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The Role of Tra1 in Cellular Stress Responses in Yeast: Implications for Human Diseases

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Abstract

Regulation of gene expression under stress conditions involves chromatin remodeling through post-translational modification of histones. One of these modifications, acetylation of lysine residues, regulates transcription initiation and is linked to a variety of essential cellular processes including cell cycle control, DNA repair, and importantly, activation of cellular stress response pathways. Dysregulation of histone acetylation has been observed in many stress-related diseases such as inflammatory diseases, cancer, neurodegenerative disorders, and fungal infections. Tra1 is the only essential component of both the highly conserved SAGA and NuA4 histone acetyltransferase (HAT) complexes that are responsible for acetylation of histones and other proteins. Tra1 has been shown to be involved in the regulation of stress response pathways; however, the underlying mechanisms remain unclear. Huntington’s disease (HD) is a neurodegenerative disease caused by the aberrantly expanded polyglutamine (polyQ) repeats within the gene encoding the Huntingtin protein (Htt), leading to misfolding and aggregation of the mutant Htt. Defect in misfolded protein stress responses is a hallmark of HD. Thus, I sought to investigate how Tra1 orchestrates the transcriptional responses to toxic misfolded polyQ expansions. First, I developed an optimized yeast model of HD to study the effects of Tra1 on polyQ toxicity. I found that polyQ expansions impair the assembly of the Tra1-containing SAGA HAT complex. This correlates with loss of Tra1/SAGA function that exacerbates polyQ toxicity. Furthermore, polyQ expansions lead to increased expression of TRA1, revealing a compensatory feedback mechanism. Interestingly, deletion of SFP1, a transcription regulator downstream of TORC1, abolished TRA1 upregulation upon polyQ expression. Thus, I identified a novel link between Tra1 and TORC1 signaling. Moreover, I showed that the increased sensitivity to heat stress in cells expressing expanded polyQ is rescued by an osmotic stabilizer sorbitol, suggesting that the defect can be traced back to the cell wall integrity. Considering that Tra1 is vital to maintain cell wall integrity and activation of the heat shock response, it appears that impaired Tra1/SAGA functions could underlie the dysregulation of these stress responses in the yeast model of HD. Finally, because of its essential role in cell wall maintenance and calcium homeostasis (two targets of antifungal drugs), Tra1 emerges as a rational target for pathological yeast infections. Indeed, I found that cells expressing a mutant tra1 allele
showed increased sensitivity to antifungal drugs. Overall, my graduate work helps characterize the global role of Tra1 in protein homeostasis and define its potential as a therapeutic target for stress-related human diseases.

Keywords

Stress response, histone acetyltransferase complex, Huntington’s disease, polyglutamine, fluorescent proteins, aggregation, heat shock response, cell wall integrity, calcineurin pathway, antifungal resistance, Tra1, TORC1, Sfp1.
Summary for lay audience

Cells respond to environmental insults by remodeling their genome. Cells require critical transcriptional machinery to regulate the expression of specific genes in response to different stressors. Impaired cellular stress response pathways can lead to accumulation of toxic misfolded proteins and major cellular dysfunctions, which are hallmarks of several human disorders from neurodegeneration to our ability to fight fungal infections. Hyperactivation of cellular stress responses, on the other hand, can cause drug resistance in cancer therapy and lead to emergence of drug resistant microorganisms. Therefore, it is crucial to investigate the regulation of cellular stress responses and their implication in stress-related diseases. Using the budding yeast as an experimental model, my thesis explores how cells respond to stress in two major health problems: Huntington’s Disease and yeast resistance to antifungal drugs. While these two issues appear quite distinct, my work established that a single protein, Tra1, plays a pivotal role in controlling how cells modulate gene expressions under stress conditions linked to these pathologies. Therefore, understanding the crucial role of Tra1 in cellular stress responses will provide better insights into the pathogenesis and therapies of stress-related disorders.
Co-Authorship Statement

Chapter 2 of the thesis was published in *Traffic* and co-authored with Sonja E. Di Gregorio, Dr. Martin L. Duennwald, and Dr. Patrick Lajoie. S. Di Gregorio performed the dot blots and SDD-AGE experiments (Figure 2.1D, 2.2D, 2.3F, S2.2). I performed all the other experiments. I co-authored the manuscript with Dr. Duennwald and Dr. Lajoie.

Chapter 3 of the thesis was published in *F1000Research* and co-authored with Maram B. Albakri, Julie Genereaux, and Dr. Patrick Lajoie. I am a co-first author. I was involved in generating the plasmids, performed dot blot and assisted with growth assays, fluorescence microscopy, and data analysis. M. Albakri generated data presented in Figures 3.1 and 3.2. J. Genereaux helped with cloning the different plasmids. Dr. Lajoie and I with M. Albakri wrote and edited the manuscript.

Chapter 4 of the thesis was published in *Traffic* and co-authored with Matthew D. Berg, Julie Genereaux, Khadija Ahmed, Dr. Martin L. Duennwald, Dr. Christopher J. Brandl and Dr. Patrick Lajoie. I completed all experiments with assistance in the β-Galactosidase assay and pull-down assay (Figure 4.4B) from M. Berg and J. Genereaux. K. Ahmed generated supplemental figure 4 (Figure S4.4). The *tra1* mutation strains were provided by the Brandl lab. Dr. Lajoie and I wrote and edited the manuscript with assistance from M. Berg, Dr. Duennwald, and Dr. Brandl.

Chapter 5 and 6 are unpublished and co-authored with Julie Genereaux, Dr. Martin L. Duennwald, and Dr. Patrick Lajoie. I performed all growth assays, microscopy, and data analysis. J. Genereaux helped with generating the plasmids and mutant yeast strains. Dr. Duennwald performed the heat shock protein western blot (Figure 5.1B). Dr. Lajoie and I wrote and edited the manuscript with assistance from Dr. Duennwald.
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<th>Full Form</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>Casp</td>
<td>Caspofungin</td>
</tr>
<tr>
<td>CDK</td>
<td>G1 cyclin-dependent protein kinase</td>
</tr>
<tr>
<td>CDRE</td>
<td>Calcineurin dependent reporter element</td>
</tr>
<tr>
<td>CFW</td>
<td>Calcofluor white</td>
</tr>
<tr>
<td>ChIP-Seq</td>
<td>Chromatin immunoprecipitation sequencing</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>CWI</td>
<td>Cell wall integrity</td>
</tr>
<tr>
<td>ETOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FLC</td>
<td>Fluconazole</td>
</tr>
<tr>
<td>FP</td>
<td>Fluorescent protein</td>
</tr>
<tr>
<td>GAL</td>
<td>Galactose</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington's disease</td>
</tr>
<tr>
<td>HDACi</td>
<td>Histone deacetylase inhibitor</td>
</tr>
<tr>
<td>HSE</td>
<td>Heat shock element</td>
</tr>
<tr>
<td>HSPs</td>
<td>Heat shock proteins</td>
</tr>
<tr>
<td>HSR</td>
<td>Heat shock response</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Htt</td>
<td>Huntingtin protein</td>
</tr>
<tr>
<td>Htt$^{ex1}$</td>
<td>region of Htt encoded by the first exon of $HTT$</td>
</tr>
<tr>
<td>IBs</td>
<td>Inclusions bodies</td>
</tr>
<tr>
<td>MET</td>
<td>Methionine</td>
</tr>
<tr>
<td>MIC</td>
<td>Miconazole</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<tr>
<td>PolyQ</td>
<td>Polyglutamine</td>
</tr>
<tr>
<td>Rap</td>
<td>Rapamycin</td>
</tr>
<tr>
<td>RiBi</td>
<td>Ribosome biogenesis</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDD-AGE</td>
<td>Semi-denaturating detergent agarose gel electrophoresis</td>
</tr>
<tr>
<td>STRE</td>
<td>Stress responsive element</td>
</tr>
<tr>
<td>TM</td>
<td>Tunicamycin</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
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<tr>
<td>UPRE</td>
<td>Unfolded protein response element</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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Chapter 1

1 Introduction

In the first chapter, I will review literature pertinent to the subject of my thesis project. Specifically, I will focus on studies that define the role of Tra1 in gene expression, transcriptional regulation, and histone modification. Then, I will provide background information on cellular stress response pathways including TORC1 signaling, the heat shock response (HSR), the cell wall integrity (CWI) pathway, and the calcineurin pathway. Furthermore, I will emphasize the importance of the dysregulation of these cellular stress responses in human diseases, especially in Huntington’s disease (HD) and fungal infections.

