress leads to a clear protein aggregation or degradation. Specially, new translated proteins are the most vulnerable targets of misfolding upon heat shock, rapidly they aggregate or they are target to degradation (ann rev 1995).

For that reason, it exists evolutionary conserved responses to heat stress from bacteria to mammal cells. Mainly there is an up-regulation of a set of genes that codes for Heat Shock Proteins (HSPs). HSPs limit stress-induced damage by avoiding the accumulation of non-native proteins at the cellular milieu, such as chaperones and UPS components. In the genome of *S. pombe* there are at least 17 genes of the HSP family. They are divided in two clusters: 4 genes that are repressed upon stress, and 13 genes that are induced. Some of these genes are *hsp9, hsp16, hsp104* or *sti1* (Lindquist 1986, Chen, Toone et al. 2003, Medicherla and Goldberg 2008).

4. Regulation of stress responses in unicellular eukaryotes

The proteome of unicellular organisms is continuously challenged due to changes in environmental conditions, to which they are directly exposed through the cell membrane. In response to extracellular stressors, yeasts have to rapidly adapt by inducing a whole new set of anti-stress proteins, and by coping with the damage exerted over the prior-to-stress proteome. Budding yeast has been widely used to study anti-stress responses and also the folding and degradation components of the proteostasis network (Sontag, Samant et al. 2017)(reviews, Luis)(Anfinsen 1973)**.

On the contrary, little is known about proteostasis control in the fission yeast *S. pombe*, and this thesis project is devoted to study the components of its network, and its characterization as an alternative proteostasis model system. We will here briefly describe the advantages of using this simple eukaryote (section 4.1), as well as the stress pathways responding to stress signals, mainly oxidative stress and heat shock, which may control the network of chaperones and degradation machineries in response to environmental challenges (section 4.2).

4.1. *S. pombe* as a convenient eukaryotic model system

S. pombe is a eukaryotic unicellular organism that was described for the first time by the German scientist Paul Linder at the end of S.XIX. He isolated the yeast from a Bantu beer from East Africa, and for this reason he called it *pombe*, which means beer in suajili. Moreover, *S. pombe* is also known as fission yeast because it grows by length extension and then it divides by fission in two cells.

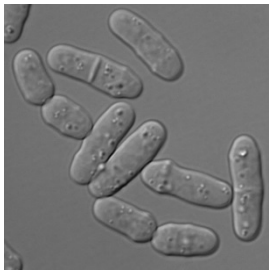

Figure 13. Fission yeast. Optical microscope image of fission yeast.

Since 1950s fission yeast has been used as model organism due to its simple growth conditions and its easy genetic manipulation. The genetic material is distributed in three chromosomes that contain 5036 genes and it is completely sequenced. In addition, *S. pombe* can grow in a haploid state which makes easier the deletion of genes by classical DNA recombination techniques and the study of its effect. These features make this model attractive for genetic studies. In addition, several laboratories and consortia have designed very useful resources such as gene strain deletion collection of 3000 non-essential genes deletion strains, or the data base "Pombase" (www.pombase.org) that contains information on genomics, proteomics, publications, etc.

Although the other yeast model organism *S. cerevisiae* has more known technological applications, *S. pombe* has evolved more slowly, for that reason fission yeast shares more features with metazoans cell.

Regarding proteostasis research, nowadays, not very many research groups use *S. pombe* as model (Kitamura, Taki et al. 2011, Coelho, Dereli et al. 2013, Poulsen, Kampmeyer et al. 2017).

4.2. Stress responses in *S. pombe*

S. pombe has developed some stress response pathways. The main ones upon oxidative stress are the Sty1/Atf1 and the Pap1 pathways. Sty1/Atf1 pathway is activated upon high level of ROSs and is involved in the survival response to this stress, whereas Pap1 is activated upon low level of ROSs and it is involved in the adaptive response to oxidative stress. Both pathways are essential for survival under oxidative stress conditions, however, they are not required for normal growth in an aerobic environment (Fig. 14).

Regarding the heat shock response, Sty1/Atf1 and Hsf1 pathways are the most important ones.

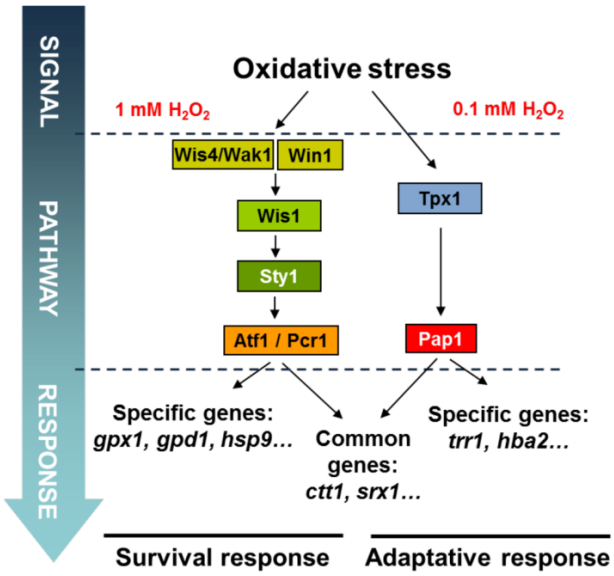

Figure 14. Two pathways can be activated upon oxidative stress in *S. pombe*. In acute oxidative stress conditions Sty1 pathway is activated, whereas upon mild oxidative stress is the Pap1 pathway.

4.2.1. Pap1 pathway

Pap1 (Pombe-AP1) is a *S. pombe* transcription factor that is involved in adaptation to different stresses: oxidative stress and drug exposure. Once activated, nuclear Pap1 binds 50-80 promoters to promote the expression of H_2O_2 detoxification or drugs-resistance response genes. Pap1 is the homologue of Yap1 in *S. cerevisiae* and AP1 in mammalians cells.

Pap1 is a 61.5 kDa protein whose sequence contains a Nuclear Localization Sequence (NLS) near the N-terminal domain and a Nuclear Export Signal (NES) in the C-terminal domain. It also has a basic region-leucine zipper (bZIP) domain that consists in a basic region that is the binding domain to specific DNA sequences, and a leucine zipper that is required for the dimerization of two DNA binding domains of Pap1 (Landschulz, Johnson et al. 1988). Moreover, Pap1 contains two clusters of cysteine residues, one located at the centre of the protein with four cysteine residues (cysteines 259,278, 285 and 290), and the second one at the C-terminal region with three cysteine residues (cysteines 501, 523 and

532). The second cluster is surrounding the NES (Castillo, Ayte et al. 2002, Calvo, Ayte et al. 2013) (Fig. 15).

Pap1 is regulated at the level of sub-cellular localization. In basal physiological conditions, Pap1 is shuttling between the cytosol and the nucleus. There is an equilibrium between the nuclear importin Imp1 that recognizes the NLS of Pap1, and the nuclear export mediated by Crm1 which recognize the NES (Calvo, Garcia et al. 2012). Export prevails, and Pap1 displays cytosolic localization.

Pap1 does not sense the increase of ROS directly. Actually, the cytoplasmatic peroxiredoxin protein Tpx1 acts as a H_2O_2 sensor and initiates the oxidation and activation of Pap1 upon mild oxidative stress (Vivancos, Castillo et al. 2005). Tpx1 is oxidised and then induces the formation of disulphide bonds between the two cysteine clusters of Pap1 causing a conformational change that hides the NES. Thereby, active Pap1 is not recognized by Crm1 and it accumulates at the nucleus. Then, Pap1 triggers gene expression of genes such as those coding for the ABC-type transporters Hba2 and Caf5, or for the peroxide scavengers catalase (Ctt1) or Srx1 (Fig. 15).

Furthermore, there is an active thioredoxin reducing system, Trx1/Trr1, that maintains reduced Pap1 in basal conditions and returns active Pap1 to the basal conformation after stress allowing the release of the NES and Pap1 shuttles again (Vivancos, Castillo et al. 2004).

Only some Pap1-dependent genes require oxidized Pap1 conformation to be activated. Genes that are expressed by oxidized Pap1 are related to stress oxidative adaptation such as *ctt1* or *srx1*, whereas the other set of genes such as *hba2* and *caf5* or *obr1* (coding for a ubiquitin-like protein) are involved in toxic drug detoxification. In addition, it was demonstrated that oxidized Pap1 can form a heterodimer with the transcription factor Prr1 to activate antioxidant genes (Calvo, Garcia et al. 2012).

The persistent accumulation of Pap1 in the nucleus has to be limited in order to prevent a toxic over-expression of Pap1-dependent genes. Besides Pap1 reduction and nuclear export, nuclear protein levels of Pap1 may be controlled by degradation. Indeed, when Pap1 is translocated to the nucleus, the UPS (particularly the E2 Ubc2 and Ubr1 RING finger E3 ligase) mark Pap1 for

degradation (Shimanuki, Saka et al. 1995, Kitamura, Taki et al. 2011, Penney, Samejima et al. 2012).

There are three interventions able to increase Pap1 activity:

I. Genetic mutations that lead to constitutively oxidized Pap1: in cells lacking thioredoxin reductase Trr1 there is an increase of oxidized Pap1 form that accumulates in the nucleus. These cells show increased drug resistance, as caffeine, compared to wild type cells.

II. Mutations promoting Pap1 nuclear accumulation: when Pap1 is lacking its NES, or *crm1* gene are truncated, Pap1 accumulates at the nucleus. These cells present a drug-resistance phenotype as well (Calvo, Garcia et al. 2012).

III. Increasing Pap1 protein levels: cells with specific UPS defects also display Pap1-dependent multi-drug resistance phenotype. For example, in cells where 26S proteasome is impaired and Pap1 is stabilized (Shimanuki, Saka et al. 1995, Penney, Samejima et al. 2012). Also in cells where the E2 *ubc2* (Penney, Samejima et al. 2012) and the E3 ligase *ubr1* that regulates the ubiquitin labelling of Pap1 is depleted (Kitamura, Taki et al. 2011).

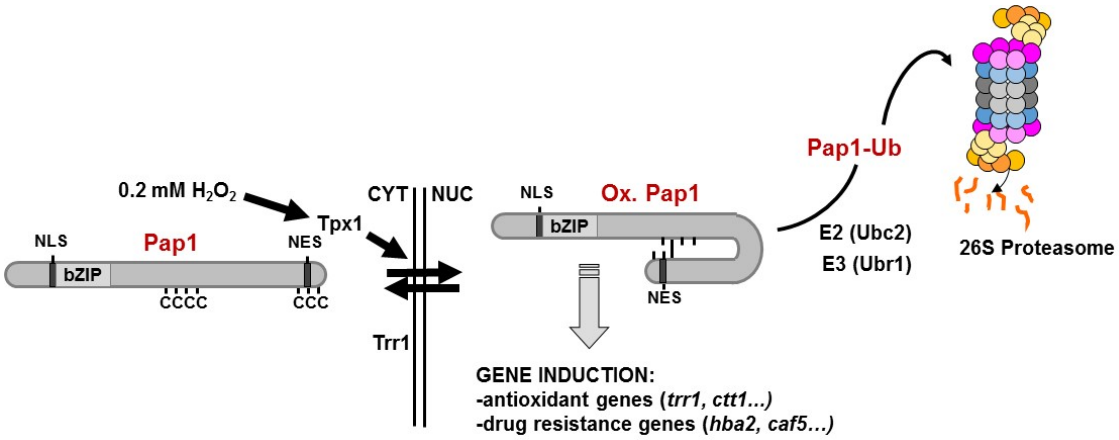

Figure 15. Pap1 structure and activation oxidative stress. Pap1 contains a Nuclear Localization Sequence (NLS), a basic-leucine zipper (bZIP) domain, two cysteine clusters and a Nuclear Export Signal. Pap1 is shuttling between nucleus and cytoplasm. Upon mild oxidative stress (0.2 mM of H_2O_2) Tpx1 is oxidized and promotes the disulphide bonds formation. Then NES is hidden and activated Pap1 is accumulated in the nucleus promoting triggering the transcription either antioxidant or drug resistance genes. Trr1 regulates the pathway as reduce active Pap1.

4.2.2. Sty1 pathway

From yeast to mammals, the Mitogen-Activated Protein Kinase (MAPK) cascades are conserved stress-response pathways. They are required for survival under diverse stress conditions such as oxidative stress, nutrient depletion, osmotic stress, heat stress or UV irradiation. The first MAPK pathway was described in 1990s in *S. cerevisiae*, where the MAPK HOG1 was shown to be activated by osmotic stress (Boguslawski 1992, Brewster, de Valoir et al. 1993, Posas and Saito 1997). Subsequently, human MAPKs were isolated: JNK (Derijard, Hibi et al. 1994, Kyriakis, Banerjee et al. 1994) and p38 (Han, Lee et al. 1994, Rouse, Cohen et al. 1994) In *S. pombe,* Shiozaki, and Millar in the laboratory of P. Russell in 1995 (Millar, Buck et al. 1995, Shiozaki and Russell 1995) identified Sty1 (also known as Spc1) as the main stress-dependent MAPK in fission yeast.

The activity of the Sty1 MAPK cascade is tightly regulated. The upstream sensors of the stress signals are Mak1, -2 and -3, which activate the phosphotransmitter Spy1/Mpr1 that inhibits the response regulator Mcs4. Then, this leads to activation of the two upstream components of the MAPK module, the redundant two MAPKKKs Wak1 (also named Wis4 or Wik1) and Win1. These MAPKKKs active the MAPKK Wis1 and Wis1 dually phosphorylates Sty1 on spell the threonine 171 and the tyrosine 173 residues. These phosphorylation sites are regulated by the tyrosine phosphatases Pyp1 and Pyp2, and the serine-threonine phosphatases Ptc1 to -4. These phosphatases maintain the basal activity of the cascade in normal conditions or return to basal conditions after stress (Vivancos, Jara et al. 2006) (Fig. 16).

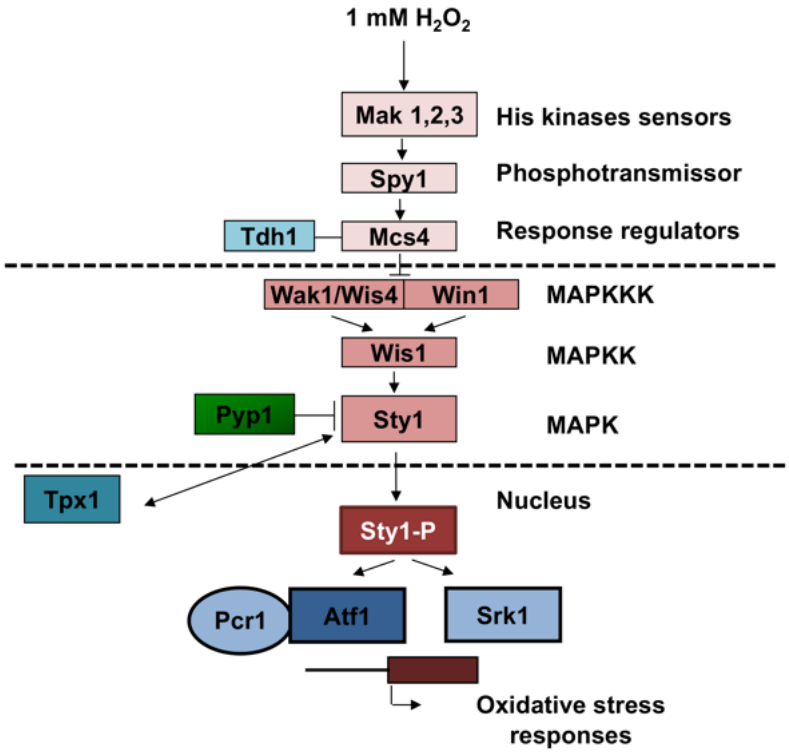

Figure 16. Activation of the Sty1 cascade pathway. Activation of the Sty1 pathway by oxidative stress and the activation series until the activation of expression of the Sty1-dependent genes.

Once Wis1 phosphorylates and releases Sty1, it is translocated to the nucleus where it phosphorylates and activates its main substrate Atf1. Atf1 is a bZIP-containing transcription factor that is constitutively nuclear. Active/phosphorylated Atf1 forms a heterodimer with Pcr1, another bZIP transcription factor, and both activate the expression of a large set of genes that are included in the core environmental stress response (CESR). CESRs genes consists of 104 transcripts that are upregulated upon at least two-fold in four out of five different insults: oxidative, osmotic, heavy metal, heat and DNA damage. On the other hand, other 106 CESRs genes are downregulated by twofold in three out of five types of stress conditions. Induced genes are involved in several processes, such as carbohydrate metabolism, detoxification of ROSs, folding and degradation, autophagy, mitochondrial functions and metabolite transport. The repressed genes are normally involved in energy consuming and growth-related processes, including RNA processing, transcription and translation, and biosynthesis of ribosome and nucleotides. Most of these genes depend on Sty1 and, to a lesser

extent, on Atf1 (Chen, Toone et al. 2003). Some of those Atf1-dependent genes are *ctt1,* the unique *S. pombe* catalase, and the sulforedoxin *srx1* that is necessary to restore Tpx1-Pap1 redox relay (Vivancos, Jara et al. 2006).