1.1 Cellular stress responses and stress-induced gene transcription

A key aspect of cellular physiology is maintaining homeostasis, a dynamic state of equilibrium of the physiological and chemical conditions within our cells and our body (Keener and Sneyd, 2009). Many extracellular and intracellular factors, such as temperature and pH changes, hypoxia, genetic mutations, and protein misfolding can perturb protein homeostasis (or proteostasis) and cause cell stress. To cope with different environmental and genetic insults and restore homeostasis, cells adjust the expression of specific genes encoding stress response proteins through activation of multiple stress response pathways (Fulda et al., 2010). These stress response pathways include the unfolded protein response (UPR), the HSR, several MAPK pathways, the TORC1 pathway, and the calcineurin pathway. The transcriptional regulation of stress-induced genes is critical for stress response activation. When increasing yeast incubation temperature from 25 to 38 °C, 52 proteins were induced in response to heat shock (Boy-Marcotte et al., 1999). Upregulation of at least 115 genes was identified during H\textsubscript{2}O\textsubscript{2} treatment in response to oxidative stress (Godon et al., 1998). During osmotic stress, the mRNA level of 186 genes raised over 3-fold (Rep et al., 2000). Dysregulation of cellular stress responses has been found in various diseases including inflammatory diseases, cancer, and neurodegenerative disorders (Chen and Xie, 2018; Leak, 2014; Lindholm et
al., 2006). Understanding the regulation of gene transcription under specific disease conditions provides insights into the fundamental mechanisms of stress-related diseases. My thesis focuses on using the budding yeast *Saccharomyces cerevisiae* as a model organism to study transcriptional regulation in cellular stress responses in two distinct major health problems, HD, an inherited neurodegenerative disease, and pathological yeast infection and antifungal drug resistance.

Stress-induced genes have specific sequences within their respective promoter that can be recognized by specific transcription factors. An example of such elements is the stress responsive element (STRE) which has a core sequence AGGGG (Ruis and Schüller, 1995). STRE is the binding site of transcription factors Msn2/4 (Görner et al., 1998; Martínez-Pastor et al., 1996). A computer search in the yeast genome predicted that 186 genes are potentially regulated through STRE (Moskvina et al., 1998). Other examples of stress response elements within gene promoters include the heat shock element (HSE), which is recognized by heat shock factors, the unfolded protein response element (UPRE), which is the binding sequence of Hac1/Xbp1 during ER stress, and the calcineurin dependent reporter element (CDRE), which is controlled by Crz1 to maintain calcium homeostasis (Morimoto and Westerheide, 2010; Stathopoulos and Cyert, 1997; Takayanagi et al., 2013). Importantly, some genes may contain both STRE and other stress response elements that can be induced by different stress-response transcription factors, such as *HSP104* which encodes a heat shock protein, and an essential osmotic stress response gene *GPD1* (Rep et al., 1999).

Transcription initiation requires the binding of transcription factors and co-activators to the DNA regulatory regions. In eukaryotes, DNA is packed with histones to form nucleosomes, the fundamental subunit of chromatin (Kornberg and Lorch, 1999). The condensed chromatin structure and interaction between DNA and histones inhibit the binding of the transcriptional machinery (Grunstein, 1990). Specific enzymes can change the state of chromatin to start gene transcription. A major class of these histone-modifying enzymes is the histone acetyltransferases (HAT) and associated complexes (Sterner and Berger, 2000). By transferring an acetyl group to certain lysine sites within histones in chromatin, HAT complexes weaken the interaction between DNA and
histones and/or change the chromatin to a more relaxed structure that allows the binding of transcription factors (Norton et al., 1989). Therefore, histone acetylation plays a vital role in gene transcription. Furthermore, many proteins of the HAT complexes have been reported as transcription co-activators (Bonnet et al., 2014). How HAT complexes regulate specific stress-induced gene transcriptions and their roles in cellular stress responses need further investigation. *My thesis focuses on the role of Tra1, an essential component of two highly conserved HAT complexes, in cellular stress responses and its implication for stress-related diseases.*

### 1.2 Tra1 and transcription initiation

Two major HAT complexes, which are highly conserved from yeast to humans, are the SAGA (Spt-Ada-Gcn5-Acetyltransferase) and NuA4 (Nucleosome acetyltransferase of H4) complexes. They respectively contain the Gcn5 and Esa1 lysine acetyltransferases, both of which have histones as a major substrate (Allard et al., 1999; Auger et al., 2008; Grant et al., 1997) (Figure 1.1A). In addition, SAGA and NuA4 have a number of non-histone target proteins with roles likely unrelated to chromatin and gene regulation (Choudhary et al., 2009; Downey et al., 2015; Henriksen et al., 2012; Mitchell et al., 2013). The SAGA complex also possesses a ubiquitin protease activity specific for monoubiquitinated histone H2B that is involved in the regulation of gene expression (Henry et al., 2003). A genome-wide microarray study first showed that SAGA contributes to the expression of about 10% of all yeast genes. Interestingly, a large amount of SAGA dependent transcripts are involved in cellular stress responses (Huisinga and Pugh, 2004). Furthermore, subsequent studies identified SAGA as a global transcription regulator that maps to the promoters of most genes (Baptista et al., 2018).

The transcription-associated protein 1 (Tra1) is homologous to the mammalian TRRAP (McMahon et al., 1998) and was first identified within the purified SAGA complex (Grant et al., 1998). Tra1 is also an essential component of the NuA4 complex (Allard et al., 1999). It is a member of the phosphoinositide-3-kinase-related kinase (PIKK) family that also includes Tor1, DNA-PKcs, ATM, ATR and SMG-1 (Lovejoy and Cortez, 2009). The PIKK proteins play multiple vital roles in maintaining cell viability, especially stress-induced signaling pathways such as DNA repair, ribosome biogenesis, and apoptosis.
Recent structural studies using cryo-electron microscopy revealed the structure of Tra1 strikingly resembles another PIKK, DNA-PKcs, thus arguing in favor of a role for Tra1 in DNA repair (Díaz-Santín et al., 2017). As opposed to other PIKK proteins such as ATM and mTOR, Tra1 is not found to have catalytic activity due to the lack of signature motifs of kinases within the PI3K domain (Mutiu et al., 2007). The function of different Tra1 domains (Figure 1.1B) was investigated in a sequence-based study (Knutson and Hahn, 2011). Similar to other PIKK family proteins, there are five different domains in Tra1 and all are essential for viability. The long N-terminal HEAT (Huntingtin, elongation factor 3, PR65/A, and TOR) domain represents about 70% of the length of Tra1, which interact with transcription activators and other subunits of the HAT complexes, followed by the FAT (FRAP, ATM, and TRRAP) and FRB (FKBP12 rapamycin binding) domains. After the PI3K domain, PIKK proteins have a small FAT C-terminal domain which is vital for the integrity of PIKK-containing complexes (Jiang et al., 2006). The authors reported the critical role of Tra1 in associating other subunits and contributing to the acetylation activity of the HAT complexes (Knutson and Hahn, 2011). Previous studies identified Tra1 as a transcription co-activator that can recruit co-activator complexes and directly interact with transcription activators during transcription initiation (Brown et al., 2001). Studies also showed that Tra1 has multiple transactivation domain binding sites within its HEAT region which allow its interaction with activators including yeast Gal4, Gcn4 or human c-myc (Lin et al., 2012; Park et al., 2001). Like Tra1 in S. cerevisiae, TRRAP in mice is essential for viability (Herceg et al., 2001). However, there are two paralogous proteins in S. pombe, Tra1 and Tra2 (Hayashi et al., 2007). Tra1 in S. pombe is not essential and associates with SAGA, while Tra2 is essential and associates with NuA4 (Helmlinger et al. 2011). The fact that only S. pombe Tra2 is essential suggests that, in other organisms, NuA4 associated function of Tra1 is essential for viability (Helmlinger et al., 2011). Thus, it may not be a coincidence that depletion of Tra1 using the anchor-away system in S. cerevisiae recapitulates with higher fidelity the transcriptional profile of esa1Δ cells (a NuA4 component) and not gcn5Δ cells (a SAGA component) (Bruzzone et al., 2018). The requirement for Tra1, therefore, appears different for SAGA and NuA4 functions.
Figure 1.1 Tra1 is an essential component of both the SAGA and NuA4 HAT complexes.

(A) Subunits of the SAGA and NuA4 HAT complexes. SAGA contains four modules, the HAT module, TAF module, deubiquitination (DUB) module, and TATA binding protein (TBP) module. The catalytic subunit of SAGA is Gcn5, whereas the acetyltransferase in NuA4 is Esa1. (B) The modular structure of Tra1 protein. Tra1 is a PIKK family protein which consists of five domains. Figure B adapted from Wang et al., 2018.
1.3 Yeast as a model organism

Since first used in the early 1900s, the budding yeast S. cerevisiae has become a compelling model organism to build the fundamental understanding of eukaryotic cell biology (Mortimer and Johnston, 1986). The advantages of employing yeast include its short life cycle, simple growth requirements, and stable expression of plasmids. Yeast also allows the possibility of extensive and relatively easy genetic manipulations (Duina et al., 2014). Since the exciting publication of the first fully sequenced eukaryotic genome database came out in 1996, S. cerevisiae continues to offer new information on eukaryotic genome organization, basic cellular mechanisms, and evolution (Goffeau et al., 1996). Numerous screening tools have been established including yeast genome-wide libraries carrying deletions of all non-essential genes and genomic plasmid banks for overexpression screen (Gelperin et al., 2005; Rose et al., 1987; Winzeler et al., 1999). These tools allow researchers to conduct high-throughput analysis of genetic interactions and protein functions. One example is the synthetic genetic array where a query mutation is crossed with the yeast deletion library. Spore inviability or growth defect of the double mutant indicates a genetic interaction between the two genes (Tong et al., 2001). More than 30% of all yeast genes have clear homologs in mammals (Botstein et al., 1997). Benefitting from the ease and rapidity of genetic screening, the yeast model is particularly amenable for genome-wide study on gene expression and signaling cascades. Most of the basic cellular mechanisms, such as the TORC1 signaling, the HSR, the MAPK pathways, and the calcineurin pathway, that I will discuss in subsequent sections, are conserved in human and widely studied in yeast.

Due to the relatively short generation time, yeast is also used for aging research (Gershon and Gershon, 2000; Kaeberlein et al., 2007). The replicative as well as chronological life span allows researchers to investigate the aging processes of proliferating and nonproliferating cells respectively. Furthermore, yeast also provides an excellent platform for assessing cellular functions affected by disease-causing proteins (Smith and Snyder, 2006). Making human point mutations in yeast orthologs, expressing human proteins in yeast, or humanizing yeast, i.e. replacing the yeast gene with its human homolog, successfully mimic human diseases’ phenotypes in S. cerevisiae (Laurent et al., 2016).
The yeast model has helped scientists gain a better understanding of different disease mechanisms such as neurodegenerations and cancer (Guaragnella et al., 2014; Miller-Fleming et al., 2008). Researchers also exploit the yeast model for large-scale drug tests. Yeast has become a powerful in vivo tool for high throughput screens to identify drug targets and demonstrate the mechanisms of action (Couplan et al., 2011; Ohnuki et al., 2010).

Similarly to other model organisms, the yeast model is not perfect. Higher eukaryotes have more complex mechanisms that are difficult to study in yeast. Because yeast is a single cell organism, tissue studies and behavioral approaches are not feasible. Nevertheless, the use of this simple organism has become widespread, especially in combination with tools available in other model organisms such as C.elegans, Drosophila, rodents, and tissue culture cell lines established from patient samples. The simple yeast model not only enables a better understanding of the fundamental mechanisms of cellular functions but also contributes to accelerating the development of therapeutic agents for human diseases. In my thesis, I used S. cerevisiae to investigate the role of Tra1 in cellular stress responses and implications for Huntington’s disease and fungal infections. In the following sections, I will review the major stress responses pathways pertinent to my thesis project.

1.4 TORC1 signaling pathway

The Target of Rapamycin (TOR) is a widely conserved serine/threonine kinase that functions in two different complexes named TORC1 and TORC2, each of which plays vital roles in various aspects of eukaryote cell growth (Loewith and Hall, 2011). TORC1 is activated under many stress conditions i.e. nutrient starvation and well studied for its role in regulating ribosome biogenesis, cell cycle, autophagy, and promoting cell growth during stress conditions (Barbet et al., 1996; Kawai et al., 2001; Noda and Ohsumi, 1998; Urban et al., 2007). TORC2, meanwhile, controls cell growth by regulating the polarization of the actin cytoskeleton (Schmidt et al., 1996). TORC2 has also been shown to regulate endocytosis and sphingolipid biosynthesis; however, the underlying mechanisms have yet to be elucidated (Tabuchi et al., 2006).
*S. cerevisiae* has two TOR genes encoding Tor1 and Tor2, which redundantly regulate growth (Loewith et al., 2002). Tor proteins are PIKK family proteins which contain a HEAT domain, binding targets for subunits of the TOR complexes; a core FAT domain, clamping onto the kinase domain and required for partners binding; an FRB domain, which contains the rapamycin binding region; and a PI3K kinase domain and FATC domain, which play critical roles in regulating protein kinase activity (Adami et al., 2007; Yang et al., 2013). The yeast TORC1 complex is formed by the proteins Kog1, Lst8, Tco89, and TOR1 or TOR2 (Loewith et al., 2002). Through fluorescence microscopy and fractionation experiments, TORC1 was found to localize to the vacuolar membrane (Reinke et al., 2004; Sturgill et al., 2008). Besides its known inhibition by rapamycin, many factors have been found to regulate TORC1 signaling including nutrient starvation, oxidative stress, heat shock, and cell wall stress (Beck and Hall, 1999; Gasch and Werner-Washburne, 2002). A yeast global protein kinase and phosphatase interaction network study identified downstream targets of TORC1 (Figure 1.2), revealing the critical role of the TORC1 signaling as described below (Breitkreutz et al., 2010).

Ribosomes play a pivotal role in protein translation and cell growth. Actively dividing yeast cells allocate up to 85% of their transcriptional activity to ribosome biogenesis (Warner, 1999). TORC1 is a vital regulator of ribosome biogenesis through phosphorylation of the transcription factors Sch9 and Sfp1 (Lempiäinen and Shore, 2009). TORC1 directly binds and phosphorylates Sch9. Sch9 supports ribosome biogenesis and ribosomal protein gene transcription by inhibiting transcriptional repressor Dot6 and its homolog Tod6 (Huber et al., 2011; Lippman and Broach, 2009). Importantly, this phosphorylation is abolished during stress conditions (Urban et al., 2007). The activation of Sch9 also leads to the inhibition of Maf1, an RNA polymerase III repressor (Vannini et al., 2010). The phosphorylation of Sfp1 by TORC1 promotes Sfp1 binding to the promoters of genes encoding ribosomal proteins and thus regulates transcription activation (Fingerman et al., 2003). Mrs6, a protein involved in ER to Golgi trafficking, directly binds Sfp1 and regulates its cellular localization. Under stress conditions, Sfp1 re-localizes from the nucleus to the cytoplasm that causes downregulation of ribosome biogenesis (Lempiäinen et al., 2009; Marion et al., 2004). Furthermore, Sfp1 or Sch9 deletion leads to reduced cell size (Jorgensen et al., 2004), suggesting that ribosome
biogenesis regulated by the TORC1 signaling pathway is critical for cell size determination.

TORC1 is essential for cell cycle regulation and growth. Treating yeast cells with rapamycin causes G1 arrest (Barbet et al., 1996). Previous literature showed that TORC1 activates G1 cyclin-dependent protein kinases (CDKs) through destabilizing the CDK inhibitor Sic1 (Moreno-Torres et al., 2015). Furthermore, TORC1 is also involved in the G2/M transition through the interaction between Kog1 and a polo-like kinase Cdc5 (Nakashima et al., 2008). Under stress or starvation conditions, cells undergo autophagy to degrade damaged proteins and organelles in order to generate biomaterials and ATP for essential protein synthesis (Reggiori and Klionsky, 2013). TORC1 has been shown to negatively regulate autophagy through the phosphorylation of autophagy-related genes (ATGs). Atg13 phosphorylation by TORC1 prevents its binding to Atg1 in yeast. The dephosphorylation and disassociation of the Atg1 mammalian homolog Ulk1 from mTORC1 increases its activity during starvation (Kamada et al., 2010; Noda, 2017; Yorimitsu et al., 2009).

The TORC1 pathway has been linked to other cellular stress response pathways. mTORC1 is crucial for HSF1 activation in HeLa cells (Chou et al., 2012). Yeast cells carrying an Hsp90 mutation, which leads to overactivation of HSF1, are more sensitive to rapamycin, suggesting that inhibition of TORC1 signaling is involved in the process (Chou et al., 2012; Millson and Piper, 2014). Rapamycin treatment has also been shown to activate the CWI pathway, suggesting the negative regulation by TORC1 on cell wall integrity (Torres et al., 2002). Therefore, it is crucial to understand the multifunctional TORC1 regulation during stress and its connection with other stress response pathways.
Figure 1.2 Model of the TORC1 signaling pathway in *S. cerevisiae*.
TORC1 is regulated by nutrients and cell stress. TORC1 level is inhibited by rapamycin. TORC1 controls ribosome biogenesis (Ribi) through phosphorylation of Sfp1 and Sch9. TORC1 negatively regulates autophagy by phosphorylating Atg13 and prevents the formation of Atg1 complex. TORC1 is also involved in cell cycle regulation.
1.5 Heat shock response

The HSR is a well-conserved transcriptional program activated when cells are exposed to stresses which induces the expression of many heat shock proteins (HSPs) (Lindquist, 1986; Richter et al., 2010). Many factors can lead to cell stress and activate the HSR including temperature changes, nutrient starvation, hypoxia, aging, and genetic mutations (Kalmar and Greensmith, 2009; Marambio et al., 2010; Murshid et al., 2013). Under stress conditions, an increased amount of misfolded or unfolded proteins accumulate within the cell, requiring a consequential increase in protein folding and quality control capacity to maintain protein homeostasis (Gidalevitz et al., 2011). The majority of HSPs function as molecular chaperones to unfold aggregated proteins, refold misfolded proteins, and target damaged proteins for degradation processes (Feder and Hofmann, 1999). Physiological changes such as the osmolyte synthesis and import, cytoskeleton remodeling, and interruption of the cell cycle regulated by HSPs also contribute to cellular survival during stress (Helmbrecht et al., 2000; Wettstein et al., 2012). Much of what we know about the HSR in eukaryotic cells has been elucidated in *S. cerevisiae* (Figure 1.3), supporting its use for investigating the underlying mechanisms of transcriptional regulation and effects during misfolded protein stress in my thesis.