Sty1 is activated upon several inputs such as: oxidative stress, heat shock, UV light, DNA damaging agents, heavy metals, osmotic stress, nitrogen or glucose starvation and aging (Wilkinson, Samuels et al. 1996, Degols and Russell 1997, Rodriguez-Gabriel and Russell 2005, Wang, Shimada et al. 2005, Zuin, Vivancos et al. 2005, Zuin, Carmona et al. 2010). However, each insult activates the pathway in a different manner, and little is known about the mechanism of activation of each one. For instance, upon oxidative or osmotic stress the upstream kinases Mak2 and Mak3 act as kinase sensors and activate the pathway (Buck, Quinn et al. 2001). Regarding heat shock, it has been described that there is a direct Pyp1 inactivation (Nguyen and Shiozaki 1999). Moreover, it has been published that other stress conditions such as nutrient depletion may activate Sty1 pathway in an indirect way (Zuin, Carmona et al. 2010) (Fig. 16**)**.

It has been observed that cells which are exposed to any mild stress are able to induce an adaptive response and they exhibit stress fitness and increased longevity phenotype. It has been demonstrated that this occurs because of prior low activation of the Sty1 pathway. For instance, cells that are treated with caloric restriction have a longer lifespan or present resistance to stronger insults (Zuin, Carmona et al. 2010, Zuin, Castellano-Esteve et al. 2010).

The Sty1 pathway participates in other non-stress-related processes such as heterochromatin assembly or homologous recombination of cell polarity (Sanchez-Mir, Salat-Canela et al. 2018).

4.2.3. Hsf1

Upon heat stress, the Heat Shock transcription Factor 1 (Hsf1) plays a pivotal role in most cell types, including fission yeast. It was discovered in the 1960s in *Drosophila melanogaster*. Later, Robert Kingston laboratory isolated Hsf1 from *S. pombe* in 1993, and they described that Hsf1 is a very conserved protein among eukaryotes. *S. pombe* Hsf1 is essential under normal growth as well as

during stress conditions (Gallo, Prentice et al. 1993), as it is in most cell types. Surprisingly, Hsf1 is not essential in *S. cerevisiae*.

In basal conditions, inactive Hsf1 displays cytosolic localization. Activation of Hsf1 depends of its phosphorylation. Apparently, many phosphorylation events (and probably different kinases) participate in Hsf1 activation. Thus, a MAPK pathway phosphorylates Hsf1 in mammalian cells (Dai and Sampson 2016). However, the detailed mechanisms leading to heat shock-dependent Hsf1 activation are unclear. One proposal is that Hsf1 is sequestered and inactivated by different chaperones during normal conditions; upon heat shock those chaperones release Hsf1 (Anckar and Sistonen 2011). Once Hsf1 is activated, Hsf1 moves to the nucleus and it forms homotrimers that bind to promoters containing the HSE (heat shock element) motif, and promote their expression. These genes code for chaperones or UPS machinery components that are involved in the PQC response to proteotoxicity.

Recently, it has been demonstrated that expression of Hsf1-dependent genes has a role in physiological processes as well, such as metabolism, aging or development (Vihervaara and Sistonen 2014).

Objectives

Objectives

Proteostasis consists in maintaining the correct balance of the concentration and molecular structure of proteins and the PQC system is in charge of taking care of it. The control of the stability of specific proteins regulates their activity, what becomes important for the regulation of cellular processes. On the other hand, the maintaining of the protein native conformation is crucial for cellular fitness. The accumulation of non-native proteins provokes stress conditions and links with several diseases. Thus, the two main goals of my thesis project are focused on (i) the study of PQC system functions related to the basal regulation of the signal transduction cascades, and (ii) the role of PQC system upon proteotoxic stress in fission yeast.

Therefore, I have developed these main objectives along my thesis project:

1.- Establishment of Pap1 as model of normal turnover of proteome.
2.- Study how the PQC affect the oxidative stress response.
3.- Characterize the PQC mechanisms of Pap1 turnover.
4.- Establishment of Htt^{NTD}.nQ-GFP and TDP-43-YFP as non-native protein reporters.
5.- Characterization of the phenotypes that promote the expression of Htt^{NTD}.nQ-GFP and TDP-43-YFP.
6.- Study the role of Hsp40 Mas5 upon proteotoxic stress.

Results

Results

This section is divided in 2 major chapters. The Chapter one is presented like a paper and one more figure added, and the second chapter is presented like a manuscript in preparation.

1. Chapter one: Identification of the Ubiquitin-proteasome system affecting the degradation of the transcription factor Pap1

In this first chapter, we focus on the role of PQC in the regulation of the protein stability.

Signal transduction cascades have to be regulated in order to limit their activity when they are not necessary in basal conditions or after cessation of the activating signal. In a screen to search for protein quality control components required for wild-type tolerance to oxidative stress in fission yeast, we have isolated eight gene deletions conferring resistance not only to H_2O_2 but also to caffeine. Here we correlate these phenotypes with the stabilization of the transcription factor Pap1. Thereby, in the following paper, we characterize which UPS components are involved in the degradation of Pap1. Furthermore, we describe the mechanism of the UPS machinery to recognize Pap1. Finally, we identify a 30 aa sequence in Pap1 as degron of Ubr1.

Marte L, Boronat S, García-Santamarina S, Ayté J, Kitamura K, Hidalgo E. Identification of ubiquitin-proteasome system components affecting the degradation of the transcription factor Pap1. Redox biology. 2020;28:101305. DOI: 10.1016/j.redox.2019.101305

1.1.1. Degradation rate of Pap1 decreases upon HS

This small section is meant to complement this first chapter of results besides the presented previous paper.

As we saw before (Fig. 3D), upon HS there is a delocalization of Rpt4 from the nuclear membrane to the Cytosol. Furthermore, in *S. cerevisiae* has been published that Hul5 is changing its localization upon HS as well (Fang, Ng et al. 2011). Thus, we want to know if others UPS elements that are involved in Pap1 degradation change their localization upon HS. We decide study the behavior of the other E3-ligase involved in Pap1 degradation, Ubr1. Ubr1 tagged with HFG is observed my microscopy and we can see that Ubr1 is localized in the nuclear membrane and it is delocalized into the cytosol upon HS (Fig. 1.1A).

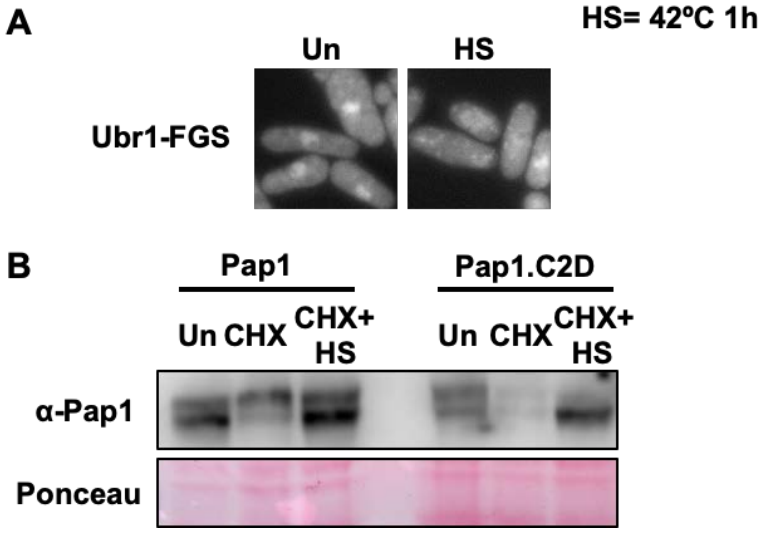

Figure 17.1. Pap1 UPS-dependent degradation is impaired upon HS. **A** Sub-cellular localization of Ubr1-FGS in basal condition and after 42ªC 1h (HS). **B** Cells expressing GFP-Pap1 and Pap1.C2D are treated with 0.1 mg/ml cycloheximide (CHX) or CHX plus 1h 42ºC (CHX+HS). The levels of GFP-Pap1 or GFP-Pap1.C2D under untreated (Un), CHX or CHX+HS conditions are analysed by WB. Ponceau staining is used as load control.

Moreover, we studied if the UPS machinery movement had a repercussion in Pap1 stability. We observed that protein levels of Pap1 and constitutive nuclear Pap1.C2D were stabilized upon HS (Fig. 1.1B), presumably because UPS elements in charge of Pap1 degradation are not in the nucleus anymore.

2. Chapter two: Expression of the aggregation-prone proteins Huntingtin and TDP-43 cause both beneficial and toxic effects in fission yeast

In this second chapter, we study the proteotoxic stress produced by the accumulation of non-native proteins and the role of the PQC system in the response to this stress. For this reason, we express the chimeras Htt^{NTD}.nQ-GFP and TDP-43-YFP that are usually used as misfolding response reporters. The expression of the chimeras induces proteotoxic stress depending on the expression levels and the facility of prone-misfolding of the chimeras. At the same time, we show that there are differences between both reporters that influences in their toxic effect. In addition, we confirm that the proteome of fission yeast seems to be more robust than the budding yeast.

Moreover, we found that chaperones Hsp104 and the Hsp70/Hsp40 couple Ssa2/Mas5 are involved in the reporter's aggregation. Surprisingly, the mild expression of Htt^{NTD}.nQ-GFP leads to promotion of the *S. pombe* lifespan. We demonstrate that this happens presumably because the recruitment of chaperones drives to the activation of stress response pathways. Therefore, cells can recognize the presence of proteotoxic stress and develop a response.

Expression of the aggregation-prone proteins Huntingtin and TDP-43 cause both beneficial and toxic effects in fission yeast

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Short title: protein aggregation and toxicity

Keywords: huntingtin, TDP-43, protein aggregation, fission yeast, neurodegenerative diseases

ABSTRACT

Many neurodegenerative disorders display protein aggregation as a hallmark, Huntingtin and TDP-43 aggregates being characteristic of Huntington disease and amyotrophic lateral sclerosis, respectively. However, whether these aggregates cause the diseases, are secondary by-products, or even have protective effects, is a matter of debate. Mutations in both human proteins can modulate the shape, number and type of aggregates, as well as their toxicity. To study the role or protein aggregates in cellular fitness, we have expressed in a simple model system different variants of Huntingtin and of TDP-43. They both display characteristic patterns of aggregation and toxicity, even though in both cases the protein has to be very highly expressed to affect cell fitness. Their aggregation properties are affected by chaperones such as Hsp104 and the Hsp40/Hsp70 couple Mas5/Ssa2, even though the proteins do not fully co-localize, suggesting that these Htt and TDP-43 derivatives have intrinsic aggregation propensity. Importantly, expression of the aggregating form of Huntingtin causes a significant extension of the fission yeast lifespan, probably as a consequence of kidnapping chaperones required for maintaining stress responses off. Our study demonstrates that in general these prion-like proteins do not harm cells under normal conditions, but rather protect them through indirect mechanisms which up-regulate cellular defense pathways.

INTRODUCTION

Even though protein aggregation is a reversible process in vivo [for a review, see (Nillegoda, Wentink et al. 2018)], proteostasis can be overwhelmed due to mutations or stress, and intra and extracellular protein inclusions are a hallmark of some pathological conditions and of aged cells. Thus, a common feature of many neurodegenerative disorders is the impairment of proteostasis, resulting in the aggregation of disease-specific proteins. The cause of toxicity and neurodegeneration may arise from a toxic gain of-function or loss-of-function of the disease protein. Genetic mutations can cause a protein to aggregate, e.g., the misfolded Huntingtin protein in Huntington's disease. However, in Parkinson's, Alzheimer's and amyotrophic lateral sclerosis (ALS), most cases cannot be associated with any known mutations, so that specific proteins appear as aggregates in the absence of genetic changes. An environmental insult, such as exposure to toxic chemicals, may be the trigger of protein aggregation. The highest risk factor for most neurodegenerative diseases is aging.

Another important issue in the aggregation of these human proteins is the shape and location of the specific foci. The general believe is that smaller, more soluble, oligomeric aggregates may be the most toxic species (Peskett, Rau et al. 2018). In recent years, several predictive algorithms have been developed to identify proteins with prion-like domains, as well as to predict their aggregation propensity (Alberti, Halfmann et al. 2009, Ross, Maclea et al. 2013, Lancaster, Nutter-Upham et al. 2014).

Regarding the molecular mechanisms that explain toxicity due to these aggregates, they are poorly understood. A first hypothesis is that these aggregates, and specifically the more heterogeneous oligomers, can display solvent-exposed hydrophobic surfaces, and these can cause aberrant interactions with other unrelated proteins; this sequestration of essential proteins could impair cell fitness (Chiti and Dobson 2006, Bolognesi, Kumita et al. 2010, Olzscha, Schermann et al. 2011). A second possibility, which may not be mutually exclusive from the former but rather occur simultaneously, is that aggregating proteins may interfere with the regular components of the protein quality control (PQC) system, so that chaperones or degrading activities [such as components of the ubiquitin-proteasome system (UPS)] may also be sequestered and leave unattended their natural substrates (Park, Kukushkin et al. 2013, Park, Hong et al. 2017). Again, both toxicity mechanisms may act in parallel.

Expansion of polyglutamine (polyQ) domains in specific proteins is the origin of several neurodegenerative diseases which are inherited as a dominant trait, with their onset and severity closely correlating with the length of the polyQ expansion (Gusella and MacDonald 1998). In Huntington disease, the aggregation of a mutated protein, named Huntingtin (Htt) depends on the number of polyQ expansion and causes the disease (1993, Andrew, Goldberg et al. 1993, Zheng and Diamond 2012). Although the presence of aggregates often correlates with toxicity (Wyttenbach, Sauvageot et al. 2002), it has been described that the oligomeric species, formed before the constitution of the mature Htt deposits, are highly toxic and responsible for the cellular alterations observed in Huntington disease (Bucciantini, Giannoni et al. 2002, Arrasate, Mitra et al. 2004). The molecular mechanism leading to Huntington disease pathogenesis are not completely understood, and numerous factors may be involved.

TDP-43 is an RNA-binding protein, with a physiological nuclear localization, which accumulates in cytoplasmic inclusions in patients of ALS and other disorders. In fact, the pathological hallmark of ALS is the presence of protein inclusions in the patients' motor neurons, containing superoxide dismutate, Tar/DNA binding protein-43 (TDP-43) and Fused in Sarcoma. Most ALS mutants do not display mutations in the TDP-43-coding gene, even though some mutations are also found in a small number of ALS patients (Mackenzie, Bigio et al. 2007, Sreedharan, Blair et al. 2008).

Most of these neurodegenerative disease-causing proteins do not have yeast orthologs. Nevertheless, the ectopic expression, or humanization, of these prion-like proteins in *Saccharomyces cerevisiae* has proven useful platforms for studying the aggregation and toxicity of these human proteins [for reviews, see (Di Gregorio and Duennwald 2018, Monahan, Rhoads et al. 2018)].

In particular, the ectopic expression of Htt.Qn and TDP-43 variants in budding yeast has demonstrated to be efficient to study the molecular bases of protein aggregation and of cellular toxicity. To provide insights in the mechanisms causing Htt protein aggregation, the laboratory of Susan Lindquist proposed in 2000 the use of a fluorescent chimera, Htt.Qn-GFP, to follow protein aggregation in yeast (Krobitsch and Lindquist 2000). Since then, many laboratories have used this heterologous system to study both aggregation propensity as well as toxicity (Giorgini, Guidetti et al. 2005, Duennwald, Jagadish et al. 2006, Mason, Casu et al. 2013, Lu, Psakhye et al. 2014). Very similarly, TDP-43 has also been expressed in budding yeast (Johnson, McCaffery et al. 2008, Braun, Sommer et al. 2011). In both cases, the isolation of deletion or over-expression suppressors of aggregation or toxicity has been used extensively, as well as the effect of small drugs on blocking either aggregate formation of cellular defects. Another classical strategy to understand aggregate formation and toxicity has been to perform quantitative mutagenesis to score aggregation propensity (and derived toxicity) of human disease proteins. This has

been recently performed expressing a collection of mutagenized TDP-43 prion domain library in budding yeast (Bolognesi, Faure et al. 2019).

Schizosaccharomyces pombe has not been extensively used as a model system to study PQC. Using misfolding reporters, we have recently described the formation and fate of protein aggregate centers, which appear after heat shock (HS) (Cabrera, Hidalgo and colleagues, unpublished results). Thus, non-irreversibly misfolded proteins, with the aid of the Hsp40/Hsp70 chaperones Mas5/Ssa2, are sequestered upon heat shock in discrete foci to escape from degradation; only when the low temperature is recovered the Hsp104 disaggregase refolds the protein aggregates. In fact, the Mas5/Ssa2 couple seems to be the master regulator of PQC in *S. pombe*: it is essential for the maintenance and folding of intrinsically unstable proteins in the absence of stress, and it also maintains the stress regulators Hsf1 (Heat Shock transcription Factor 1 (Vjestica, Zhang et al. 2013) and Sty1 (a MAP kinase regulating the common environmental stress response) (Sanso, Vargas-Perez et al. 2011) inactive unless heat shock is applied (Boronat, Hidalgo and colleagues, unpublished).

Regarding the humanization of *S. pombe* to study the bases of neurodegenerative diseases, Supattapone and colleagues have expressed Htt in fission yeast, to conclude that Htt.Q103 can aggregate but cannot exert toxicity in this unicellular eukaryote (Zurawel, Kabeche et al. 2016, Zhao, Zurawel et al. 2018). The distribution of polyQ proteins varies from one species to another, being surprisingly small in the fission yeast proteome (0.07% of all proteins in *S. pombe* display long polyQ stretches). The fact that expression of Htt with long (but not short) polyQ tails in fission yeast produces intracellular aggregates but not toxicity would be consistent with the idea that the bases of the problems caused by protein aggregation is the aberrant sequestration of endogenous and essential prone-to-aggregate proteins. Whether other non-polyQ-containing human proteins, such as TDP-43, cause or not toxicity in *S. pombe* has not been studied yet.

Here, we express not only Htt variants but also TDP-43 derivatives in fission yeast. In both cases we have been able to detect toxicity caused by aggregation, but only when the proteins are expressed at very high levels. Some TDP-43 derivatives, more soluble and oligomeric than others, can also elicit more damaging effects in fission yeast that Htt variants, suggesting that the shape and type of aggregates is critical for damage. When toxicity occurs, the protein degradation machinery, namely UPS, is not affected, while chaperones required to assist protein refolding seem to be partially sequestered into aggregation foci. We suggest that in fission yeast, where endogenous aggregation-prone proteins are not abundant, the toxicity caused by massive over-expression of Htt.Q103 or TDP-43 variants is due to the sequestrations of important chaperones of the PQC, but not of UPS components. Importantly, moderate expression of Htt.Q103 can sequester only the Hsp40 Mas5, and this causes the unexpected activation of a stress response cascade and a beneficial lifespan extension.

RESULTS AND DISCUSSION

Expression of Htt.Qn-GFP in fission yeast

Although the expression of Htt in budding yeast has been extensively studied, little is known about the expression of Htt in fission yeast. We have expressed in S. pombe the Htt chimeras described in 2000 by the Lindquist lab, which include the N-terminal domain of Htt fused to 25, 47 and 103 polyQ repeats, followed by a proline-rich domain of Htt and the GFP tag (Krobitsch & Lindquist, 2000) (Fig. 1A). These proteins were expressed from constitutive promoters of different strengths (Fig. 1A). For constitutive expression, the Htt^{NTD}.nQ-GFP-coding genes were integrated into the S. pombe genome and expressed by the constitutive tpx1 and sty1 promoters. As expected from the intrinsic promoters' strengths, the protein levels from the tpx1 promoters was 3-5 higher than from the sty1 promoter (Fig. 1B). Based on

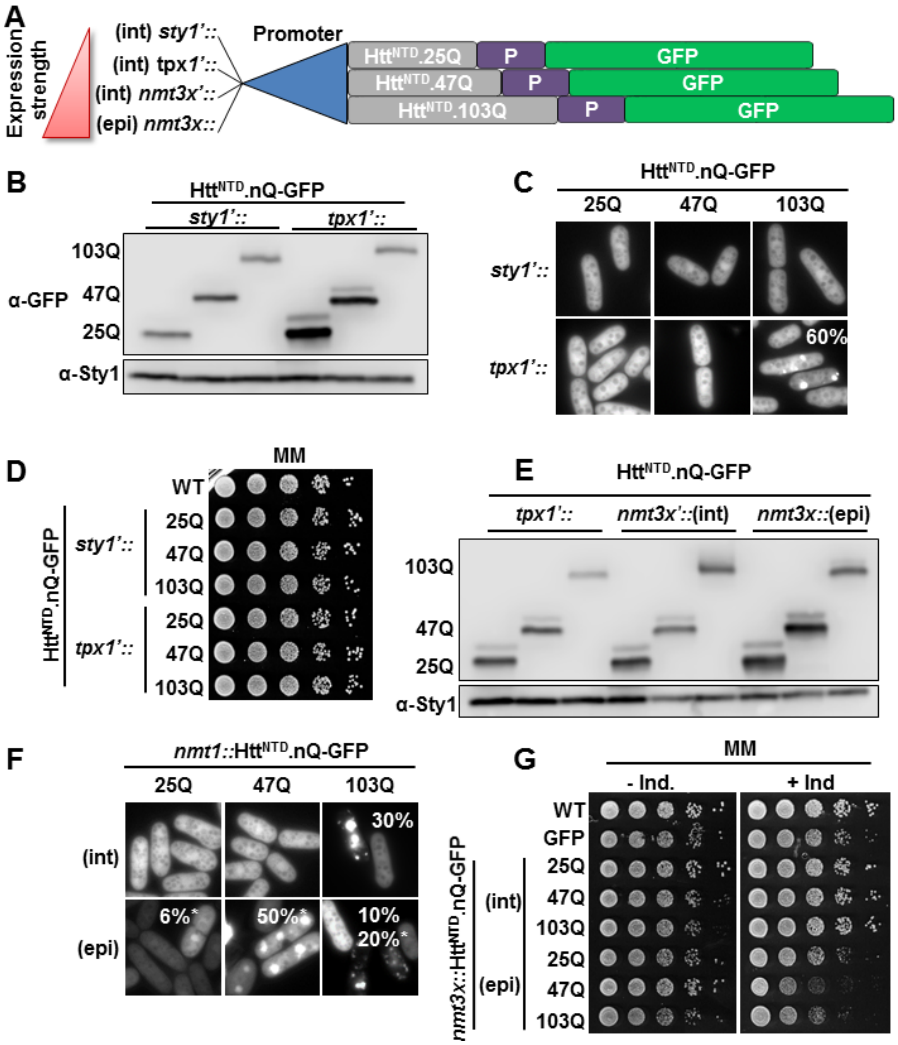

Figure 1. Characterization of the expression of Htt^{NTD}.nQ-GFP in fission yeast. **A** Schematic representation of the Htt^{NTD}.nQ-GFP constructs used in this project: the red triangle indicates the increase of the force of expression of the different used promoters; the blue triangle, promoter; gray box, N-terminal region of human HTT containing a stretch of 25, 47 o 103 glutamines; green box, GFP. **B** Steady-levels of Htt^{NTD}.nQ-GFP determined by WB of TCA extracts from SG295.25Q, LM30.47 and 103Q (*sty1'* promoter) and AB1.25,47 and 103Q (*tpx1'* promoter). Sty1 was used as loading control. **C** Fluorescent microscopy of strains expressing Htt^{NTD}.nQ-GFP under the control of sty1' and *tpx1'* promoters SG295.25Q, LM30.47 and 103Q (*sty1'* promoter) and AB1.25Q, AB1.47Q and AB1.103Q (*tpx1'* promoter). Percentage of number of cells with aggregates is indicated in white. **D** 972 (WT) strain and previous strains used in B and C were serial diluted and spotted on solid MM plates. **E** Steady-levels of Htt^{NTD}.nQ-GFP determined by WB of TCA extracts from AB1.25Q, AB1.47Q and AB1.103Q (*tpx1'* promoter); LM208.25Q, LM208.47Q and LM208.103Q (integrative *nmt1'* promoter); and HM123 transformed with p659.25Q, p659.47Q and p659.103Q (expressing episomal Htt^{NTD}.nQ-GFP under the control of episomal *nmt1* promoter). Sty1 was used as loading control. **F** Fluorescence microscopy of the strains used in E expressing Htt^{NTD}.nQ-GFP under the control of *nmt1'* promoter. Percentage of number of cells with aggregates is indicated in white, and the accumulates with (*). **G** 972 (WT), HM123 transformed with p690 (expressing GFP under the control of the *nmt1* promoter) and previous strains used in F were serial diluted and spotted on repressing (-ind. MM+ Thiamine) or inducing (+ ind. MM) solid plates.

western blot analysis using internal endogenous proteins and polyclonal antibodies, we determined that the intracellular protein concentrations of the chimeras from the sty1 and tpx1 promoters are in the order of 1 and 12 µM, respectively (Table 1).

We analyzed by fluorescent microscopy the aggregation profiles of the different Htt^{NTD}.nQ-GFP. The 25Q and 47Q chimeras display soluble cytosolic fluorescence when expressed either from the tpx1 or the sty1 promoters. On the contrary, Htt^{NTD}.103Q-GFP expressed from the strong tpx1 promoter aggregates into discrete foci (Fig. 1C). 60% of tpx1' promoter driven Htt^{NTD}.103Q-GFP-expressing cells contained aggregates.

Table1. Cellular concentration of the different constructs**.**

Promoter	[Htt^{NTD}.nQ-GFP] (μM)	[TDP-43-YFP] (μM)
(int) *sty1'*	4	-
(int) *tpx1'*	12	-
(int) *nmt1'*	19	9
(epi) *nmt1*	36	18 (WT) 36 (M322K)

In order to determine whether the constitutive expression of Htt^{NTD}.nQ-GFP could affect cell fitness, we spotted cells expressing Htt^{NTD}.nQ-GFP, and we did not observe any significant growth defects compared to the wild-type strain (Fig. 1D). Moreover, it has been described that depletion of the proline rich region of Htt is required to induce toxicity in budding yeast (Dehay & Bertolotti, 2006). However, we expressed Htt chimeras lacking this region without any effect on cell growth (Fig. S1A).

We next decided to study the expression of Htt^{NTD}.nQ-GFP but now under the control of the inducible nmt1 (no message in thiamine) promoter, which triggers the expression of downstream genes in the absence of thiamine. In order to achieve different protein levels, we decided to express the construct from integrative and episomal plasmids, which are present in 7-8 copies per cell. We compared by WB the protein levels of these conditional systems, using as control extracts from the tpx1 promoter. As shown in Fig. 1E, expression of the chimeras from the nmt1-driven episomal plasmids reaches the highest levels (around 30 μM), about three times higher than the tpx1 constitutive expression (12 μM), whereas the integrative conditional expression allows an intermediate level (19 μM) (Fig. 1E) (Table 1). Next, we visualized by fluorescence microscopy the aggregation pattern of Htt^{NTD}.nQ-GFP under the control of these two new systems. We obtained similar results between integrative nmt1' expression and tpx1', we observed a diffuse GFP signal in 25Q and 47Q, and only 103Q leads to aggregation 30% of the cells. However, when induced at 15 μM from the nmt1 promoter and episomal plasmids, fluorescence foci were detected from the 25, 47 and 103Q-expressing cells. The percentage of cells with these type of rounded inclusions also depends on the polyQ length: 25Q showed a 6%*, 47Q a 50%* and 103Q a 10%* of cells with aggregates. However, in 103Q expressing cells we also observed aggregate-like foci in 20% of the cells (Fig. 1F). Unexpectedly, the percentage of cells with aggregates in both conditional systems is lower, although the size of aggregates is bigger compared to the expression under the constitutive tpx1 promoter. We speculate that higher expression levels of aggregation-prone Htt^{NTD}.103Q-GFP allow the formation of larger protein inclusions and limits spreading.

Next we investigated the effect of the inducible expression of the chimeras on cell growth. As shown in Fig. 1F, proteins expressed from the integrative plasmids did not have any impact on fission yeast growth, while the high levels of expression of the chimeras jeopardized cell growth to different extents. Thus, Htt^{NTD}.25Q-GFP exerts some toxicity but it is comparable to the expression from the same promoter and episomal plasmids of only GFP. Both Htt^{NTD}.47Q-GFP and Htt^{NTD}.103Q-GFP seem to be toxic at these very high levels. Surprisingly, expression of 47Q is more toxic that 103Q (Fig. 1G). We think that it happens because cells expressing 47Q show more accumulates than 103Q. Probably, these accumulates are more oligomeric and soluble that 103Q aggregates, which explains the higher toxicity.

In conclusion, aggregate formation of the Htt^{NTD}.nQ-GFP chimeras depends on both the concentration and the polyQ length. In addition, the expression of Htt^{NTD}.nQ-GFP can lead to toxicity but only if they are expressed at extremely high levels, about 30 μM (Table 1), higher than any other protein of the S. pombe proteome.

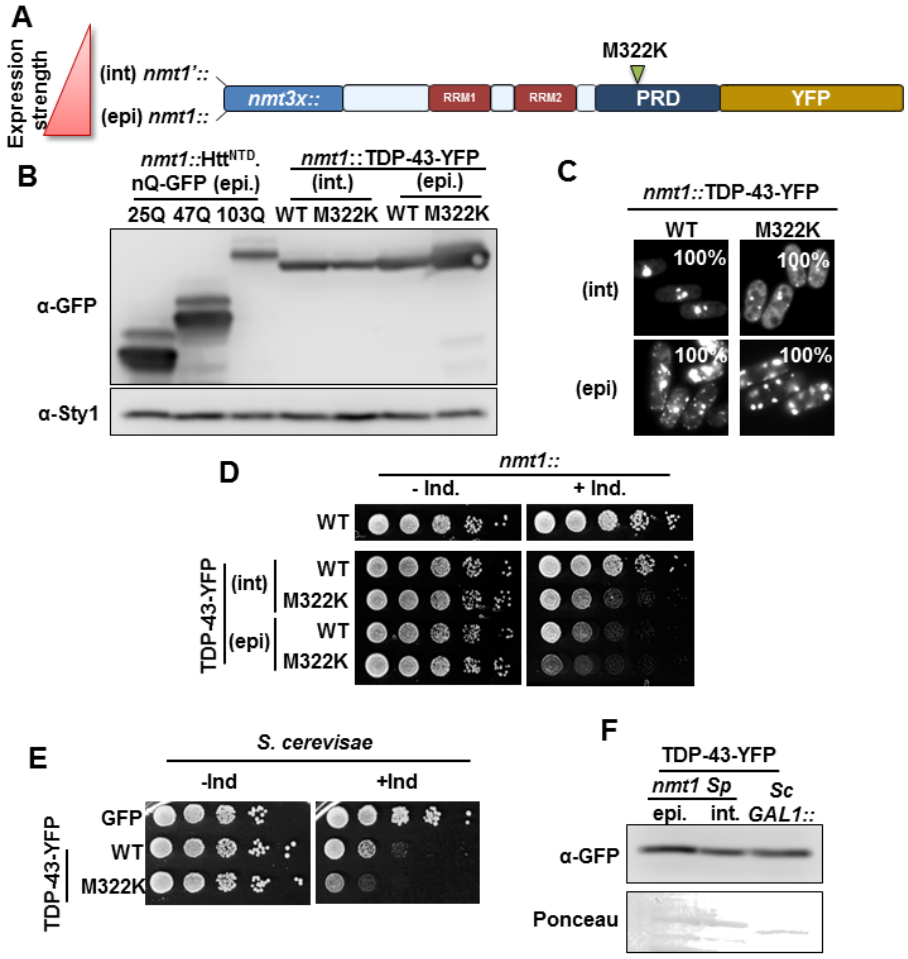

Figure 2. Characterization of the expression of TDP-43-YFP in fission yeast. **A** Schematic representation of the TDP-43-YFP constructs used in this thesis: the red triangle indicates the increase of the force of expression of the promoters; the light blue box, promoter; red box, RNA recognition motive 1 and 2 (RRM1-2); dark blue box, prion-like domain (PRD); yellow box (YFP); green triangle indicates the position of the mutation M322K. **B** Steady-levels of Htt^{NTD}.nQ-GFP and TDP-43-YFP determined by WB of TCA extracts from HM123 transformed with p659.25Q, p659.47Q and p659.103Q (expressing Htt^{NTD}.nQ-GFP under the control of episomal *nmt1* promoter), LM218 (integrative *nmt1*-driven WT), LM218.M322K (integrative *nmt1*-driven M322K) and HM123 transformed with p660 (WT) and p660.M322K (M322K) (express episomal TDP-43-YFP and TDP-43.M322K-YFP under the control of *nmt1* promoter). Sty1 was used as loading control. **C** Fluorescent microscopy of strains expressing TDP-43-YFP used in B. Percentage of number of cells with aggregates is indicated in white. **D** 972 (WT) strain and previous used strains in C were serial diluted and spotted onto repressing MM plus thiamine (-Ind.) or inducing MM (+Ind.). **E** Strains BY4741 expressing: pRS426 plasmid containing GFP (GFP) and pRS416 containing TDP-43-YFP (WT) or TDP-43.M322K-YFP (M322K), all under the control of *GAL1* promoter; were serial diluted and spotted onto repressing glucose (Ind.) or inducing galactose (+Ind.) solid media. **F** Steady-levels of TDP-43-YFP or TDP-43.M322K-YFP determined by WB of native extracts from *S. pombe* strains used in C and D; and *S. cerevisiae* used strains expressing TDP-43-YFP in E. Ponceau staining was used as loading control.