The key regulators of the HSR are heat shock factors (Akerfelt et al., 2010). Although mammals have multiple heat shock factors providing redundancy and specialization of the stress signals, Heat shock factor 1 (HSF1) is the main heat shock factor and is well conserved from yeast to human (Morimoto, 1998). During stress, HSF1 binds to a specific domain (nGAAAn) termed the heat shock element (HSE) within the promoter of its targets, thereby regulating downstream gene transcription. The initial activation of the HSR requires HSF1 trimerization, phosphorylation at specific sites, and other post-translational modifications (Chu et al., 1996; Holmberg et al., 2001; Westerheide et al., 2009). As previously mentioned, the transcriptional regulation of HSPs also depends on co-activators. Tra1 has been shown to be involved in HSP activations (Lebedeva et al., 2005). In addition, two other transcription factors, Msn2 and Msn4, were also found to be activated by heat shock in *S. cerevisiae*, mainly targeting enzymes involved in carbon metabolism and anti-oxidants (Boy-Marcotte et al., 1999).
HSF1 regulates the activation of HSPs which are critical for protein quality control. Well-characterized HSPs include Hsp70, Hsp90, Hsp104, and small heat shock proteins (sHSPs) family. Hsp70 chaperones support about 30% of proteins to maintain their correct folding by binding transiently to hydrophobic polypeptides of unfolded proteins to prevent non-specific interactions that may cause misfolding and aggregation (Sharma and Masison, 2009). Compared to Hsp70, Hsp90 tends to interact with its clients at later and more mature folding stages (Taipale et al., 2010). Targets of Hsp90 are mainly transcription factors and protein kinases (Karagöz and Rüdiger, 2015). The binding of Hsp70 and Hsp90 to Carboxyl terminus of Hsc70-interacting protein (CHIP) leads to unfolded protein degradation (Kundrat and Regan, 2010). Hsp104 promotes damaged protein refolding by solubilizing protein aggregates in cooperation with Hsp70 and Hsp40 (Glover and Lindquist, 1998; Okuda et al., 2015). The sHSPs are usually 12-43kDa and are also essential for cell survival under stress due to their chaperone activity and anti-apoptotic effects (Bakthisaran et al., 2015). HSP26 and HSP42 deletion strains can survive under heat shock but displayed a significant cell morphology change and cell wall damage (Haslbeck et al., 2004). Furthermore, HSPs, such as Hsp70 and Hsp90, can directly interact with HSF1 and repress its activation (Abravaya et al., 1992; Zou et al., 1998). The feedback mechanism provides an autoregulatory system that controls the extent of the HSR. In S. cerevisiae, HSF1 C-terminal domain deletion has been shown to increase cell sensitivity to high temperature. Notably, the growth defect at a high temperature can be rescued by treating the cells with an osmotic stabilizer, sorbitol. This phenotype is further linked to the interaction between Hsp90 and Slt2, the mitogen-activated protein kinase (MAPK) required for cell integrity (Truman et al., 2007). Moreover, inhibiting Hsp90 led to increased sensitivity to cell wall stressor caspofungin in another yeast species Aspergillus fumigatus (Lamoth et al., 2012) These results demonstrate the crosstalk between the HSR and the cell wall integrity (CWI) pathway in regulating cell survival during stress.
Figure 1.3 Model of the heat shock response pathway in *S. cerevisiae*. The HSR is crucial for proteostasis. HSF1 is phosphorylated and forms a trimer during stress. Then HSF1 translocates into the nucleus and binds to the HSE of its downstream targets to activate transcription. HSPs assist with protein refolding or target damaged proteins for degradation through the interaction with CHIP ubiquitin ligase. Overexpression of HSPs inhibits HSF1 activation to reduce the level of HSR.
1.6 Cell wall integrity

The yeast cell wall is a layered dynamic structure outside of the cell membrane which occupies more than 20% of a yeast cell’s dry weight (Orlean, 1997). The outer layer of the yeast cell wall is comprised of glycoproteins protecting the inner layer from wall-degrading enzymes (Zlotnik et al., 1984), whereas the inner layer is mainly made of glucan polymers and chitin that regulate cell wall elasticity and remodeling (Smits et al., 1999). In *S. cerevisiae*, the cell wall is critical for budding and cell division, response to environmental stress, and cell-cell contact (Cid et al., 1995; Hohmann, 2002; Reynolds and Fink, 2001).

The CWI pathway is a MAPK signaling cascade that maintains cell shape and cell wall integrity during various cellular stresses (Levin, 2011). Briefly, cell surface sensor proteins transmit stress signals to the Rho1 GTPase, the master regulator of the CWI pathway. During stress, Rho1 interacts with Pkc1 and activates the MAPK cascade: Bck1 (MEKK), Mkk1/2 (MEKs), and Slt2 (MAPK) (Figure 1.4). Deletion of any CWI kinase causes cell growth defects at 37 °C and also increases cell sensitivity to cell wall stressors and mating pheromone (Gustin et al., 1998; Kirchrath et al., 2000; Martin et al., 2000). These results indicate the essential role of the MAPK signaling in regulating cellular stress responses.

Cell surface sensors Wsc1/2/3 and Mid2 located on the plasma membrane recruit Rom1/2, the guanine nucleotide exchange factor of Rho1, to activate its GTPase activity. The yeast Pkc1 is homologous to the mammalian protein kinase C (Levin et al., 1990). Deletion of *PKC1* is lethal but cells can grow with the support of sorbitol. The cell walls were found to be thinner in the *pck1Δ* strain (Roemer et al., 1994). When Pkc1 is activated by Rho1, it phosphorylates specific residues of Bck1, leading to the phosphorylation of Mkk1/2 and Slt2, which is functionally homologous to human ERK5 (Truman et al., 2006). The ERK5 signaling is crucial for oxidative stress and environmental stress through regulation of the serum and glucocorticoid-induced kinase (SGK) (Chao et al., 1999; Wang and Tournier, 2006). Downstream targets of the MAPK cascade include transcription regulators Rlm1 and Swi4/6. Rlm1 target genes are involved in cell wall synthesis (Jung et al., 2002). Meanwhile, Swi4/6 mainly regulate transcription of genes required for cell
G1 to S phase (Harrington and Andrews, 1996). Swi4/6 are also found to activate FKS1/2 transcription, which encode proteins regulating β-glucan and chitin synthesis (Roemer et al., 1994)

The CWI pathway is shown to be activated during heat shock stress to regulate cell survival (Truman et al., 2007). Previous literature indicated that Tra1 is involved in the CWI activation. However, the underlying mechanisms are not fully understood (Hoke et al., 2008). Thus, it will be interesting to investigate how Tra1 regulates cellular stress response to high temperature. Furthermore, high calcium concentration can also activate Slt2 phosphorylation (Mizunuma et al., 1998). In parallel with the CWI pathway, Fks2 is also regulated by Crz1, a transcription activator controlled by the calcium-calcineurin signaling (Stathopoulos and Cyert, 1997). All evidence suggests a connection between the CWI pathway and regulation of calcium homeostasis.
Figure 1.4 Model of the cell wall integrity pathway in *S. cerevisiae.*
The CWI pathway is a MAPK signaling pathway in response to cell wall stress. Sensors on the plasma membrane transmit the signal to Rho1 GTPase and activate the MAPK Slt2. Downstream targets of Slt2 are transcriptional regulators Swi4/6 and Rlm1 which activate the transcription of gene involved in cell wall synthesis and cell cycle. Figure modified from Levin, 2005.
1.7 Calcium-calcineurin pathway

Calcium ions play a vital role in cell signal transduction pathways. The regulation of calcium is crucial for cell survival, membrane fusion, cytoskeleton organization, and cell polarization (Cao et al., 2012; Foskett and Spring, 1985; Portis et al., 1979; Shirayoshi et al., 1983). Under normal growth condition, the level of the yeast cytosolic Ca\(^{2+}\) is between 50 to 200 nM (Miseta et al., 1999) and maintained stably by pumping Ca\(^{2+}\) into vacuoles through a vacuolar calcium ATPase Pmc1. Part of the cytosolic Ca\(^{2+}\) is transported into ER and Golgi through Pmr1, a secretory pathway calcium ATPase, which also helps pump extra Ca\(^{2+}\) out of the cells (Cunningham and Fink, 1994). However, calcium concentration can be abnormally increased resulting from environmental changes as well as intracellular stresses such as cell wall stress or ER stress (Bonilla et al., 2002; Chen et al., 2012). To maintain calcium homeostasis, the calcium-calcineurin pathway is activated (Crabtree, 2001) (Figure 1.5).