Variants of TDP-43 cause more toxicity in fission yeast than Htt.Q103

We generated a series of constructs of human gene TDP-43 in its wild-type version and with the mutation M322K in the C-terminal prion-like domain (PRD), fused to a C-terminal yellow fluorescent protein (YFP). We expressed these constructs in S. pombe under the control of the nmt1 promoter, using again integrative and episomal plasmids (Fig. 2A). We confirmed that the protein levels with the episomal version of the constructs are higher compared to those accomplished from the integrative version. Furthermore, when using the same expression system (nmt1 promoter and episomal plasmid), the protein levels of the TFP-43-YPF variants and of the Htt^{NTD}.nQ-GFP chimeras are similar (Fig. 2B).

We studied the sub-cellular distribution and aggregation propensity of TDP-43 in S. pombe by fluorescence microscopy. 100% of the cells expressing TDP-43-YFP from the integrative plasmid present nuclear aggregates, but when TDP-43-YFP is expressed at high concentrations from episomal plasmids the aggregates

are also present in the cytosol. On the other hand, cells expressing the mutated TDP-43.M322K-YFP present nuclear and cytosolic foci in both the integrative and the episomal version (Fig. 2C).

We next tested whether expression of TDP-43 causes toxicity in S. pombe. We performed a spotted dilution on solid media, and we observed that cells expressing either wild-type or mutant TDP-43 under the highest expression version show growth defects, whereas under the control of the integrative nmt1 promoter, only cells expressing the mutated construct show growth defects (Fig. 2D). Therefore, we suggest that the presence of cytosolic inclusions of TDP-43 is linked to toxicity. In addition, we also conclude that the mutant form TDP-43.M322K, as in S. cerevisiae, shows more toxicity than the wild-type construct.

Accomplishing toxicity through the expression of Htt and other aggregation-prone proteins in S. cerevisiae is a rather controversial issue, with many laboratories claiming that not even 103Q can impair cell fitness. To shed light into this issue and to determine whether budding and fission yeast are differentially affected by the expression of TDP-43, we have compared the effect of TDP-43 expression in S. pombe and S. cerevisiae. First, we expressed the same constructs under the control of the galactose-inducible GAL1 promoter in budding yeast. We performed dilution spots on solid media confirming that the growth of S. cerevisiae is impaired by expression of TDP-43 and the M322K mutation enhances the toxic effect (Bolognesi et al., 2019, Johnson et al., 2008) (Fig. 2E).

This result is similar to the toxicity exerted by high levels of TDP-43 in S. pombe (expressed from the episomal plasmids, nmt1 promoter). Then, we wanted to know if the same protein levels lead to the same growth defects in both yeast models. Upon total induction of these systems, we observed that the protein levels of TDP-43-YFP under the control of the integrative promoter nmt1 in S. pombe were similar to that seen with the GAL1 promoter in S. cerevisiae (Fig. 3F). These results suggest that the expression of TDP-43 can drive to toxicity in both yeast species and the M322K mutation enhance this toxic effect. However, comparing S. pombe and S. cerevisiae there is a higher toxic effect in S. cerevisiae at the same levels of TDP-43-YFP. The same happens with the expression of Htt^{NTD}.nQ-GFP (Zurawel et al., 2016). This supports the idea of S. pombe has a "stronger" proteome than S. cerevisiae.

Therefore, after analysing the expression of Htt^{NTD}.nQ-GFP and TDP-43-YFP, we confirm that toxicity is linked to aggregation. Moreover, in both sets of reporters, it is necessary to overcome a concentration of 20 µM, depending on the protein chimera, to detect toxic effects, except TDP-43.M322K that confers toxicity in lower levels, 7 µM. This indicates that TDP-43.M322K is very toxic itself, presumably because the solubility properties of this reporter is different from the others. Likewise, our results indicate that the expression of TDP-43-YFP variants is more dangerous than the expression of Htt^{NTD}.nQ-GFP variants, since at the same levels, TDP-43 confers more growth defects. In addition, the expression of TDP-43-YFP drives to aggregation in the 100% of the cells, while expression of Htt^{NTD}.nQ-GFP does not. Thereby, the two reporters display characteristic and differential features regarding both aggregation propensity and capacity to trigger damage.

The intracellular concentrations of Htt.Qn and TDP-43 derivatives is not dependent on the proteasome or autophagy

Now we wanted to study the molecular mechanisms that drive to toxicity due to aggregates. It has been reported that aggregates can impair the normal activity of the PQC components, affecting the proper proteostasis of cells. First, we tested if the UPS degrades our reporters and, in that case, they could be overflowing the UPS machinery and then, impairing the normal degradation activity of the cell. To test this, we treated with cycloheximide cells expressing Htt^{NTD}.nQ-GFP under the control of the constitutive *tpx1'* promoter. We observed that after overnight treatment there is no decrease of protein levels (Fig. 3A). Same happens in cells expressing TDP-43-YFP under the control of the episomal *nmt1* promoter (Fig. 3B). Therefore, the UPS is not removing them. Then, we decided to test if autophagy is involved in the elimination of Htt^{NTD}.nQ-GFP or TDP-43-YFP. WT and Δ*atg1* (autophagy is abolished) cells present the same protein levels of the reporters

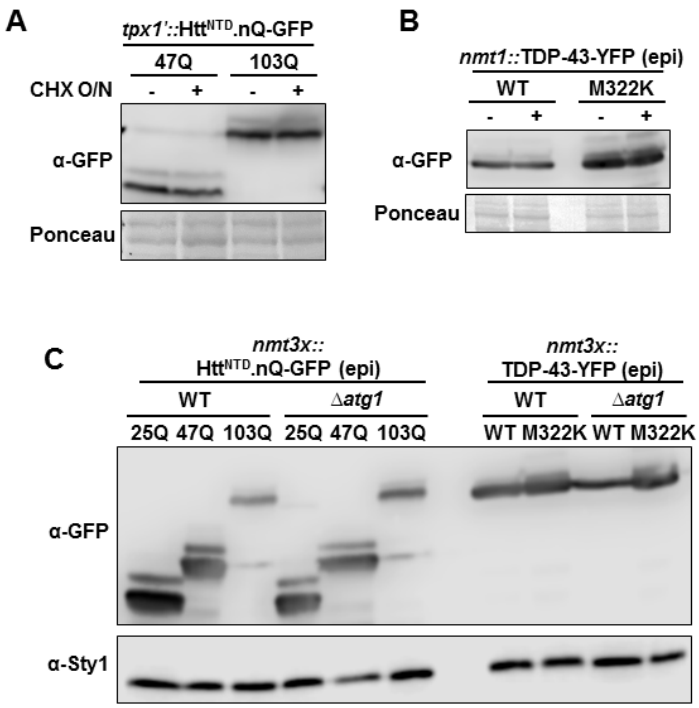

Figure 3. Htt^{NTD}.nQ-GFP and TDP-43-YFP are not degraded by the UPS or autophagy. **A** Strains AB1.47Q and AB1.103Q were overnight treated with 0.1 mg/ml CHX. The Htt^{NTD}.nQ-GFP levels under the control of the constitutive promoter *tpx1'* were analyzed by Western Blot of TCA extracts of untreated (-) or treated (+) cells. Ponceau staining is used as loading control. **B** HM123 transformed with p660 (WT) and p660.M322K (M322K) (express episomal TDP-43-YFP and TDP-43.M322K-YFP under the control of *nmt1* promoter) were overnight treated with 0.1 mg/ml CHX. The TDP-43-YFP levels were analyzed by Western Blot of TCA extracts of untreated (-) or treated (+) cells. Ponceau staining is used as loading control. **C** Steady-levels of Htt^{NTD}.nQ-GFP and TDP-43-YFP determined by WB of TCA extracts from both HM123 and SK1 transformed with p660 (WT) and p660.M322K (M322K) to express episomal TDP-43-YFP and TDP-43.M322K-YFP, and p659.25Q, p659.47Q and p659.103Q to express episomal Htt^{NTD}.nQ-GFP under the control of *nmt1* promoter were serial diluted and spotted onto repressing MM plus thiamine (-Ind.) or inducing MM (+Ind.).

(Fig. 3C), so autophagy neither is involved in the degradation of Htt^{NTD}.nQ-GFP or TDP-43-YFP. Nevertheless, there is a little increase of toxicity in Δatg1 strains expressing 103Q or both TDP-43 variants (Fig. S2A). These results point to autophagy have a role in avoiding proteotoxic stress independently of the degradation of proteotoxic non-native proteins.

Actually, to finish confirming whether the degradation machinery is affected, we are working to determine if the degradation of known substrates of the UPS is affected in the presence of toxic levels of Htt^{NTD}.nQ-GFP or TDP-43-YFP.

Several PQC components modulate the aggregation propensity of Htt.103Q but not TDP-43

Besides UPS components, aggregates can affect the normal activity of the other most important group of the PQC components: the molecular chaperones. We generated a series of strains containing deletions of some PQC components and expressing Htt^{NTD}.nQ-GFP constitutively under the control of *tpx1'* promoter. Then, we were looking for significantly variations in the number of cells with aggregates. We found a dramatically change of aggregation in the deletions of *hsp104* and the Hsp40 *mas5*.

The deletion of *mas5* exacerbates significantly the number of cells with aggregates. Cells expressing 47Q already showed aggregates (75%) and the 100% of cells expressing 103Q have aggregates. Even there are aggregates in cells expressing Htt.103Q under the weak constitutive *sty1'* promoter in a Δ*mas5* background (Fig. 4A). On the contrary, cells lacking Hsp104 and expressing 103Q did not present aggregates anymore. Surprisingly, cells overexpressing Hsp104 neither show any aggregate (Fig. 4C). Moreover, the aggregation of the double deletion cells has an intermediate phenotype (Fig. S3A). This points to an independent role of Mas5 and Hsp104 in the proteostasis of Htt.nQ, and that Hsp104 is not necessary for Htt.nQ aggregation. In the same way, in cells lacking Ssa2, the Hsp70 of Mas5, there are aggregates in 47Q as well as cells lacking Mas5 (Fig. 4B). Therefore, these results suggest that the couple Ssa2/Mas5 (Hsp70/Hsp40) avoid the default formation of aggregates, whereas Hsp104 promote their formation.

Then, we tested what happens when we express TDP-43-YFP under the control of the integrative *nmt1* promoter in cells lacking Hsp104 or Mas5. In this case, we did not detect any variation in the aggregation phenotype (Fig. 4D).

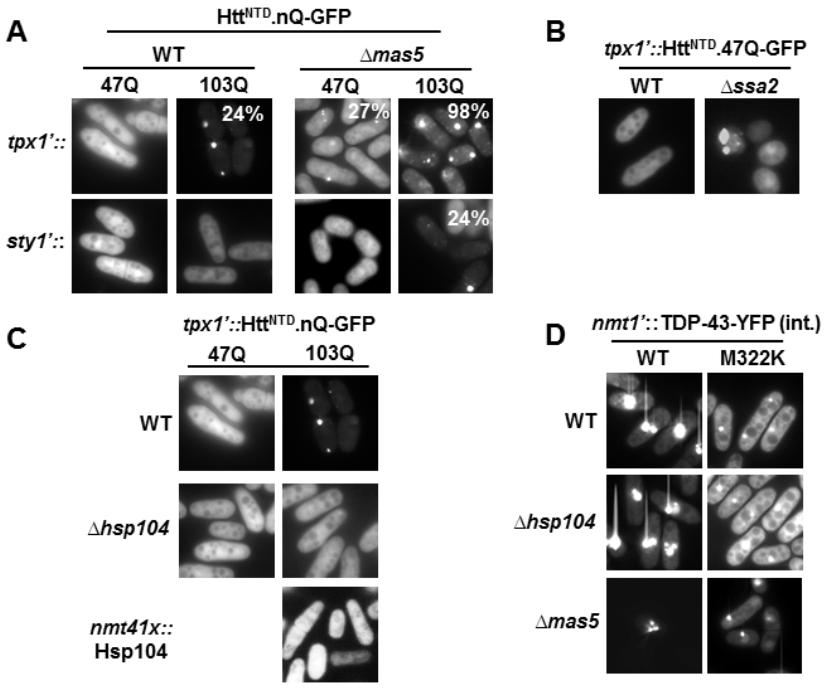

Figure 4. Components of PQC system are involved in Htt^{NTD}.nQ-GFP and TDP-43-YFP aggregation. A Fluorescent microscopy of strains expressing Htt^{NTD}.nQ-GFP under the control of constitutive *tpx1'* AB1.47Q, AB1.103Q (WT), and SB413.47Q, SB413.103Q (Δ*mas5*); or *sty1'*promoter LM30.47Q, LM30.103Q (WT), LM18.47Q and LM18.103Q (Δ*mas5*). Percentage of number of cells with aggregates is indicated in white. **B** Fluorescent microscopy of strains expressing Htt^{NTD}.47Q-GFP under the control of constitutive *tpx1'* AB1.47Q (WT), AB10.47Q (Δ*ssa2*). **C** Fluorescent microscopy of strains expressing Htt^{NTD}.nQ-GFP under the control of constitutive *tpx1'* AB1.47Q, AB1.103Q (WT), AB2.47Q, AB2.103Q (Δ*hsp104*) and AB1.103Q transformed with p520 to over express Hsp104 under the control of the episomal nmt41 promoter. **D** Fluorescent microscopy of strains expressing Htt^{NTD}.nQ-GFP under the control of the conditional integrative *nmt1* LM218 (WT), LM218.M322K (WT); LM219 (WT), LM219.M322K (Δ*hsp104*), LM225 (WT) and LM225.M322K (Δ*mas5*).

We tested the effect of the deletion of other PQC components. We did no detect any effect even in the expression of 103Q in cells lacking Hsp16 or Sti1 (Fig. S3B). On the contrary, even at 47Q expression we observed aggregation in cells lacking Def1 or the Hsp40 SPBC17A3.05c. However, we did not see any difference in cells lacking the Hsp70 Ssa1 (Fig. S3C). Interestingly, we found that cells lacking the E2 Ubc14 have a similar phenotype to Δ*mas5* cells. Under the expression of *sty1'* promoter is difficult to see aggregates, though (Fig. S3D).

Therefore, our results suggest that Hsp104 and Mas5 genetically interact with Htt.nQ aggregates. Then, these chaperones can be overworked in cells expressing Htt^{NTD}.nQ-GFP that may lead in toxic effects in the cell. On the contrary, Hsp104 and Mas5 do not seem to have any function in the aggregation of TDP-43-YFP. This goes in the direction that TDP-43 leads to toxicity affecting other cellular process.

Hsp104 and Mas5 do not fully co-localize with Htt.103Q

We next study if the function of Mas5 or Hsp104 on Htt.nQ aggregation is direct. First, by fluorescent microscopy we visualized if Mas5 tagged to mCherry in the N-terminal controlled by the constitutive *sty1'* promoter co-localizes with 103Q aggregates. In basal conditions there are no even aggregates of Mas5. Upon HS, Mas5 form cytosolic aggregates of which only a 10% co-localize with103Q aggregates (Fig. 5A).

However, the deletion of *mas5* drives to the drastic increase of Htt.nQ aggregation. Then, Mas5 could be bound the soluble Htt.nQ and prevent it from aggregation. So that, we decided to perform a Co-immunoprecipitation of Htt^{NTD}.nQ-GFP and Mas5 tagged with HA in the C-terminal, which allows us to study the interaction in the soluble state. The results showed that there is a physic interaction between Mas5 and 47Q and 103Q but not with 25Q in basal conditions (Fig. 5B). Therefore,

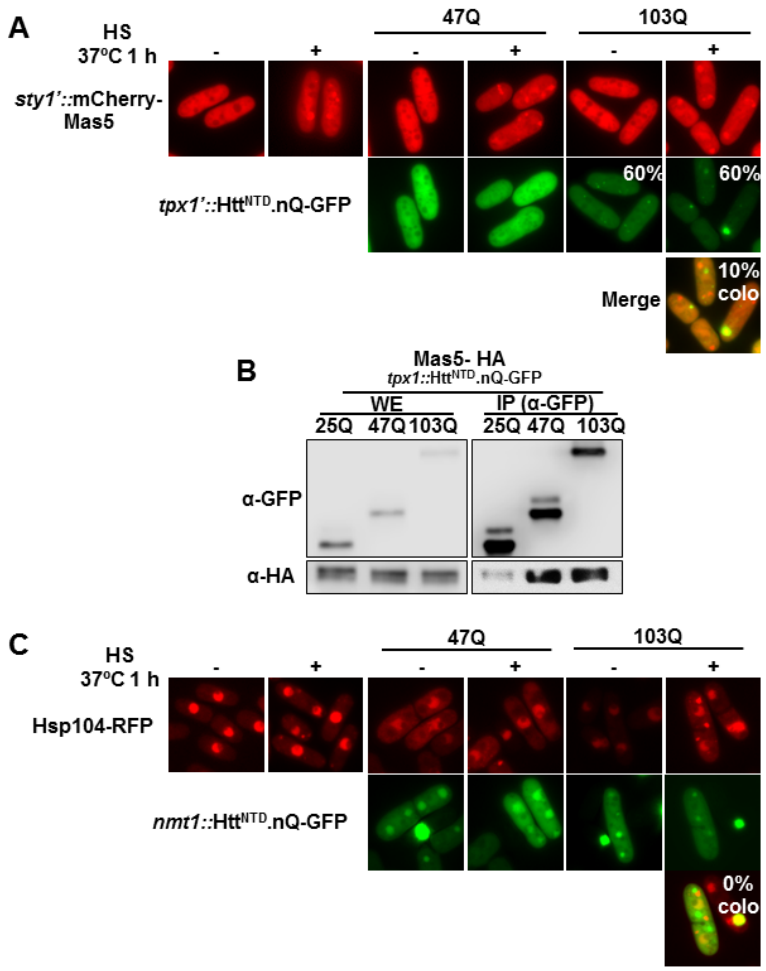

Figure 5. Co-localization of Mas5 and Hsp104 with Htt^{NTD}.nQ-GFP. **A** Fluorescent microscopy of strains expressing mCherry-Mas5 under the control of *sty1'* promoter SB644 or strains expressing also Htt^{NTD}.nQ-GFP under the control of *tpx1'* promoter LM217.47Q and LM217.103Q in basal conditions (-) and after 1h 37ºC (+). mCherry-Mas5 is visualized by mCherry channel, Htt^{NTD}.nQ-GFP by the GFP channel and merge of both channels. Percentage of number of cells with aggregates and percentage of aggregates from both channels than co-localize are indicated in white. **B** Interaction between Mas5-HA and Htt^{NTD}.nQ-GFP determined by co-immunoprecipitation of native extracts of strains LM121.25Q, LM121.47Q and LM121.103Q. IP was assayed with GFP beads, Htt^{NTD}.nQ-GFP and Mas5-HA proteins were detected in the Whole cell extracts (WCE) and pull-down (IP) after immunoblotting with an antibody against GFP and HA. **C** Fluorescent microscopy of strains expressing Hsp104-RFP SB386 or LM2 transformed with p659.25Q, p659.47Q and p659.103Q to express episomal Htt^{NTD}.nQ-GFP under the control of *nmt1* promoter in basal conditions (-) and after 1h 37ºC (+). Hsp104-RFP is visualized by RFP channel, Htt^{NTD}.nQ-GFP by the GFP channel and merge of both channels. Aggregates from both channels than co-localize are indicated in white

.

Mas5 is bound to those Htt forms that their aggregation is enhanced in Δmas5 strains.

In the same way, we performed the same experiments to test if there is an interaction between Htt.nQ and Hsp104. First, by fluorescence microscopy we discarded the co-localization of Htt^{NTD}.nQ-GFP and Hsp104-RFP in basal or HS conditions. Interestingly, the expression of 47Q abolishes the formation of Hsp104 aggregates after HS (Fig. 5C). Also, we did not see any interaction by co-immunoprecipitation (data not shown).

Previously we saw that Mas5 has a role in Htt.nQ aggregation. Now we confirm that Mas5 joins directly to molecular Htt.nQ in order to avoid aggregation. These results together the role of Mas5 in the reversible aggregation upon HS of misfolded proteins suggests that the Ssa2/Mas5 couple seems to be the key component of PQC system in the management of non-native proteins in *S. pombe*. Actually, these roles may look like opposites, but we have to think that the cellular conditions upon HS are very different compare to basal conditions.

Regarding Hsp104, it is clear that Hsp104 has a role in Htt.nQ aggregation, but it may have an indirect function.

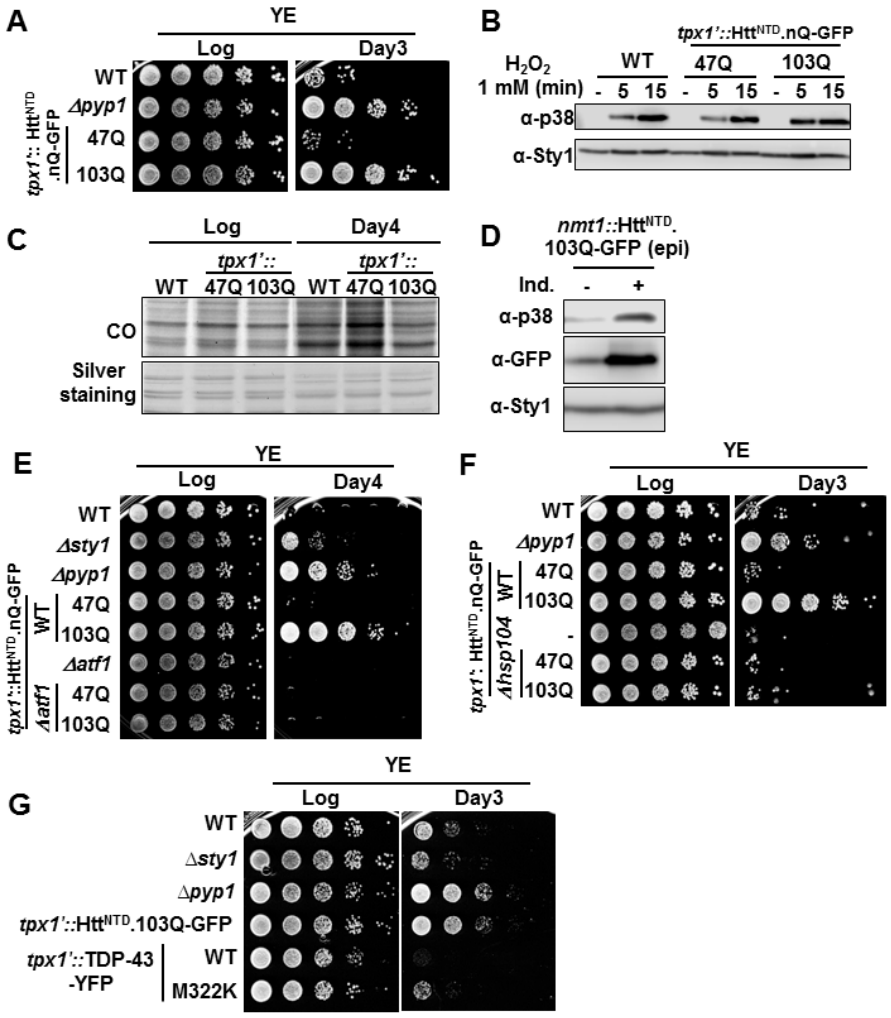

Figure 6. Expression of 103Q under the control of the constitutive *tpx1'* promoter provides a lifespan promotion. A Strain 972 (WT), EP48 (Δ*pyp1*), AB1.47Q (47Q) and AB1.103Q (103Q) were grown in YE. Serial dilution of logarithmic (Log) or stationary phase (Day3) were spotted onto YE plates. **B** Sty1 activation was analyzed in TCA extracts from strains 972 (WT), AB1.47Q (47Q) and AB1.103Q (103Q) untreated (-) or treated with H_2O_2 1 mM during 5 or 15 min by WB with antibody α-p38. Sty1 was used as loading control. **C** Protein carbonyl (CO) determination in extracts from strain 972 (WT), AB1.47Q (47Q) and AB1.103Q (103Q) of logarithmic (Log) or stationary phase (Day4). Silver staining was used as loading control. **D** Sty1 activation and steady levels of 103Q were analyzed in TCA extracts form HM123 + p659.103Q (episomal *nmt1::*Htt^{NTD}.103Q-GFP) grown in repressing MM plus Thiamine (-) or inducing MM (+) by WB with antibody α-p38 and α-GFP. Sty1 was used as loading control. **E** Strain 972 (WT), AV18 (Δsty1), EP48 (Δ*pyp1*), AB1.47Q (47Q), AB1.103Q (103Q), EP48 (Δ*atf1*), LM23.47Q (Δ*atf1* 47Q) and LM23.103Q (Δ*atf1* 103Q) were grown in YE. Serial dilution of logarithmic (Log) or stationary phase (Day4) were spotted onto YE plates. **F** Strain 972 (WT), EP48 (Δ*pyp1*), AB1.47Q (47Q), AB1.103Q (103Q), SG287 (Δ*hsp104*), AB2.47Q (Δ*hsp104* 47Q) and AB2.103Q (Δ*hsp104* 103Q) were grown in YE. Serial dilution of logarithmic (Log) or stationary phase (Day3) were spotted onto YE plates. **G** Strain 972 (WT), AV18 (Δ*sty1),* EP48 (Δ*pyp1*), expressing *tpx1'*-driven 103Q AB1.103Q and strains expressing *tpx1'*-driven TDP-43-YFP LM210 (WT) and LM210.M322K (M322K) were grown in YE. Serial dilution of logarithmic (Log) or stationary phase (Day3) were spotted onto YE plates.

Therefore, the expression of Htt^{NTD}.nQ-GFP have a repercussion in the chaperones network, which can be affecting the normal cellular activity. Particularly, the toxic expression of Htt.47Q prevents the normal aggregation of Hsp104 upon HS, which can be impairing cellular fitness.

Expression of non-toxic concentrations of Htt.Q103 extends lifespan

It was already published that in *S. pombe* the expression of non-toxic levels of Htt^{NTD}.nQ-GFP does not exacerbate the growth defects of several stresses such as heat stress, or oxidative stress (Zurawel, Kabeche et al. 2016). Thereby, we decided to test aging as stressor for the first time. Surprisingly, we observed for

spotting assay that cells expressing constitutive 103Q under the control of *tpx1'* promoter shown resistance to stationary phase (Fig. 6A).

Previous work of our group demonstrated that cells that were exposure to a mild stress induce a cellular response that results in stress fitness and longevity. This was due to the previous activation of the Sty1 pathway that prepares the cell for a subsequent higher stress condition (Zuin, Carmona et al. 2010). Thus, we wanted to test if there is an early or higher activation of the Sty1/Atf1 pathway in cells expressing 103Q construct. First, we observe that cells expressing the constitutive 103Q under the control of *tpx1'* have an earlier phosphorylation of Sty1 than WT and 47Q-expressing cells upon oxidative stress (Fig. 6B). In addition, a classical hallmark of aging produced by oxidative stress is the protein carbonylation. Indeed, we found that 103Q-expressing cells do not show carbonylated proteins accumulation after four days, unlike WT and 47Q strains (Fig. 6C).

In order to know if the only expression of Htt^{NTD}.nQ-GFP could induce this activation in non-stress conditions, we measured the activation of Sty1 in absence of stress in cells expressing the highest 103Q protein levels. We confirmed that 103Q expression facilities the activation of Sty1 (Fig. 6D). Moreover, we demonstrated that this phenotype depends on the Sty1/Atf1 pathway, because in cells lacking Atf1 there is no long live phenotype anymore (Fig. 6E).

We next investigated if the aggregation drives to this phenotype. The mutant deletion of *hsp104* prevents the aggregation of 103Q under the control of *tpx1'* promoter. Thus, we tested if cells expressing Htt^{NTD}.nQ-GFP and lacking *hsp104* without aggregates maintain the resistance to stationary phase. We observed by spot dilution that there is no resistance phenotype anymore (Fig. 6F).

As we know, Mas5 is limiting the basal activation of Sty1 (Data not published). Therefore, our results indicate that the recruitment of Mas5 by Htt.103Q drives the activation of Sty1. Then, as we expected, the non-toxic expression of TDP-43 do not promote the long live phenotype (Fig. 6G), since Mas5 is not involved in TDP-43 aggregation.

Here we identify Mas5 as sensor of the proteotoxic stress, besides as key component of PQC system in fission yeast.

MATERIALS AND METHODS

Growth conditions, yeast strains and plasmids

*S. pombe c*ells were grown in rich medium (YE), synthetic minimal medium (MM) or MM plus thiamine (2 µM) as described previously (Alfa, Fantes et al.). *S. cerevisiae* cells were grown in MM Ura-. Origins and genotypes of strains and plasmids used in this study are outlined in Table S1 and Table S2 respectively. C-terminal tagging of genes was done using homologous recombination with PCR fragments, using as templates pFA6a plasmid derivatives (Bahler, Wu et al. 1998). Most deletions were obtained by crossing deletion mutants from the Bioneer collection (Kim, Hayles et al. 2010) with wild type strain 972 to remove auxotrophies. To express mCherry-Mas5, we constructed an integrative plasmid, p705′, to express the chimera under the control of the constitutive *sty1'* promoter. Plasmids expressing Htt^{NTD}.nQ-GFP were generated from p426.PQn-GDP plasmids (Krobitsch and Lindquist 2000) and cloned into empty pRep plasmids with the different promoters. In the same cloning strategy, plasmids expressing TDP-43-YFP and TDP-43.M322K-YFP were generated from pRS416 containing these constructs ceded by Dr. B. Lehner like *S. cerevisiae* strain (Bolognesi, Faure et al. 2019). Plasmids p723.47'Q and 103Q' were generated adding insert of *kan:MX6* resistance gene to p503 plasmids after the terminator using SacI restriction site. Plasmid p520 was generated amplifying Hsp104 from genomic DNA and then it was cloned into empty episomal plasmid with nmt41 promoter.

Solid sensitivity assay

For survival on solid plates, *S. pombe* strains were grown in YE or MM plus thiamine (2 µM), diluted and spotted in MM plus thiamine (20 µM) or MM medium agar plates as described previously (Calvo, Gabrielli et al. 2009). *S.* cerevisiae strains were grown in MM (Ura-) plus glucose to an OD_{600} of 0.5 then they are

serial diluted 1:10 six times from initial concentration of 0.5 and spotted in MM (-Ura) with glucose or galactose. Plates were incubated at 30ºC during 2 to 3 days.

Determination of total protein carbonyls

Yeast cells were grown in YE to an OD_{600} of 0.5, at which point they were treated with 2.5 mM H_2O_2 for 4 hours. Pellets from 50 ml cultures were washed with H_2O, resuspended in carbonylation buffer (8 M urea, 20 mM Na-phosphate buffer pH 6, 1 mM EDTA and protease inhibitors) and lysed by vortexing for 5 minutes. Protein extracts were incubated with 1% streptomycin sulphate (Sigma, S6501) in ice for 5 minutes and centrifuged for 5 minutes. Supernatants were recovered and protein concentration was calculated by Bradford assay and diluted to 1 µg/µl with carbonylation buffer. 100 µg of protein were incubated with 4 µl 50 mM fluorescein 5-thiosemicarbazide (FTC) (Sigma) at 37°C for 2 hours protected from light. Proteins were then precipitated with 10% trichloroacetic acid (TCA), incubated at -20°C for 10 minutes and centrifuged 10 minutes. Pellets were washed three times with chilled ethanol:ethyl acetate (1:1) and let to air dry. To visualize protein carbonyls by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE), pellets were resuspended in 50 µl dilution buffer (8 M urea, 20 mM Na-phosphate buffer pH 8, 1 mM EDTA). Protein concentration was determined by Bradford assay. 5 µg of protein were loaded with 5-fold sample buffer without any dye. Gels were scanned using Typhoon 8600 Variable Mode Imager scanner (Molecular Dynamics) with a 526 nm short pass filter at 800V. Gels were then fixed, and total protein was visualized by silver staining. Where indicated, protein carbonyl levels were quantified using the ImageQuant 5.2 program (GE healthcare, Little Chalfont, Buckinghamshire, United Kingdom) for carbonyls and ImageJ software for total protein.

Fluorescence microscopy

S. pombe cells were grown in MM or YE to an OD_{600} of 0.5. *S. cerevisiae* cells were grown in MM (-Ura) plus raffinose to an OD_{600} of 0.5, then then it added galactose for 4-6 hours. At which point, both were harvested by centrifugation 1 min at 3,000 rpm and visualized at room temperature. Images were acquired using a Nikon Eclipse 90i microscope equipped with differential interference contrast optics, a PLAN APO VC 100x 1.4 oil immersion objective, an ORCA-II-ERG camera (Hamamatsu), excitation and emission filters GFP-4050B and mCherry-C (Semrock) and image image acquisition software Metamorph 7.8.13 (Gataca Systems). Processing of all images was performed using Fiji (ImageJ, National Institutes of Health) (Schindelin, Arganda-Carreras et al. 2012).

TCA Extracts and Western Blot

S. pombe cells were grown in MM or YE to an OD_{600} of 0.5. *S. cerevisiae* cells were grown in MM (-Ura) plus raffinose to an OD_{600} of 0.5, then then it added galactose for 4-6 hours. Modified trichloroacetic acid (TCA) extracts were prepared as previously described (Vivancos et al, 2005). Samples were separated by SDS-PAGE and detected by immunoblotting. GFP-tagged and YFP-tagged proteins are visualized with monoclonal anti-GFP (Roche), phosphorylated Sty1, with polyclonal α-p38 (Cell Signaling) and anti-Sty1 polyclonal antibody (Jara et al, 2007) was used as loading control.