Calcineurin is a highly conserved protein phosphatase in eukaryotes (Aramburu et al., 2000). Calcineurin is a heterodimeric protein containing a catalytic subunit calcineurin A together with a Ca\(^{2+}\) binding and regulatory subunit calcineurin B (Rusnak and Mertz, 2000). In *S. cerevisiae*, CNA1 and CNA2 encode calcineurin A, whereas CNB1 encodes calcineurin B. Deletion of each component is viable but cells displayed growth defect under stress conditions. Double deletion of CNA1 and CNA2 show increased cell sensitivity to mating pheromone (Cyert et al., 1991). Furthermore, deletion of CNB1 leads to no detectable calcineurin activity, even though Cna1 and Cna2 protein levels remain unchanged, indicating that the essential role of subunit B on enzymatic activity (Cyert and Thorner, 1992). Under stress conditions, a rapid influx of Ca\(^{2+}\) into the cytosol through the high affinity Ca\(^{2+}\) influx system (HACS) and low affinity Ca\(^{2+}\) influx system of the plasma membrane is sensed by calmodulin (Muller et al., 2001). External calcium ions and calmodulin then bind to calcineurin and activates its phosphatase activity.

Next, the critical target Crz1, a transcriptional regulator, gets dephosphorylated and translocates to the nucleus to activate the transcription of downstream targets including PMC1 and PMR1 (Cyert, 2003; Li et al., 2011). The localization of Crz1 is dependent on its phosphorylation state. Nuclear import of Crz1 requires binding to Nmd5p. Only
dephosphorylated Crz1 can bind to Nmd5p in vitro (Polizotto and Cyert, 2001). Moreover, the export to the cytoplasm is achieved by Msn5p that exclusively recognize phosphorylated proteins (Boustany and Cyert, 2002). Calcineurin is shown to bind Crz1 in a PIISIQ sequence and regulates its dephosphorylation (Kim et al., 2003).

When imported into the nucleus, Crz1 recognizes and binds to the calcineurin-dependent response elements (CDRE), a unique 24 bp sequence, within the target gene promoters and regulates transcription (Stathopoulos and Cyert, 1997). A genome-wide microarray has identified 163 genes which are directly regulated by Crz1, mainly involved in ion transport (e.g. PMC1), vesicle transport (e.g. CVT19), cell wall synthesis (e.g. FKS2), lipid synthesis (e.g. SURI), protein degradation (e.g. APG5), and transcription (e.g. RCNI) (Cyert, 2003; Yoshimoto et al., 2002). These results suggest the cross-talk between the calcineurin signaling and other cellular processes such as plasma membrane synthesis, the CWI pathway, and autophagy besides modulating ion homeostasis.

The calcineurin signaling has been studied as a new target for antifungal drugs through its essential role in regulating fungal cell survival and virulence (Karababa et al., 2006; Soriani et al., 2008). Thus, it is crucial to understand the cellular mechanisms that regulate the calcineurin pathway. Histone acetylation has been linked to the regulation of the calcineurin pathway in fungi. Researchers observed that the C. neoformans gcn5A mutant strain is more sensitive to calcineurin inhibitors (O’Meara et al., 2010). As mentioned previously, Tra1 is vital for histone acetylation. Accordingly, a synthetic genetic array showed that cells with tra1SRR3413 and cnb1Δ double mutation showed a slow growth phenotype (Hoke et al., 2008). Cells carrying a mutant Tra1 allele displayed a growth defect under conditions of high calcium, indicating that calcium homeostasis pathways are impaired (Berg et al., 2018). These results suggest that functional Tra1 is required for the calcium-calcineurin pathway activation. Chapter 6 of my thesis will study the role of Tra1 in regulating the calcineurin pathway and the effects on antifungal resistance.
Figure 1.5 Model of the calcium-calcineurin pathway in *S. cerevisiae*. Calcineurin is activated by the binding of calmodulin and Ca\(^{2+}\). Calcineurin dephosphorylates Crz1 which controls its nucleus localization. Crz1 binds to CDRE of its target genes and activates transcription of genes involved in the transport of calcium. Figure modified from Liu et al., 2015.
1.8 Tra1 and cellular stress responses

The nature of the Tra1 protein and its critical role within the SAGA/NuA4 complexes indicate that Tra1 may be involved in the regulation of cellular stress responses. Systematic genetic array analysis of \textit{tra1\textsubscript{SRR3413}} identified 114 negative genetic interactions where 30 interacting genes directly play a role in stress responses, with some genes implicated in stress-related signaling pathways (Hoke et al., 2008). Moreover, Tra1 incorporation into the SAGA complex requires Tti2, a subunit of the Triple T complex responsible for PIKK family protein stabilization (Elias-Villalobos et al., 2019; Genereaux et al., 2012). Loss of Tti2 function leads to defect in SAGA-dependent transcription that correlates with increased sensitivity to various stresses (Genereaux et al., 2012; Hoffman et al., 2016). Interestingly, the addition of an osmotic stabilizer sorbitol ameliorates the growth defect, suggesting that Tra1 is involved in cell wall function. Indeed, proper SAGA function through Gcn5 is required for the cell wall integrity transcriptional program (Sanz et al., 2016) and Tra1 mutants are hypersensitive to cell wall perturbing agents such as caffeine and calcofluor white (Berg et al., 2018; Helmlinger et al., 2011; Hoke et al., 2008; Mutiu et al., 2007). Studies of Tra1 deletions in \textit{S. pombe} showed that Tra1 recruits SAGA to a subset of promoters, mainly controlling genes involved in stress response (Helmlinger et al., 2011). Also, NuA4 was shown to directly interact with the stress-induced transcription factors Msn4, supporting a role for the complex in the general stress response pathway (Mitchell et al., 2008). The link between Tra1 and cellular stress response pathways is summarized in Figure 1.6.

One of the most characterized roles of Tra1 in the response to stress is its role in the activation of the HSR in response to heat shock. The HSR is an evolutionary conserved stress response pathway to maintain protein homeostasis. The transcription of genes encoding HSPs requires heat shock factor HSF1 as well as co-activators such as SAGA (Cotto and Morimoto, 1999; Lindquist, 1986; Vinayachandran et al., 2018; Zanton and Pugh, 2004). Acetylation and displacement of histone H3 is observed at the promoters of HSPs during heat stress (Erkina and Erkine, 2006). Moreover, a loss-of-function mutation within Tra1 leads to growth inhibition at high temperature and in the presence of the cell wall perturbing agent (Berg et al., 2018; Hoke et al., 2010; Mutiu et al., 2007).
phenotypes correlate with a defective activation of the HSR. Similar phenotypes are observed in cells carrying a mutant HSF1 (1-583) lacking the C-terminal domain (Truman et al., 2007). In support of a role for Tra1 in Hsf1 function, a physical interaction between Hsf1 and Tra1 was observed by mass spectrometry (Breitkreutz et al., 2010). Moreover, studies in Drosophila found that Hsp70 transcription requires the recruitment of Tra1 homolog Nipped-A to its promoter (Lebedeva et al., 2005). In mammals, HSP genes are downregulated in TRRAP deficient cells (Herceg et al., 2003). Collectively, these results indicate that Tra1 is involved in HSF1-dependent transcription of HSP genes across species.

Dysregulation of Tra1 has been observed in many stress-related diseases which I will introduce in the following section. Our recent study reported elevated TRA1 mRNA and protein levels in a yeast model of HD (Jiang et al., 2019). Increased TRRAP expression is also observed in a mammalian HD cell line (Kim et al., 2016). Furthermore, TRRAP has been described as a potential biomarker of Alzheimer’s disease (AD) and is upregulated in hippocampal tissues of AD patients (Ho Kim et al., 2015). TRRAP also plays a critical role in cancer. TRRAP was identified to interact with several cancer-related proteins such as c-Myc, P53, E2F transcription factors, and viral oncoprotein E1A (Jethwa et al., 2018; Lang and Hearing, 2003; McMahon et al., 1998). Overall, Tra1/TRRAP could be an important modifier of HD and other stress-related diseases through its role in regulating cellular stress responses.
Figure 1.6 Tra1 and cellular stress response pathways.
Under stress conditions, cells activated stress response pathways to retain homeostasis. The main stress response pathways include in this thesis are the TORC1 signaling which regulates cell cycle, autophagy, and ribosome biogenesis; the heat shock response, which assists with protein folding and degradation; the cell wall integrity pathway that controls cell wall synthesis; and the calcineurin pathway, which maintains calcium homeostasis. Tra1 is critical for cellular stress response activations. Direct interaction between Tra1 and transcription regulator Sfp1, and HSF1 has been reported in yeast. Tra1 mutation leads to increased cell sensitivity to different stressors such as cell wall stressor caffeine and extra Ca^{2+}. 
1.9 Cellular stress responses and human diseases

Dysregulation of cellular stress responses has been observed in various stress-related diseases including neurodegenerative disease, cancer and chemotherapy resistance in cancer therapy, and antifungal resistance (Chen and Xie, 2018; Cowen et al., 2014; Kim et al., 2015; Leak, 2014; Liu et al., 2017). Insufficient cellular stress responses can lead to impaired protein homeostasis and defects in multiple cell functions. In contrast, hyper-activated stress responses result in drug resistance and limit disease treatments. Therefore, investigating the mechanisms of cellular stress responses is crucial for understanding pathological mechanisms and developing target-specific therapies. My thesis focuses on the regulation of cellular stress responses in HD and antifungal resistance, which I will describe in the following sections.