Co-immunoprecipitation

Cells from 50 ml culture at OD_{600} 0.5 grown in YE were harvested by centrifugation and cell pellets resuspended in 250 µl of lysis buffer (NP-40 0.5%, NaCl 300mM, Tris-HCl 10 mM pH7.5, 0.5 mM EDTA, 1 mM PMSF and protease inhibitor cocktail). After addition of glass beads, cells were lysed using a Precellys cell disrupter (Bertin Technologies). The lysate was centrifuged at 6000 *g* for 10 min and the supernatant (1 mg of protein) was incubated with 20 µl of GFP-trap beads (Chromotek) for 1 h at 4°C. Beads were isolated by centrifugation at 2000 rpm for 1 min at 4°C and washed with lysis buffer three times. Bound proteins were eluted with SDS sample buffer, boiled for 15 min at 95°C, loaded onto SDS-PAGE gels, and subjected to immunoblot analysis.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY INFORMATION

Expression of the aggregation-prone proteins Huntingtin and TDP-43 cause both beneficial and toxic effects in fission yeast

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Includes:

3 supplementary figures

2 supplementary Tables

Supplementary References

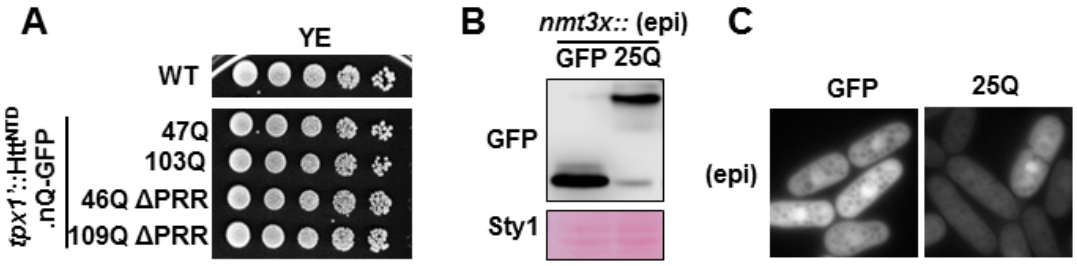

Fig. S1. Characterization of the expression of Htt^{NTD}.nQ-GFP in fission yeast. **A** Strain 972 (WT), cells expressing *tpx1'*-driven Htt^{NTD}.nQ-GFP AB1.47Q (47Q), AB1.103Q (103Q) and cells expressing *tpx1'*-driven Htt^{NTD}.nQΔP-GFP AB19.46Q (46QΔPRR), AB19.109Q (109QΔPRR), were spot diluted onto YE plates. **B** Steady-levels of HM123 transformed with p690 (expressing episomal GFP under the control of *nmt1* promoter) and with p659.25Q (expressing episomal Htt^{NTD}.25Q-GFP under the control of episomal *nmt1* promoter). Ponceau staining is used as loading control. Fluorescence microscopy of the strains used in B.

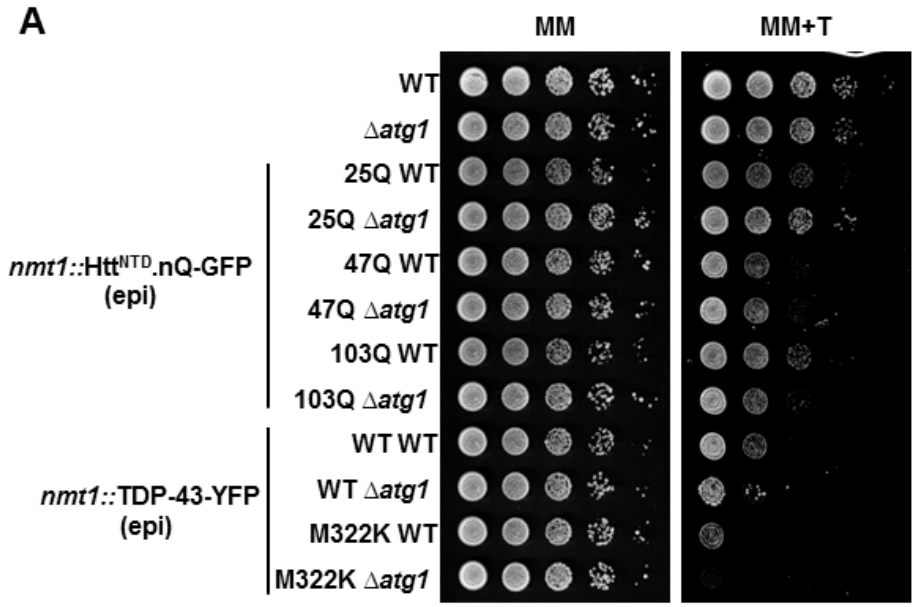

Fig S2. Htt^{NTD}.nQ-GFP and TDP-43-YFP are not degraded by the UPS or autophagy. A Strains 972 (WT), CC11 (Δ*atg1*) and both HM123 and SK1 transformed with p660 (WT) and p660.M322K (M322K) to express episomal TDP-43-YFP and TDP-43.M322K-YFP, and p659.25Q, p659.47Q and p659.103Q to express episomal Htt^{NTD}.nQ-GFP under the control of *nmt1* promoter were serial diluted and spotted onto repressing MM plus thiamine (-Ind.) or inducing MM (+Ind.).

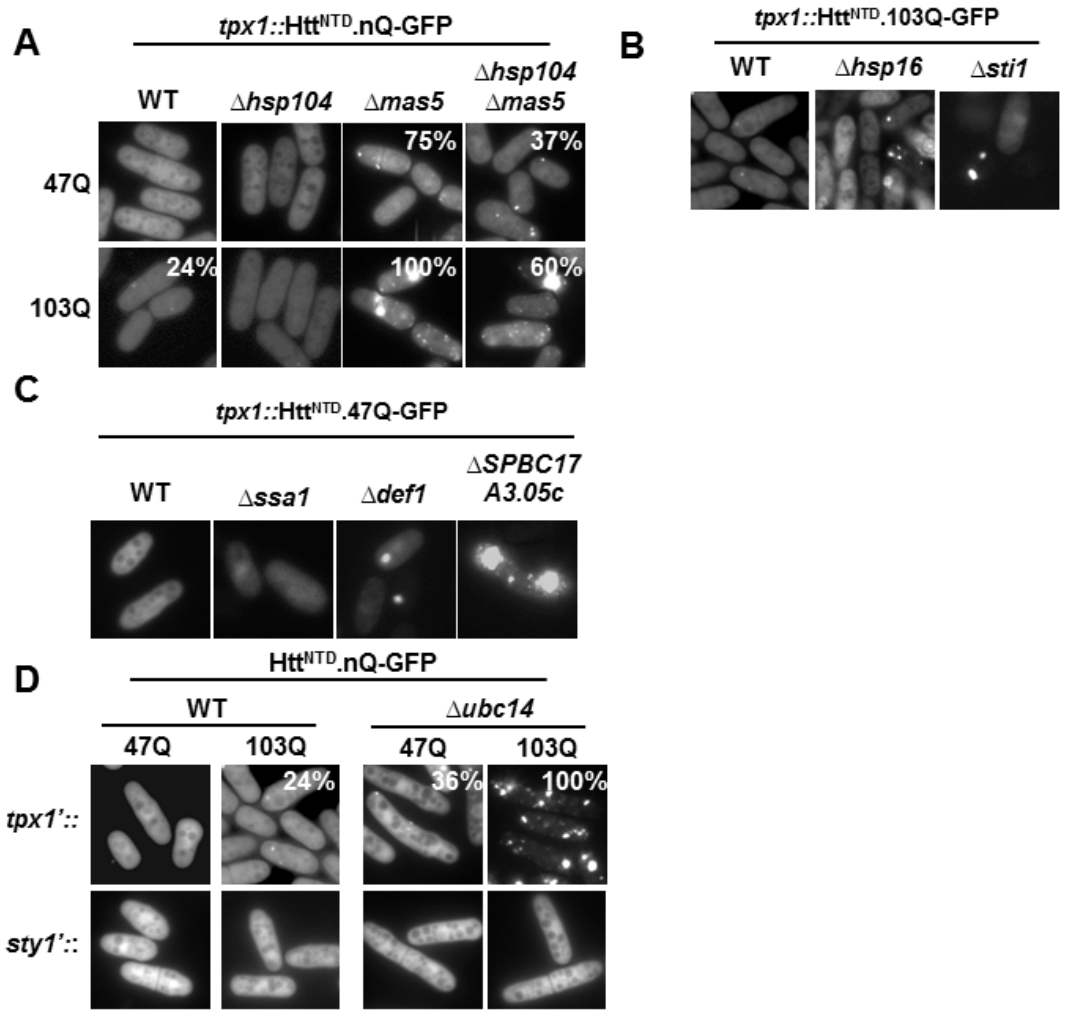

Fig. S3. Components of PQC system are involved in HttNTD.nQ-GFP and TDP-43-YFP aggregation. **A** Fluorescent microscopy of strains expressing HttNTD.nQ-GFP under the control of constitutive tpx1' promoter AB1.47Q, AB1.103Q (WT); AB2.47Q, AB2.103Q (Δhsp104); SB413.47Q, SB413.103Q (Δmas5); LM207.47Q, LM207.103Q (Δhsp104Δmas5). Percentage of number of cells with aggregates is indicated in white. **B** Fluorescent microscopy of strains expressing HttNTD.103Q-GFP under the control of constitutive tpx1' promoter AB1.103Q (WT), LM52.103Q (Δhsp16), AB11.103Q (Δsti1). C Fluorescent microscopy of strains expressing HttNTD.47Q-GFP under the control of constitutive tpx1' promoter AB1.47Q, AB9.47Q (Δssa1), AB8.47Q (Δdef1) and AB7.47Q (ΔSPBC17A3.05c). **C** Fluorescent microscopy of strains expressing HttNTD.nQ-GFP under the control of constitutive tpx1' AB1.47Q, AB1.103Q (WT), and SB313.47Q, SB313.103Q (Δubc14); or sty1'promoter LM30.47Q, LM30.103Q (WT), LM41.47Q and LM41.103Q (Δubc14). Percentage of number of cells with aggregates is indicated in white.

Table S1. Strains used in this study

Strain	Genotype	Origin
972	*h⁻*	(Leupold 1970)
HM123	*h⁻ leu1-32*	(Moreno, Klar et al. 1991)
LM2	*h⁺ hsp104::RFP::kanMX6 leu1-32*	This work
AB1.25Q	*h⁻ tpx1'::htt^{NTD}.25Q-GFP::leu1*	This work
AB1.47Q	*h⁻ tpx1'::htt^{NTD}.47Q-GFP::leu1*	This work
AB1.103Q	*h⁻ tpx1'::htt^{NTD}.103Q-GFP::leu1*	This work
SG293.25Q	*h⁻ sty1'::htt^{NTD}.25Q-GFP::leu1 ura4-D18*	This work
LM30.47Q	*h⁻ sty1'::htt^{NTD}.47Q-GFP::leu1*	This work
LM30.103Q	*h⁻ sty1'::htt^{NTD}.103Q-GFP::leu1*	This work
LM208.25Q	*h⁻ nmt1'::htt^{NTD}.25Q-GFP::leu1*	This work
LM208.47Q	*h⁻ nmt1'::htt^{NTD}.47Q-GFP::leu1*	This work
LM208.103Q	*h⁻ nmt1'::htt^{NTD}.103Q-GFP::leu1*	This work
AB2.47Q	*h⁻ hsp104::kanMX6 tpx1'::htt^{NTD}.47Q-GFP::leu1*	This work
AB2.103Q	*h⁻ hsp104::kanMX6 tpx1'::htt^{NTD}.103Q-GFP::leu1*	This work
AB6.103Q	*h⁻ leu1-32 tpx1'::htt^{NTD}.Q103-GFP::leu1 leu1::natMX6*	This work
AV18	*h⁻ sty1::kanMX6*	(Zuin, Vivancos et al. 2005)
EP48	*h⁺ pyp1::natMX6*	This work
MS98	*h⁻ atf1::natMX6*	(Fernandez-Vazquez, Vargas-Perez et al. 2013)
LM23.47Q	*h⁻ atf1::kanMX6 tpx1'::htt^{NTD}.47Q-GFP::leu1*	This work
LM23.103Q	*h⁻ atf1::kanMX6 tpx1'::htt^{NTD}.103Q-GFP::leu1*	This work
SG287	*h⁺ hsp104::natMX6*	This work
LM218	*h⁻ nmt1'::tdp-43-YFP::leu1*	This work
LM218.M322K	*h⁻ nmt1'::tdp-43.M322K-YFP::leu1*	This work
SK1	*h⁻ atg1::ura4+ ura4-C190T leu1-32*	(Mukaiyama, Kajiwara et al. 2009)
SB413.47Q	*h⁻ mas5::kanMX6 tpx1'::htt^{NTD}.47Q-GFP::leu1*	This work
SB413.103Q	*h⁻ mas5::kanMX6 tpx1'::htt^{NTD}.103Q-GFP::leu1*	This work
LM18.47Q	*h⁻ mas5::kanMX6 sty1'::htt^{NTD}.47Q-GFP::leu1*	This work
LM18.103Q	*h⁻ mas5::kanMX6 sty1'::htt^{NTD}.103Q-GFP::leu1*	This work
AB10.47Q	*h⁻ ssa2::kanMX6 tpx1'::htt^{NTD}.47Q-GFP::leu1*	This work
LM219	*h⁻ hsp104::kanMX6 nmt1'::TDP-43-YFP::leu1*	This work
LM219.M322K	*h⁻ hsp104::kanMX6 nmt1'::TDP-43.M322K-YFP::leu1*	This work
LM225	*h⁻ mas5::kanMX6 nmt1'::TDP-43-YFP::leu1*	This work
LM225.M322K	*h⁻ mas5::kanMX6 nmt1'::TDP-43.M322K-YFP::leu1*	This work

SB644	*h- sty1'::mCherry-mas5::leu1*	This work
SB387	*h- hsp104::RFP::kanMX6*	This work
LM217.47Q	*h- sty1'::mCherry-mas5::leu1 tpx1'::htt^{NTD}.47Q-GFP::leu1::kanMX6*	This work
LM217.103Q	*h- sty1'::mCherry-mas5::leu1 tpx1'::htt^{NTD}.103Q-GFP::leu1::kanMX6*	This work
LM121.25Q	*h- mas5::natMX6 tpx1'::htt^{NTD}.25Q-GFP::leu1*	This work
LM121.47Q	*h- mas5::natMX6 tpx1'::htt^{NTD}.47Q-GFP::leu1*	This work
LM121.103Q	*h- mas5::natMX6 tpx1'::htt^{NTD}.103Q-GFP::leu1*	This work
LM207.47Q	*h- mas5::natMX6 hsp104::kanMX6 tpx1'::htt^{NTD}.47Q-GFP::leu1*	This work
LM207.103Q	*h- mas5::natMX6 hsp104::kanMX6 tpx1'::htt^{NTD}.103Q-GFP::leu1*	This work
LM210	*h- tpx1'::tdp-43-YFP::leu1*	This work
LM210.M322K	*h- tpx1'::tdp-43.M322K-YFP::leu1*	This work
LM52.103Q	*h- hsp16::kanMX6 tpx1'::htt^{NTD}.103Q-GFP::leu1*	This work
SB313.47Q	*h+ ubc14::kanMX6 tpx1'::httNTD.103Q-GFP::leu1 ura4-D18 ade6-M210.*	This work
SB313.103Q	*h^{+} ubc14::kanMX6 tpx1'::htt^{NTD}.103Q-GFP::leu1 ura4-D18 ade6-M210.*	This work
LM41.47Q	*h- ubc14::kanMX6 sty1'::htt^{NTD}.47Q-GFP::leu1*	This work
LM41.103Q	*h- ubc14::kanMX6 sty1'::htt^{NTD}.103Q-GFP::leu1*	This work
AB11.103Q	*h- sti1::kanMX6 tpx1'::htt^{NTD}.103Q-GFP::leu1*	This work
AB9.47Q	*h- ssa1::kanMX6 tpx1'::htt^{NTD}.47Q-GFP::leu1*	This work
AB8.47Q	*h- def1::kanMX6 tpx1'::htt^{NTD}.47Q-GFP::leu1*	This work
AB7.47Q	*h- SPBC17A3.05c::kanMX6 tpx1'::htt^{NTD}.47Q-GFP::leu1*	This work
CC11	*h- atg1::ura4+ ura4-C190T sty1'::GFP-atg8::leu1*	This work
BY4147	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Benedetta Bolognesi
Sc TDP-43-YFP	*MATα GAL1:: TDP-43-YFP::ura3*	Benedetta Bolognesi
Sc TDP-43.M322K-YFP	*MATα GAL1:: TDP-43.M322K-YFP::ura3*	Benedetta Bolognesi

Table S2. Plasmids used in this study

Plasmid	Genotype	Origin
p503.25Q'	*tpx1'::htt^{NTD}.25Q-GFP::leu1*	This work
p503.47Q'	*tpx1'::htt^{NTD}.47Q-GFP::leu1*	This work
p503.103Q'	*tpx1'::htt^{NTD}.103Q-GFP::leu1*	This work
p499.25Q'	*sty1'::htt^{NTD}.25Q-GFP::leu1*	This work
p499.47Q'	*sty1'::htt^{NTD}.47Q-GFP::leu1*	This work
p499.103Q'	*sty1'::htt^{NTD}.103Q-GFP::leu1*	This work
p688.25Q'	*nmt1'::htt^{NTD}.25Q-GFP::leu1*	This work
p688.47Q'	*nmt1'::htt^{NTD}.47Q-GFP::leu1*	This work
p688.103Q'	*nmt1'::htt^{NTD}.103Q-GFP::leu1*	This work
p659.25Q	*nmt1::htt^{NTD}.25Q-GFP::leu1 (episomal)*	This work
p659.47Q	*nmt1::htt^{NTD}.47Q-GFP::leu1 (episomal)*	This work
p659.103Q	*nmt1::htt^{NTD}.103Q-GFP::leu1 (episomal)*	This work
p710'	*nmt1'::tdp-43-YFP::leu1*	This work
p710.M322K'	*nmt1'::tdp-43.M322K-YFP::leu1*	This work
p660	*nmt1::tdp-43-YFP::leu1 (episomal)*	This work
p660.M322K	*nmt1::tdp-43.M322K-YFP::leu1 (episomal)*	This work
p.723.47Q'	*tpx1'::htt^{NTD}.47Q-GFP::kanMX6*	This work
p.723.103Q'	*tpx1'::htt^{NTD}.103Q-GFP::kanMX6*	This work
p690	*nmt1::GFP::leu1 (episomal)*	This work
p705'	*sty1'::mCherry-mas5::leu*	This work
P520.41x	*nmt41::hsp104::leu1 (episomal)*	This work
p416-GFP	*GAL1::GFP::ura3*	Benedetta Bolognesi

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Discussion

Discussion

1. Proteostasis in fission yeast

The activity of each protein depends on two key factors: the molecular structure and the concentration. Proteostasis is the proper balance of these two factors and the PQC system is responsible for keeping it. On the contrary, the disbalance of this system can lead to several diseases.