1.9.1 Cellular stress responses in neurodegeneration and cancer

A hallmark of neurodegenerative diseases is protein misfolding (Soto and Estrada, 2008; Sweeney et al., 2017). Upon accumulation of misfolded proteins, neurons require increased activity of molecular chaperones, mainly heat shock proteins, to cope with the protein misfolding defect. In Alzheimer's disease (AD), Hsp70 and Hsp90 overexpression have been shown to inhibit Aβ formation and ameliorate disease phenotypes (Luo and Le, 2010; Sun et al., 2017). Impaired HSR has been shown in Parkinson’s disease (PD) and HD, which may explain the mechanism of amyloid-like aggregations formed in neurons and the toxicity. For example, Hsp70 levels are decreased in Parkinson’s disease brain sample, suggesting the reduced HSR activation (Alvarez-Erviti et al., 2010). Moreover, due to the high demand for oxygen, the brain is especially vulnerable to the accumulation of reactive oxygen species (ROS) and oxidative stress. Increased oxidative stress has been found in different neurodegenerative disorders that may cause DNA damage and mitochondria dysregulation (Wang and Patterson, 2015). Literature has reported Aβ-induced oxidative stress in AD (Wang et al., 2014). Oxidative imbalance was also shown to appear at early stage of AD, which could be a leading cause of the disease (Butterfield et al., 2006). Dysregulation of complex I of the respiratory chain and increased ROS accumulation has also been observed in PD (Blesa et al., 2015). Overall, impaired cellular stress responses contribute to neurodegenerative disease pathology and could be potential
therapeutic targets for drug development. Indeed, several drugs targeting the HSP proteins or oxidants have been screened for the treatment of AD and PD (Repalli and Meruelo, 2015; Wojsiat et al., 2018).

Many studies have reported the role of cellular stress responses, especially the HSR, in cancer development. HSF1 overexpression is observed in a variety of cancer types (Santagata et al., 2011). It was shown that activation of HSF1 helps maintain the cellular malignant phenotypes by increasing resistance to apoptosis (Mendillo et al., 2012; Meng et al., 2010). The HSPs were also shown to promote tumorigenesis and cancer cell survival by stabilizing oncoprotein mutant p53 and regulating glycolysis (Dai and Whitesell, 2005; Zhao et al., 2009). Furthermore, activation of the HSR has been linked to resistance to chemotherapy. Increased expression of HSPs correlated with increased drug resistance in breast cancer (Vargas-Roig et al., 1998). Importantly, the HSR plays a critical role in tumor metastasis. Epithelial-mesenchymal transition (EMT) was reduced when knocking down HSF1 in the ovarian cancer spheroids (Powell et al., 2016). Furthermore, expression of Hsp10 was found in ascites of ovarian cancer patients which could be involved in T-lymphocyte inhibition (Akyol et al., 2006). High expression of Hsp70 correlates with the aggressiveness of ovarian cancer and leads to poor overall survival (Elstrand et al., 2009). Activation of the HSR provides tumor cells with a cytoprotective effect to survive in the anoxic, acidic, and nutrient-deprived microenvironment. These results delineate the critical role of the HSR in tumor survival and metastasis, further emphasize the potential to target this pathway for cancer therapy.

As such, HSF1 and HSPs have been studied as targets for drugs and immunotherapy in cancer treatment. One exciting result showing that inhibiting Hsp90 with a geldanamycin analog 17AAG43 benefits multiple cancer types in clinical trials (Wang et al., 2016; Workman, 2002).

1.9.2 Impaired heat shock response in Huntington’s disease

Huntington’s disease (HD) is an autosomal recessive neurodegenerative disorder associated with mutations in a gene encoding the Huntingtin protein (Htt) (Whalley, 2015). The cause of HD is an abnormal increase of polyglutamine repeats (polyQ) within a region of Htt encoded by the first exon of HTT (Httex1) (Gusella and MacDonald, 2009).
Normally, the \textit{HTT} gene encodes less than 35 CAG repeats. However, an increased number of polyQ leads to the appearance of HD symptoms in patients, such as chorea, cognitive loss, involuntary movements, and diminished coordination (Roos, 2010). The disease is inherited, and the length of polyQ repeats can increase over consecutive generations. Physical symptoms usually occur at around 40 years of age. However, a higher number of CAG repeats leads to a juvenile HD in which symptoms start before the age of 20 (Ghosh and Tabrizi, 2015).

At the cellular level, increased PolyQ expansions cause the Htt protein to incorrectly fold, and aggregate into cytoplasmic, non-soluble, amyloid-like fibrils termed inclusion bodies (IB) (Arrasate and Finkbeiner, 2012). While most studies focus on how polyQ toxicity leads to neuronal cell death, the effects at early time points have not been well established. A series of cellular processes have been found to be impaired in HD, including dysfunctional ER stress response, protein degradation pathways, transcription activation, mitochondrial activity, and calcium signaling (Duennwald and Lindquist, 2008; Jiang et al., 2016; Kumar et al., 2014; Papsdorf et al., 2015). Thus, expression of mutant Htt impairs normal cellular functions, especially the ability of cells to properly regulate stress response pathways.

The principal regulator of proteostasis, the HSR, is impaired during polyQ expansion. Reduced HSF1 activation and HSPs expression were observed in striatal cells expressing full-length polyQ-expanded Htt and HD knock-in mice (Chafekar and Duennwald, 2012). Overexpressing Hsp26, Hsp42, and Hsp104 attenuated polyQ toxicity in the yeast HD model (Cashikar et al., 2005). To study the mechanisms by which polyQ expansions affect the HSR, a chromatin immunoprecipitation sequencing (ChIP-Seq) assay was performed to identify changes in HSF1 targets during expanded polyQ expression. About 30\% of genes binding to HSF1 during heat shock lost the interaction in the presence of polyQ. Surprisingly, genes identified were not enriched in classical HSF1 targets but involved in other functions such as GTPase regulation, cytoskeleton remodeling, focal adhesion, and cell proliferation (Riva et al., 2012). These results suggest that polyQ may also impair non-canonical HSR targets.
Compounds that induce HSR activation have shown therapeutic effects against HD. Treating PC12 cells expressing 81Q with celastrol, which upregulates HSPs, significantly reduced the neuronal death rate. Another drug, geldanamycin, which can activate the HSR, was shown to inhibit Htt aggregation in COS-1 cell line (Sittler et al., 2001). Furthermore, a study in an HD mice model demonstrated that the beneficial effects of the HSR activation were transient and diminished during disease progression (Labbadia et al., 2011). The same study also found that the impairment may result from histone H4 hypoacetylation which reduces chromatin accessibility and ability of HSF1 to bind to the HSP promoters. This result further links impaired the HSR in HD to histone acetylation. In the fifth chapter of my thesis, I will study the role of Tra1 in regulating the HSR in the presence of toxic polyQ. Investigating how polyQ expansions impair the HSR will provide us a better insight into the molecular pathogenesis of HD and help identify potential therapeutic targets.

1.9.3 Cellular stress responses in antifungal resistance

The ability for fungi to survive and adapt to a variety of environmental stressors is formidable. Nowadays, fungal infection has become a huge problem for human health. Around 20 to 25% of the world’s population has skin fungal infections (Havlickova et al., 2008). Due to the lack of understanding of fungal stress responses, the development of effective and target-specific antifungal reagents is still challenging. Approximately 300 fungal species have been found to be pathogenic to humans (Casadevall, 2018). *Candida, Aspergillus, Pneumocystis*, and *Cryptococcus* are the most common fungi that have been found to cause human diseases such as chronic skin infections including athlete’s foot and ringworm. Severe infection may cause death especially in people with immune diseases (Brown et al., 2012; Johnson, 2018). The widely used antifungal drugs are mainly azoles, polyenes, and echinocandins. Azoles and polyenes target ergosterol that leads to plasma membrane defects in fungi. Echinocandins (caspofungin and anidulafungin), on the other hand, blocks the synthesis of β(1,3)-d-glucan, a major component of the fungal cell wall (Groll et al., 1998; Kathiravan et al., 2012). The mechanisms of action of common antifungal agents are shown in figure 1.7. Resistance to common drugs has been observed in many fungal pathogens, specifically
among *Candida* species. Patients also suffer from strong side effects (Cowen, 2008; Cowen et al., 2014). Therefore, it is crucial to understand the mechanism of antifungal resistance and identify new targets for fungal infection therapy.