Yeast model has proven to be a great system to understand cellular process associated with proteome integrity. Furthermore, yeast model has been used successfully as important diseases model.

The proper concentration of the proteome is maintained with specific lifetime of each protein. Cells have developed many mechanisms for detecting and eliminating proteins by the UPS. These mechanisms usually are related with keeping a certain protein concentration to limit the protein activity. Proteostasis mechanisms are involved in the turnover of either conditionals or constitutive proteins. Therefore, constitutive proteins never accumulate, and conditional proteins do not act when are not necessary anymore. Describing these mechanisms is very interesting to understand the regulation of cellular processes as we do with transduction cascade signals.

The molecular function of one protein depends on its structure as well. However, external and internal inputs can lead to non-native proteins apparition. The PQC system is in charge to protect the native conformation and avoid the accumulation of the non-native proteins. When PQC is not able to prevent non-native protein accumulation can be toxic for the cell, which is associated to several diseases.

Along this thesis we have developed successfully three protein models to study these two key factors that maintain proteostasis and their relation to the stress response: the normal turnover of proteins related to the transduction signaling cascades and the proteotoxic stress in fission yeast.

We have demonstrated the role of the PQC in the signaling of stress response. We have characterized the basal turnover of the transcription factor

Pap1. We have identified the PQC machinery that is involved in this process. Furthermore, we successfully understand the mechanisms of E3-ligase action.

Secondly, we have characterized the response upon proteotoxic stress in in fission yeast expressing two different mutated proteins models: Htt and TDP-43. In fact, this is an important issue, since these proteins are involved in different human diseases.

Moreover, our results support the idea of the PQC system can have a regulator role in cellular process besides integrity proteome protector. Particularly, during the develop of my thesis projects, I have observed the participation of the PQC system in different regulator and signaling mechanisms of the stress response:

I. the limitation of the activity of the stress response Pap1 pathway
II. the cellular response upon proteotoxic stress

Thereby, we suggest that the PQC machinery must be subject of study to understand different cellular processes.

2. Pap1

Oxidative stress leads in protein damage, resulting in the accumulation of non-native proteins that aggregates or are degraded.

Here we performed a search screening looking for PQC proteins than could be involved in the elimination of oxidized proteins. Surprisingly, we found that the mutant deletion of some PQC components results in the decrease of carbonylated protein levels upon oxidative stress. Then, we observed that those mutant cells have an increased antioxidant power than WT cells. We realized that the elimination of different elements of the UPS promotes the increase of the steady-levels of Pap1 what increased the expression levels of Pap1-dependent genes that are involved in ROSs detoxification.

Therefore, the PQC has an important role in basal condition in absence of proteotoxic stress. The activity of several proteins must be limited in order to prevent toxic effects for the cell. For example, the constitutive activation of signal transduction pathways as Pap1 pathway, can be deleterious for the cell. Thus, it seems that cells have developed a degradation strategy to limit Pap1 pathway activity.

2.1. Pap1 turnover occurs in basal conditions

Here we demonstrate that Pap1 turnover is constant, it is not necessary Pap1 is active for degradation. Pap1.C278A mutant leaks the essential cysteines residues for disulphide formation that allows Pap1 activation have low Pap1 basal protein levels that points to Pap1.C278A is being degraded.

Moreover, cells lacking the Pap1 importin Imp1 present high protein levels of Pap1, so we confirm that in basal conditions Pap1 is degraded in the nucleus by the cytosol-nucleus shuttling. Furthermore, it has been described that Yap1, the homologous of Pap1 in *S.* cerevisiae, has to be bound to DNA for be degraded. We observed that Pap1 degradation does not depend on DNA binding, mutations of Pap1 b-ZIP domain impairs the Pap1 DNA binding and still continues to degrade. Thus, we conclude that Pap1 has localization-dependent constant turnover.

2.2. Determination of a Ubr1-dependent degron

Although UPS system has been intensively studied, little is known about how the proteins are selected for degradation mechanism. Being the E3-ligase recognition of the substrate the rate-limiting step, from minutes to days. It has already reported that E3-ligase Ubr1 is crucial for Pap1 degradation (Kitamura, Taki et al. 2011). Here we isolate an only 30 aa sequence from the Pap1 sequence that acts as specific degron of Ubr1. Also, it has been found others Ubr1 substrates as meiotic inducer Mei2, but in this case it has been described a 428 aa long sequence as essential domain for UPS recognition (Kitamura, Katayama et al. 2001).

Surprisingly, those sequence do not have significant similarities. This points that is not the sequence *per se* but characteristics of the structure that provides this sequence what is recognized by Ubr1.

The N-end rule is an important mechanism for regulated controlled degradation in the cell. We dismiss that N-end rule is involved in the obtained results

of degradation of fused GFP. We observe that cells lacking the essential Ubr11 for N-end rule degradation in *S. pombe* do not show degron stabilization.

2.3. Several E3 ligases participate in Pap1 degradation

In *S. cerevisiae*, Ubr1 is involved in the degradation of misfolded cytosolic proteins, whereas in *S. pombe* it has been related to controlled degradation pathways. Nevertheless, it has been reported that in *S. cerevisiae* Ubr1 cooperates with other E3-ligases (Samant, Livingston et al. 2018). Here we show that other E3-ligases have a role in Pap1 stability: SPBC14f5.10c and Hul5. Although the depletion of these two proteins provide a little increase of Pap1 steady-levels, the double deletion of *ubr1* and *hul5*, and not *ubr1* and *SPBC14f5.10c*, present more Pap1 protein levels than single *ubr1* deletion. This demonstrate the cooperation between Hul5 and Ubr1. SPBC14f5.10c would have an independent role of Pap1 ubiquitination labelling.

Now, it will be interesting to understand the cooperation and the differences between Ubr1 and Hul5 specific roles in Pap1 degradation.

At recognition level, when we isolate the 30 aa degron, we first determine a bigger 60 aa sequence termed Deg1 as essential sequence for the Pap1 UPS degradation. Then, we divided Deg1 in two parts, Deg2 and Deg3, we observed that Deg3 is the essential degron for Pap1 degradation and that in cells lacking Ubr1 this Deg3 is totally stable. Surprisingly, Deg1 is not stable in Δ*ubr1* cells. Part of the Deg2 plus part of the Deg3 sequences must be recognized by Hul5 as well. This support the idea of the necessary cooperation among E3-ligases for proper degradation labelling.

Then, at functional level, Ubr1 function has a more influence than Hul5 in the Pap1 degradation. Maybe Hul5 and Ubr1 have the same function, but Hul5 has a lower affinity for Pap1. As well as it has been reported for cytosolic misfolded proteins (Samant, Livingston et al. 2018), Ubr1 and Hul5 can ubiquitin label with different Lysine residue links. Being Ubr1 labelling more efficient for degradation.

Furthermore, Hul5 has been described also as a E4-ligase. These UPS proteins elongate a previous ubiquitin label. So, Hul5 can improve the recognition of Ubr1-ubiquitinated Pap1 by the proteasome.

2.4. Double functionality of the E3-ligases

In *S. cerevisiae* has been reported that Ubr1 and Hul5 have a cytosolic role in proteotoxic stress conditions. However, in basal conditions, Hul5 it is localized in the nucleus and it has a role in degradation of short-lived proteins. In the other hand, in *S. pombe* Ubr1 has a nuclear localization and it is involved in short-lived protein turn-over. in basal conditions. Nevertheless, Ubr1 is translocated to the cytosol upon HS. Regarding Hul5, we were not able to confirm if it is translocated to the cytosol upon HS as well as Ubr1. However, it has been reported that Hul5 in *S. cerevisiae* also does it. This can be a useful PQC strategy for regulate transcription signals. During HS is important for fitness the clearance of non-native proteins that are accumulating. Moreover, at the same time could be useful that nuclear proteins that transduce the HS response do not be degraded. Although Pap1 is not involved in HS response, upon HS nuclear Pap1 is not degraded. So, UPS machinery involved in Pap1 degradation is not available upon HS, presumably because it is in the cytosol. Thereby, this can be a strategy to enhance the HS response through proteins with similar regulation to Pap1 that are involved in HS. Now we should study if this PQC machinery re-localization occurs in oxidative stress conditions.

2.5. Mapping the turnover of Pap1

We identified eight UPS mutant strains that present resistance phenotype to drugs and H_2O_2. All of them except Ltn1, presented an increased protein steady levels of Pap1, although the double deletion of Ltn1 and Pap1 leads to oxidative stress sensitivity recover. Ltn1 is an E3-ligase associated to 60S subunit of the ribosome and it is involved in degradation of aberrant nascent proteins. Could be that any Pap1-dependent gene has a misfolded-prone sequence, so part of

its new synthetized copies are ubiquitinated by Ltn1. We should continue our investigation to understand the participation of Ltn1 in Pap1 pathway.

Regarding to the proteasome, we know that mutations of essential proteasome subunits leads on drug resistance in a Pap1-dependent manner (Penney, Samejima et al. 2012). Here identify the proteasome scaffold protein Nas6 and the non-essential subunit of 19S lid Rpt4. We think that the increase of Pap1 steady-levels and the resulting stress resistance is due to a general proteasome misfunction. Because neither Rpt4 nor Nas6 seem to have a substrate-specific function.

However, Rpt4 seems to be regulated in a localization level upon HS. This Rpt4 behaviour is different of other proteasome subunits such as Pad1 or Pre6. This could be associated, as I mentioned before, to a PQC system strategy for stress fitness which correlates with the E3s delocalization upon HS.

The rest of the identified genes in our screening are involved in the ubiquitin labelling. Ubi1 is one of the several genes that codifies for ubiquitin molecule; Ubc2 that is a E2-conjugating enzyme whose function in Pap1 selected degradation is already known, and the E3-ligases Ubr1, Hul5 and SPBC14f5.10c that I discussed before.

2.6. Concluding remarks

We demonstrated that Pap1 has turned out to be a great model to better understand PQC mechanisms for selective substrates as strategy to maintain proteostasis of some proteins. We achieved to describe a complex map of the specific Pap1 degradation that depends on the UPS (Fig 1).

Therefore, we characterize another cellular mechanism to limit the action on proteins and regulate cellular processes in basal conditions. Which has given us more knowledge about how to alter the activity of some proteins through its concentration.

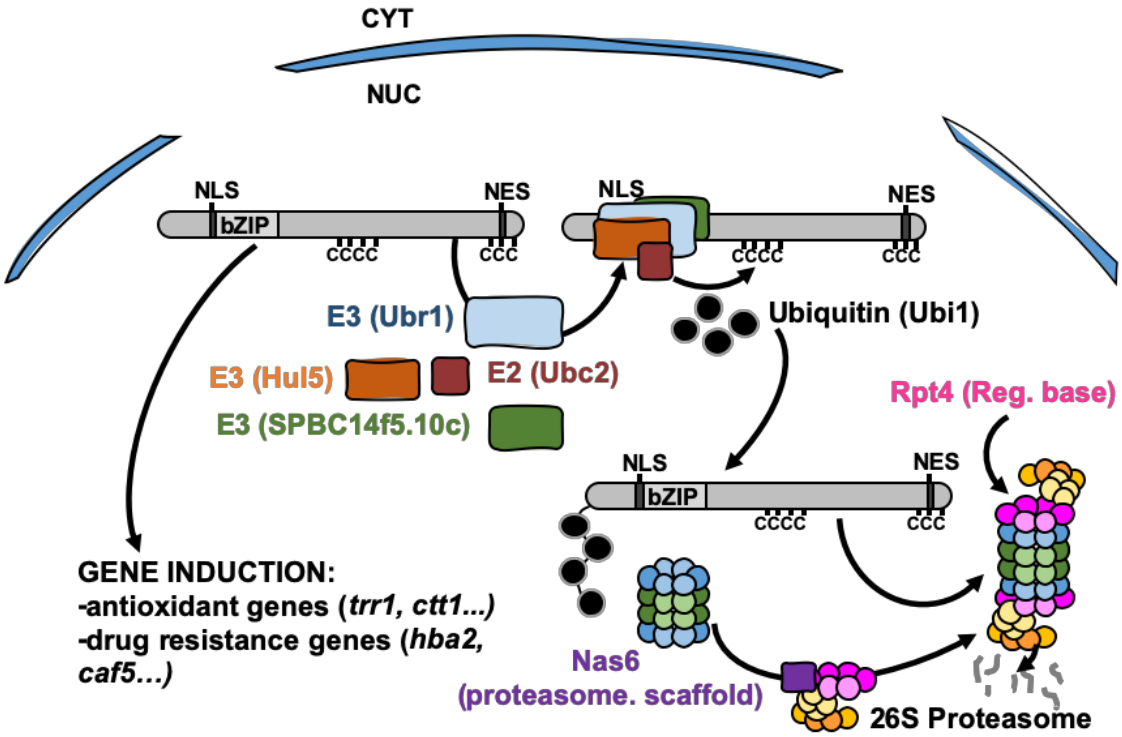

Figure 18. Pap1 degradation pathway. Schematic pathway of how UPS elements mediate the Pap1 degradation in the nucleus.

3. Htt and TDP-43

Non-native proteins can accumulate for many reasons, one of them is the mutated amino acids sequence that leads to degradation or aggregation of new synthetized peptides. Thereby, the collapse of PQC system carrying out these tasks can end in several neuronal diseases, aging or cancer. HD and ALS are canonical diseases caused by the accumulation of non-native proteins enhanced by mutations. Indeed, both diseases have provided two extensive human mutated non-native protein models: Htt.nQ and TDP-43.

In this thesis project, we have characterized the expression of these systems in *S. pombe.* Although during several years both systems have been studied in yeast, especially in *S. cerevisiae*, little is known about the expression of Htt in *S. pombe* and we have been the first group that studies the expression of TDP-43 in *S. pombe*.

Here, we have confirmed that *S. pombe* has more resistance than *S. cerevisiae* to proteotoxic stress. Both expression of Htt and TDP-43 seem to have fewer toxic effects in fission yeast. Moreover, we tested the effect of the expression of Htt added to aging. Surprisingly, we observed that the expression of Htt induces an earlier activation of the stress response resulting in an expanded lifespan. Furthermore, we identified the Hsp40 Mas5 and Hsp104 as important chaperones involved in aggregates formation.

Therefore, this thesis reaffirms the use of yeasts as model for human diseases. Furthermore, we suggest a role of PQC machinery in signalling.