The cell wall is a promising target in the study of antifungal drugs for many reasons. First, cell walls are unique structures in fungi so targeting cell wall synthesis can avoid side effects to human and animal host cells. Furthermore, as mentioned previously, cell wall integrity is vital for fungi survival under stress conditions (Levin, 2005). Thus, the CWI pathway may be a novel target for killing the fungal pathogens. Mutant *Aspergillus* strains with defects in the cell wall have shown reduced virulence in mouse infection models (Valiante et al., 2015). Moreover, AfM KK2 deletion leads to increased sensitivity to antifungals (Dirr et al., 2010). In *Candida albicans*, mutations in CaCAS5, a transcriptional regulator of CWI targets, made cells more sensitive to caspofungin (Bruno et al., 2006). These findings suggest that the CWI pathway is critical for fungal virulence and antifungal resistance. Since Tra1 may be required for the CWI pathway activation (Berg et al., 2018), it could be a potential target for antifungal drug development.

Another cellular stress response pathway that has been found relevant to pathogen virulence and drug resistance is the calcineurin pathway. Calcineurin is vital for fungal virulence and survival in the host environment. A *cna1* mutation *Cryptococcus neoformans* strain is viable but cannot survive at 37 °C or alkaline pH and is not toxic in a rabbit model (Odom et al., 1997). Reduced virulence of *C. albicans* carrying a calcineurin mutation was also seen in a mouse model of systemic infection (Blankenship et al., 2003). Furthermore, calcineurin inhibitors FK506 and cyclosporine A and their analogs have been shown to have protective effects against fungal infections (Lee et al., 2018; Pandit, 2003). Treatment with a calcium channel blocker amlodipine, in combination with fluconazole, caused significant cell death in fluconazole-resistant *C. albicans* strain (Liu et al., 2016). All the evidence indicates that the calcineurin pathway is critical for antifungal resistance. To further study the mechanism, whole proteomic analyses were performed in the well-studied *S. cerevisiae* to identify calcineurin targets. Seventy candidates were found to interact with the calcineurin signaling pathway, involved in membrane structure, polarized growth, protein trafficking, and cell wall
integrity (Goldman et al., 2014), indicating the multifunctional role of calcineurin in regulating fungal survival. As previously demonstrated, Tra1 has been linked to calcineurin signaling regulation. Therefore, I will study the mechanisms on how Tra1 regulates the calcineurin pathway activation and its role in antifungal resistance.
Figure 1.7 Mechanism of action of common antifungal agents.
Azoles inhibit the cytochrome P450 dependent enzyme lanosterol 14α-demethylase, which interrupt the synthesis of ergosterol, the main sterol in fungal cell membrane. Polyenes bind to ergosterol, leading to membrane depolarization and leakage of intracellular cations. Echinocandins inhibit β(1,3)-d-glucan synthesis and cause cell wall defect. Calcineurin inhibitors which suppress calcineurin activation also prevent fungal infections. Figure modified from Alzohairy et al., 2014.
1.10 Fluorescent-tagged yeast model of Huntington’s Disease

The baker's yeast *S. cerevisiae* has been widely studied as a model for neurodegeneration such as AD, PD, ALS, and HD (Bharadwaj et al., 2010; Fushimi et al., 2011; Outeiro and Lindquist, 2003). Although there is no *HTT* ortholog in the yeast genome, expressing the first 17 amino acids of Htt\textsuperscript{ex1} followed by different length of polyQ repeat sequences successfully mimic the toxic phenotype and protein aggregation observed in mammalian models of HD (Duennwald, 2011; Krobitsch and Lindquist, 2000). Several CAA codons are interrupted into the CAG repeats to prevent the expansion and contraction of the accurate number of polyQ repeats. Removal of the proline-rich region and adding a FLAG-tag is also required for polyQ toxicity in the yeast model (Duennwald et al., 2006). Furthermore, our study demonstrated that different fluorescent tag can also differentially affect polyQ toxicity and aggregation morphology in yeast (Jiang et al., 2017). A series of cellular phenotypes have been reported using the yeast model of HD, including transcriptional dysregulation, ER stress and ER-associated degradation defects, vesicle trafficking defects, and impaired mitochondrial function (Duennwald and Lindquist, 2008; Kumar et al., 2014; Nath et al., 2015; Quintanilla and Johnson, 2009). Overall, yeast provides us with an excellent model to investigate cellular mechanisms in misfolded protein stress responses and understand polyQ toxicity in HD.

1.10.1 Yeast model used in this thesis

The yeast model of HD used in this thesis is optimized from previous studies (Duennwald and Lindquist, 2008; Krobitsch and Lindquist, 2000). Strains used in this thesis are derivatives of W303a or BY4741/4742. Briefly, the polyQ high expression model uses the human Htt\textsuperscript{ex1} fragment carrying non-pathological 25Q or HD-associated 46Q/72Q expansion regions designed under a GAL promoter, and tagged with a yeast optimized fluorescent protein (FP). The high expression model mimics the toxic polyQ condition. Cells show an obvious growth defect when 72Q is induced by switching the carbon source from glucose to galactose. Aggregation is observed in cells expressing 46Q and 72Q. The polyQ low expression strain uses the human Htt\textsuperscript{ex1} fragment with 25Q/103Q regions designed under a MET25 promoter. Cells display little growth defect when
induced in synthetic complete media lacking methionine in standard growth condition. The model successfully mimics the cellular mechanisms of HD at early time points before neuronal cell death which provides us a good tool to investigate the underlying mechanisms to cope with mutant Htt accumulation and polyQ toxicity under stress conditions.

1.10.2 Flanking sequence affects polyQ toxicity in yeast

Previous literature reported that the endogenous C-terminal polyproline region of Htt\textsuperscript{ex1} has profound effects on polyQ toxicity in yeast (Duennwald et al., 2006). PolyQ toxicity requires the deletion of the proline-rich region. In the presence of the proline-rich region, polyQ only formed one or two tight fluorescent foci, whereas they displayed multiple aggregates throughout the cytosol without the proline-rich sequence. The large aggregate formed by mutant Htt containing proline-rich region was found to co-localize with aggresome markers (Wang et al., 2009). Chaperones can be recruited to aggresomes to help with protein refolding and degradation. Autophagy was found to facilitate aggresome degradation (Johnston et al., 1998). Thus, the proline-rich sequence may direct Htt into the aggresome to prevent misfolding. Furthermore, many proteins have been found to interact with proline-rich regions including molecular chaperones and protein remodeling proteins (Kay et al., 2000), so the mechanism may involve the recruitment of chaperone proteins that need further investigations. On the other hand, a FLAG-tag (DYKDDDDK) is critical for mutant Htt toxicity. The hypothesized mechanism is that FLAG-tag mimics the sumoylation of polyQ proteins because SUMO proteins are also enriched in negative charged amino acid at the C-terminus. And polyQ sumoylation has been shown to enhance its toxicity (Steffan et al., 2004).

Previous studies including ours, suggested that the fluorescent tag can alter polyQ toxicity and aggregation (Duennwald et al., 2006; Jiang et al., 2017). Tagging polyQ with mCherry and TagBFP significantly reduce its toxicity in yeast. The full results are shown in chapter two and three of this thesis. Since the first fluorescent protein (FP), wild-type green fluorescent protein (GFP) discovered in \textit{Aequorea Victoria} jellyfish, was used to highlight sensory neurons in \textit{C.elegans} (Tsien, 1998), FPs have become a powerful tool in the molecular toolbox in studying protein localization, function, and interactions. A
series of modified FPs which are brighter, exhibit enhanced photostability and cover a broader spectral range have come out and provided scientists with better choices in monitoring different proteins and cellular structures (Chudakov et al., 2010). For instance, a yeast GFP clone integrated into the yeast chromosome has been made to obtain a better result when labelling proteins in yeast (Niedenthal et al., 1996).

The most commonly used FPs are enhanced version of wild-type GFP (EGFP) as well as the original blue (BFP), cyan (CFP) or yellow (YFP) derivatives. Another group is the red fluorescent proteins (RFP) cloned from corals which have a longer excitation wavelength. However, choosing which fluorescent tag to use must consider different experimental conditions and the misfolding of the fusion proteins. Different FPs have different structures and thus chromophores. The GFP family has a barrel structure where an alpha-helix runs through eleven beta-sheets (Evdokimov et al., 2006; Henderson and Remington, 2005). The GFP-like chromophore in *Aequorea victoria* GFP is achieved by cyclization at positions 65–67 (Ser-Tyr-Gly) (Barondeau et al., 2003). Although RFPs have a similar structure with GFP, the post-translational modification is diverse. For example, the bond between the alpha-carbon and nitrogen of 66 Gln in DsRed is dehydrogenated, which extends the GFP chromophore by forming $\text{C==N==C==O}$ at the 2-position of the imidazolidinone (Gross et al., 2000; Yarbrough et al., 2001). FPs usually have a tendency to oligomerize, which can interfere with their fusion partners (Loening et al., 2007; Wall et al., 2000). Through mutation studies and protein engineering techniques, better FPs have been developed (Table 1.1). However, it also raises the question on which FPs to use and how to assess their impact on targeted proteins. Most assays available were performed *in vitro* or not suitable for large-scale analysis. Therefore, a rapid and high-throughput assay is imperative to assess the impact of FPs on their fusion partners. Overall, the ideal FP would have sufficient photostability and brightness to provide a detectable signal (Shaner et al., 2005). In addition, using monomeric versions of FPs could avoid aberrant oligomerization and toxicity (Campbell et al., 2002). Erik Snapp and his group have developed an array of monomeric oxidizing environment-optimized FPs (moxFPs). These moxFPs could avoid non-native disulphide bond formation between cysteine residue, providing a brighter signal and more accurate protein localization of ER and secretory proteins (Costantini et al., 2015). It also needs to be noted that FPs must
have minimal crosstalk in excitation and emission channels during multiple-labelling experiments (Lata et al., 2006).