3.1. Characterization of HttNTD.nQ-GFP expression in S. *pombe*

Several years ago, Susan Lindquist's group reported that in *S. cerevisiae* the aggregation of Htt^{NTD}.nQ-GFP depends on the length of the polyQ stretch. Now, we express in *S. pombe* the chimera Htt^{NTD}.nQ-GFP under two constitutive promoters, *sty1'* and *tpx1'*, because the constitutive expression is more approximate to HD affected neurons. In *S. pombe* Htt^{NTD}.nQ-GFP expression under the control of the *sty1'* promoter does not promote aggregation with any polyQ length,

whereas *tpx1'*-driven more protein levels and presents aggregates only in the 103Q form. Therefore, we demonstrated that aggregation in *S. pombe* does not just depend on polyQ length but also in concentration.

However, human cells present aggregates when polyQ has more than 35Q, and the intracellular concentration of Htt in human cells is much lower than we are expressing (Baldo, Sajjad et al. 2018). On the contrary, the constitutive expression of Htt^{NTD}.nQ-GFP (n>30Q) is not toxic in *S. pombe* nor *S. cerevisiae*. These results suggest that the PQC system of yeasts is stronger than human cells', since to observe deleterious effects in yeast, a more acute stress is needed. However, we cannot forget that Htt has a function in human cells, so there is a loss of function which can make the negative effects in human cells more evident, that in yeasts does not.

In previous publications it has been reported that the expression of Htt^{NTD}.nQ-GFP in *S. pombe* does not lead toxicity (Zurawel, Kabeche et al. 2016, Zhao, Zurawel et al. 2018). Here we show that the constitutive expression of the chimera neither promotes any toxic effect. However, we observed growth defects in cells expressing the chimera under the control of the inducible *nmt1* promoter in episomal system, where we reach the highest protein levels. 47Q and 103Q already showed growth defects, but the 47Q form presents higher toxicity than the 103Q. By microscope, we can see that the expression under the control of episomal *nmt1* leads to an accumulation of protein in circles shape, different from "normal" aggregates. Our results suggest that these protein hosts could be a toxic form. We can see that cells expressing the 47Q have more percentage the cells with hosts. 103Q would form aggregates that would alleviate the toxicity besides host accumulates. Moreover, there are a lot of cells with an aberrant morphology that links with the toxicity and present host.

The constitutive expression of Htt^{NTD}.nQ-GFP promotes a higher number or cells with aggregates compared with the inducible expression, however the inducible aggregates are bigger presumably because the protein levels are higher than the constitutive promoters. Thereby, is easier for the cell form bigger aggregates upon higher Htt^{NTD}.nQ-GFP expression which is preventing aggregates spreading.

Therefore, here we demonstrate that the expression of Htt^{NTD}.nQ-GFP in *S. pombe* can be toxic and it depends on an extreme protein expression, although.

3.2. Characterization of TDP-43-YFP expression in *S. pombe*

ALS is a neurodegenerative disease that has been related to the failure of TDP-43 proteostasis. TDP-43 expression has already characterized in *S. cerevisiae*. Nevertheless, here it is the first time that is expressed in *S. pombe*. We observed the same results than in *S. cerevisiae* and mammalian cells: the toxicity of TDP-43 is related to its cytosolic inclusions. TDP-43 aggregates have nuclear localization and upon increase of the protein levels they translocates to the cytosol.

In addition, we also express the mutated TDP-43.M322K form. Recently, it has been reported that this mutation leads to more dynamics aggregates which is increasing the deleterious effects of TDP-43 expression (Bolognesi, Faure et al. 2019). In *S. pombe* obtained also observed that the mutation promotes more toxicity in the same way that in *S. cerevisiae*. Moreover, M322K show a cytosolic localization instead of nuclear. Thus, in fission yeast is also important that non-native proteins form compact compartments to avoid the impairment of the normal cellular activity.

Like Htt^{NTD}.103Q-GFP, TDP-43 toxicity also depends on concentration. The high protein levels of TDP-43 leads to cytosolic inclusions that promotes toxicity. Moreover, like Htt^{NTD}.103Q-GFP, it is necessary higher expression levels than in budding yeast to see toxicity. However, TDP-43 seems to be more toxic *per se* than Htt, besides TDP-43 is not related to the same PQC components than Htt. We must investigate now the relation between *S. pombe* PQC and TDP-43.

3.3. Role of Mas5 and Hsp104 in Htt aggregation

We tested the effect of deletion of different PQC components in the aggregation of Htt^{NTD}.nQ-GFP. We have highlighted the role of Hsp104 and the Ssa2/Mas5 Hsp70/40 couple.

As we showed, cells lacking Hsp104 and overexpressing 103Q do not present any aggregates. Which is occurring in the same way in *S. cerevisiae*. That points to Hsp104 is necessary first for aggregates formation and later for disaggregate in *S. pombe*. This points to Hsp104 may maintain a balance between soluble and insoluble Htt^{NTD}.nQ-GFP.

On the other hand, cells lacking Mas5 or Ssa2 enhance dramatically the aggregates formation. Htt^{NTD}.47Q-GFP aggregates appears in *tpx1'*-driven and even appears aggregates in the weakest promoter *sty1'*-driven Htt^{NTD}.103Q-GFP in cells lacking Mas5. That both mutant deletions strains show similar phenotypes makes sense because Ssa2 is the Hsp70 of Mas5. Also, we show that upon HS Mas5 aggregates are not colocalizing with the 103Q aggregates, but it is co-immunoprecipitating with the 47 and 103Q forms. That points to Mas5 bind soluble 47Q and 103Q. Moreover, there is no binding of Mas5 to 25Q. We suggest that this is because this length not present a non-native conformation, as it can work normally in human cells. Therefore, Mas5 bound non-native prone-aggregates proteins like 47Q and 103Q, in order to prevent aggregation.

When we study the phenotypes of cells lacking Mas5 and Hsp104 we observed an intermediate phenotype. This points to Hsp104 and Mas5 act independently. Moreover, Hsp104 induces aggregation more than disaggregation, because in cells with de double deletion see fewer aggregates than in single mutation of *mas5*. These results contrast with hat was published in *S. cerevisiae*, where the Hsp104 function is facilitated by Hsp40/70 (Bosl, Grimminger et al. 2006).

Therefore, we think that non-native molecules of 47Q and 103Q are recognized by Mas5/Ssa2 that avoids their aggregation or by Hsp104 that promotes their aggregation. At the same time, the Htt would have more affinity for Mas5 than Hsp104, since in cells lacking Mas5 there is more aggregates, because Hsp104 would have more facility for aggregates formation. Thus, Hsp104 could be easing the workload of Hsp70/40 system. Likewise, in absence of Mas5 and Hsp104, 47Q and 103Q molecules can aggregate, but we do not know if they do passively by them self or by other chaperones that we have not found yet (Fig 2).

On the other hand, surprisingly, Mas5 and Hsp104 do not seem to have any function on TDP-43-YFP proteostasis. That points to a difference between features of the TDP-43 and Htt aggregates.

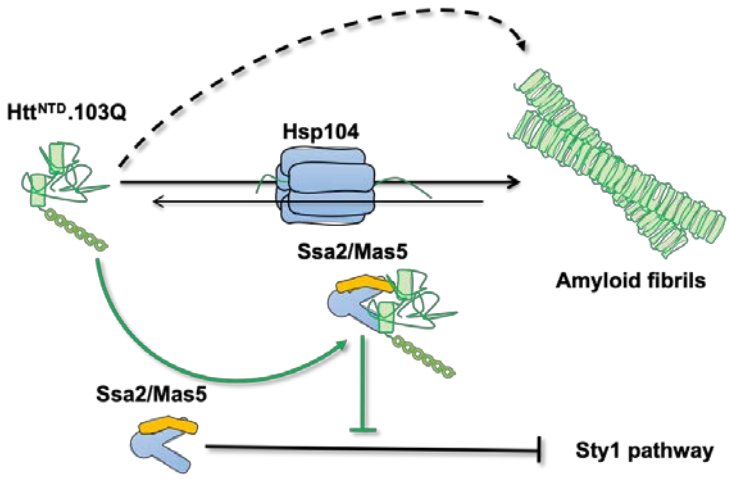

Figure 19. Model of Htt proteostasis and possible activation of Sty1 pathway. In basal conditions, couple Mas5/Ssa2 are inhibiting basal activation of the Sty1 pathway. Upon expression of Htt.103Q, it is initially recognized by Hsp104 or Mas5. Hsp104 promotes the aggregation of Htt.103 and then, to a lesser extent, the disaggregation. On the other hand, Mas5 in cooperation with Ssa2 keep Htt.103Q in a soluble state and stop inhibiting Sty1 pathway. Dashed arrow *indicates* the default aggregation of Htt103Q in absence of those chaperones. Green arrows indicate reactions occurring that affect Sty1 pathway regulation in cells expressing 103Q.

3.4. Expression of 103Q promotes longer lifespan through Sty1 pathway activation

It has been reported (Zurawel, Kabeche et al. 2016) and we have also checked (data not shown), that the proteotoxic stress produced by Htt^{NTD}.nQ-GFP expression does not increase the sensitivity of cells upon several stresses. However, stationary phase never has been tested. During aging, it has been published that PQC system declines, so we wanted to know if the addition the expression of Htt^{NTD}.nQ-GFP leads to sensitivity to stationary phase. Surprisingly, cells that express constitutively Htt^{NTD}.103Q-GFP expression under the control of *tpx1'* promoter show a longer lifespan. Moreover, cells expressing Htt^{NTD}.103Q-GFP do not accumulate carbonylated proteins. That points to a decrease the aging-produced ROSs. In our lab we demonstrated that the previous exposition to a mild stress like nutrient starvation promotes the resistance to a posterior acute stress like stationary phase because there is an early activation of the Sty1 pathway(Zuin, Carmona et al. 2010). Here we demonstrate that the expression of Htt^{NTD}.103Q-GFP facilitates the activation of the Sty1 pathway.

Furthermore, we observed that this depends on the aggregate formation, since cells lacking Hsp104, where there are no aggregates, there is no lifespan promotion.

Now we have to keep investigate to clarify the mechanisms of the transduction of the proteotoxic stress signal. However, it has already been published the mechanisms of activation of Hsf1 upon proteotoxic stress. Is postulated that in basal conditions Mas5 binds and inhibits Hsf1. Upon HS the increase of non-native proteins in the cellular milieu promotes the Hsf1 release and the consequent upregulation of the Hsf1-dependent genes (Vjestica, Zhang et al. 2013). Then, in the same way, the expression of Htt^{NTD}.nQ-GFP induces a proteotoxic stress that promotes the Mas5 recruitment that can be triggering the activation of Sty1 pathway. However, the expression of Htt^{NTD}.47Q-GFP does not confer stationary phase resistance but does recruit Mas5. This occurs because the 47Q form does not need as Mas5 as 103Q does to keep soluble. For that reason, cells lacking Mas5 have a significant lower amount of aggregates expressing 47Q than 103Q. We should perform more experiments to resolve this.

Therefore, we propose a signaling role of the PQC. The PQC machinery must have some mechanisms to sense and transduce the proteotoxic stress signal.

3.5. Difference between *S. pombe* and *S. cerevisiae* in the expression of reporters

It has already reported that the same Htt expression levels can be toxic in *S.* cerevisiae, whereas it is not in *S. pombe*. Moreover, in *S. cerevisiae* the expression of TDP-43 has different characteristics than Htt. So, here we study if the effect of TDP-43 expression in fission yeast has also a different effect comparing with budding yeast. Our results showed that the effect of TDP-43 expression is similar to what happens with Htt expression. At the same levels of expression Htt and TDP-43 have a higher toxic effect in *S. pombe* than in *S. cerevisiae*. Moreover, it has been reported that the bigger amount of polyQ proteins in the proteome of *S. cerevisiae* comparing with *S. pombe* is the reason because expression of Htt produces toxicity (Zurawel, Kabeche et al. 2016). In fact, this could answer also the high toxic effect of TDP-43 expression in budding yeast. Therefore, we postulate that this means that the proteome of budding yeast is more “fragile” than the proteome of fission yeast.

In addition, the expression of 103Q leads to 100% of cells with aggregates in *S. cerevisiae* (Krobitsch and Lindquist 2000), whereas in *S. pombe* only cells lacking Mas5 show a 100% of aggregation. We demonstrated that *S. pombe* keep soluble Htt through Mas5. However, in *S. cerevisiae*, is the Hsp40 Sis1 which has been related to Htt aggregates. On contrary of Mas5, Sis1 function is involved in the recognition and degradation via UPS of misfolded proteins (Park, Kukushkin et al. 2013, Samant, Livingston et al. 2018). This suggest that both yeast models performed different strategies to dilute with non-native proteins.

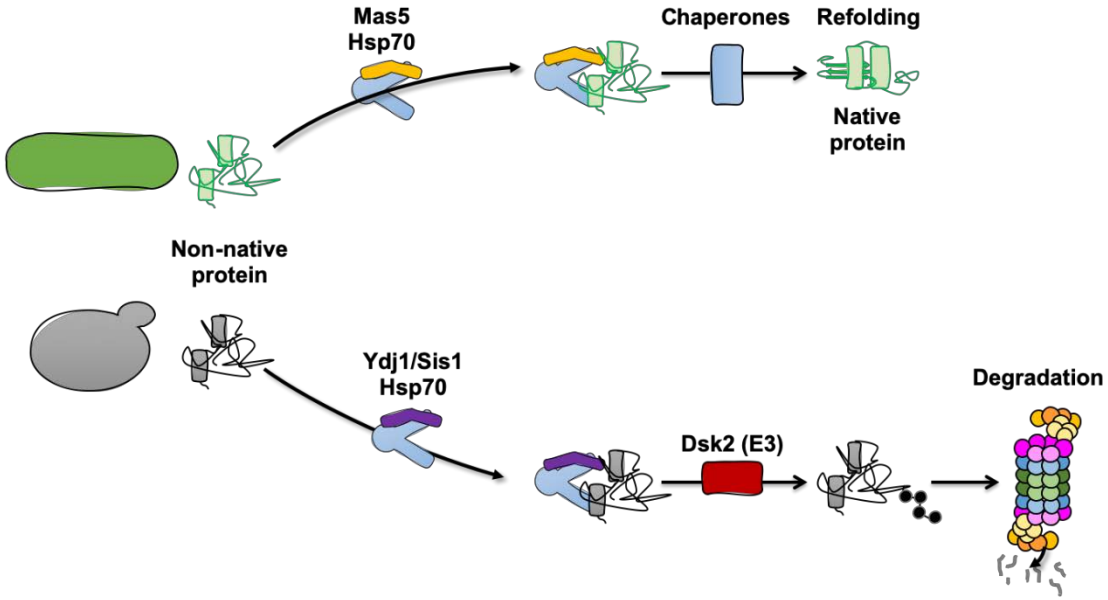

Figure 20. PQC system acts differently in *S. pombe* than *S. cerevisiae*. Model of the different fates of non-native proteins depending on the organism. The PQC in *S. pombe* tends to refold, whereas in *S. cerevisiae* tends to degrade.

All together suggests that *S. cerevisiae* have developed a removing strategy, whereas *S. pombe* have developed a strategy where Mas5 has a pivotal role and where non-native proteins tends to be refolded. Since the risk of keeping non-native proteins at the cellular milieu is less than in *S. cerevisiae* (Fig 3).

Anyway, what is happening in both scenarios is similar: upon the proteotoxic stress produced by Htt expression, the huge Htt cannot be degraded or refolded, so the Hsp40s are overflowed in both species. However, there are a different cellular repercussion.

To make sure this theory we should express Mas5 in *S. cerevisiae* in order to see a similar behavior to *S. pombe* upon proteotoxic stress. On the contrary, in cells lacking Mas5, non-native proteins would tend to be degraded o aggregated more than WT cells.

3.6. Concluding remarks

In this second chapter of this thesis, we show that *S. pombe* is an appropriate model for studying the proteotoxic stress induced for proteins involved in human diseases. *S. pombe* show differences with *S. cerevisiae* that could be interesting for understanding the proteostasis of those disease-reporters.

Moreover we suggest that Mas5 is a key component of the PQC system in *S.pombe* what also could work as sensor of proteotoxic stress for the cell.

Conclusions

Conclusions

1.- Pap1 has a constant turnover through the UPS which depends on its nuclear localization.
2.- Ubr1 degradation depends on a 30 aa degron.
3.- The E3-ligases Ubr1 and Hul5 cooperate in the ubiquitin labeling of Pap1.
4.- Maintaining certain steady levels of proteins is a correct way to limit their function.
5.- The aggregation of Htt depends on concentration and polyQ length.
6.- The expression of Htt or TDP-43 in *S. pombe* produces toxic effects depending on the concentration.
7.- TDP-43 toxicity links to cytosolic inclusions.
8.- Fission yeast is less sensitive to proteotoxic stress than budding yeast.
9.- Mas5/Ssa2 couple has a crucial role preventing Htt aggregation.
10.- Hsp104 participates in the aggregation and disaggregation of Htt.
11.- The constitutive permissive expression of Htt.103Q lengthens the lifespan through activation of Sty1 pathway.
12.- The PQC system has an important role in the stress response signaling in basal and stress conditions in fission yeast.

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