Using modern FPs, we are able to optimize the previous yeast model of HD as a compelling tool to study cellular functions during polyQ expansion through fluorescence microscopy techniques. Furthermore, the yeast polyQ model provides researchers a high throughput tool for assessing the impact of FPs on their target in vivo.
### Table 1.1 Modern fluorescent proteins

<table>
<thead>
<tr>
<th>FPs</th>
<th>Origin</th>
<th>Peak Excitation</th>
<th>Advantages</th>
<th>Oligomerization</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>sfGFP</td>
<td>Aequorea Victoria GFP</td>
<td>485nm</td>
<td>High folding efficiency</td>
<td>Monomer</td>
<td>(Pèdelacq et al., 2006)</td>
</tr>
<tr>
<td>NeonGreen</td>
<td>B. lanceolatum LanVFP</td>
<td>506nm</td>
<td>Bright, monomeric</td>
<td>Monomer</td>
<td>(Shaner et al., 2013)</td>
</tr>
<tr>
<td>mCherry</td>
<td>Discosoma DsRed</td>
<td>587nm</td>
<td>High photostability</td>
<td>Monomer</td>
<td>(Shaner et al., 2004)</td>
</tr>
<tr>
<td>mScarlet</td>
<td>Synthetic</td>
<td>569nm</td>
<td>Truly monomeric</td>
<td>Monomer</td>
<td>(Bindels et al., 2017)</td>
</tr>
<tr>
<td>FusionRed</td>
<td>Entacmaea quadricolor sea anemone RFP</td>
<td>580nm</td>
<td>Low toxicity in vivo</td>
<td>Monomer</td>
<td>(Shemiakina et al., 2012)</td>
</tr>
<tr>
<td>TagBFP2</td>
<td>Entacmaea quadricolor sea anemone RFP</td>
<td>399nm</td>
<td>high pH stability, fast chromophore formation</td>
<td>Oligomer</td>
<td>(Subach et al., 2011)</td>
</tr>
<tr>
<td>moxBFP</td>
<td>Aequorea Victoria GFP</td>
<td>385nm</td>
<td>Optimized for oxidizing environments</td>
<td>Monomer</td>
<td>(Costantini et al., 2015)</td>
</tr>
</tbody>
</table>
1.11 Objectives and hypothesis

Cellular stress responses are crucial for cell survival and growth under stress conditions. Dysregulation of cellular stress responses is linked to different diseases including inflammation (Muralidharan and Mandrekar, 2013), cancer (Herr and Debatin, 2001), neurodegenerative diseases (Chafekar and Duennwald, 2012; Puspita et al., 2017), and fungal infections (Brown et al., 2014). The activation of stress response pathways requires the transcription of stress-induced genes encoding molecular chaperones, metabolic enzymes, and proteins involved in biomass synthesis (Estruch, 2000). However, the transcriptional regulations of cellular stress responses, especially the roles of transcription coactivators and post-translational modifications of histones, remain elusive. Available high throughput approaches and comparative genomics to human make S. cerevisiae an excellent model to study the activation of cellular stress response pathways. The transcriptional co-activator Tra1 within the SAGA and NuA4 HAT complexes has been found to be critical during cell stress (Hoke et al., 2008). How Tra1 regulates cellular stress responses and its implication in stress-related diseases is poorly understood. Therefore, my thesis investigates Tra1-dependent regulation of stress response pathways in a yeast model of HD, determining its role in cellular stress responses such as the TORC1 pathway, the HSR, and the CWI pathway, as well as their implications for antifungal drug resistance.

My overall hypothesis is that Tra1 is a key regulator of cellular stress responses through its role in the SAGA acetyltransferase complex, activating transcription, and represents a potential therapeutic target for stress-related human diseases such as Huntington’s Disease and fungal infections.

1.11.1 Chapter 2. Polyglutamine toxicity in yeast uncovers phenotypic variations between different fluorescent protein fusions.

Rationale: Flanking sequences could alter polyQ toxicity in yeast (Duennwald et al., 2006). FPs differ in their physical properties and can affect protein function of their fusion partners. Therefore, we provide a novel series of vectors based on the latest yeast-optimized FPs for efficient investigation of polyQ toxicity in yeast. Furthermore, there
are no rapid and high throughput methods available to assess the effects of FPs on their fusion partners. We may be able to develop an efficient assay to evaluate new FPs using the yeast model of HD.

**Hypothesis:** Different FPs alter the polyQ aggregation morphology and toxicity. The yeast polyQ model is an ideal *in vivo* tool to assess the impact of fluorescent tags, especially on naturally oligomeric or misfolded proteins.

1.11.2 Chapter 3. Polyglutamine toxicity assays highlight the advantages of mScarlet for imaging in *Saccharomyces cerevisiae*.

**Rationale:** RFPs are the most popular alternatives to GFP in fluorescent imaging. Different monomeric versions of RFP have been developed over the years that provide a stable and robust signal. Observed from our polyQ yeast model, unlike what is reported for green FP variants, yemRFP tagged polyQ expansions show reduced toxicity and a different pattern of aggregates (Jiang et al., 2017). Thus, it is crucial to test the effect of two latest versions of RFP, mScarlet, and FusionRed, on their binding partners using the method we developed.

**Hypothesis:** The ymScarlet has significant advantages over the previous generation of red FPs for use in fluorescent fusions in yeast.

1.11.3 Chapter 4. Sfp1 links TORC1 and cell growth regulation to the yeast SAGA-complex component Tra1 in response to polyQ proteotoxicity.

**Rationale:** Acetylation of histones has been shown to be impaired by polyQ expansions in different HD models (Gray, 2011; Steffan et al., 2001). Tra1 is an essential component of both the SAGA and NuA4 HAT complexes. Our preliminary data showed that Tra1 mutant cells are more sensitive to polyQ toxicity. Moreover, previous papers have shown the interaction between Tra1 and Sfp1, linking the regulation of Tra1 to TORC1 pathway and cell growth (Lempiäinen et al., 2009). Importantly, Tra1 is considered functionally equivalent to TRRAP in mammals and TRRAP was identified as a cofactor for c-Myc in
regulating oncogenic transformation (McMahon et al., 1998). Thus, the results may indicate a novel pathway impaired by polyQ in HD.

**Hypothesis:** PolyQ impairs Tra1 signaling through dysregulation of the TORC1/Sfp1 pathway, leading to defect in SAGA HAT complex and transcription activation.

### 1.11.4 Chapter 5. Impaired Tra1-dependent transcription during heat shock in the presence of polyQ expansions

**Rationale:** Impaired HSR by expanded polyQ expression has been found in both yeast and mammalian system (Chafekar and Duennwald, 2012). Importantly, upon exposure to stress, neuronal cells accumulate damaged proteins and lack a fully functional proteostasis network to cope with this damage. Therefore, the impaired protein homeostasis may lead to dysfunction of multiple cellular functions and reveal the pathological hallmarks of HD and other neurodegenerative diseases.

**Hypothesis:** Heat shock sensitivity of cells expressing polyQ expansions can be traced back to the defect of Tra1/SAGA which regulates the activation of the heat shock response and the cell wall integrity pathway.

### 1.11.5 Chapter 6. Tra1 is a novel potential target for antifungal therapy.

**Rationale:** The calcineurin pathway regulates cell growth and drug resistance in many fungal pathogens (Juvvadi et al., 2014; Liu et al., 2015). Our preliminary data suggest that Tra1 plays a vital role in regulating calcineurin signaling and cell sensitivity to antifungal drugs. Thus, Tra1 could be a new target for antifungal drug development.

**Hypothesis:** Tra1 mediates cell growth and antifungal drug resistance through the activation of the calcium-calcineurin pathway in yeast.
1.12 References


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component Tra1 to multiple cellular processes. *BMC Genet.* 9, 46.


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Chapter 2

2 Polyglutamine toxicity in yeast uncovers phenotypic variations between different fluorescent protein fusions

Fluorescent proteins (FPs) have revolutionized the work of scientists by allowing the direct visualization of protein trafficking and localization in living cells. However, FPs are too often seen as interchangeable building blocks than can be easily swapped to accommodate a specific imaging condition. In this study, we will monitor polyQ toxicity and aggregation in a yeast model of HD to highlight the differential impact of the most common FPs on the behavior of fluorescent reporters.

2.1 Abstract

The palette of fluorescent proteins (FPs) available for live-cell imaging contains proteins that strongly differ in their biophysical properties. FPs cannot be assumed to be equivalent and in certain cases could significantly perturb the behavior of fluorescent reporters. We employed *Saccharomyces cerevisiae* to comprehensively study the impact of FPs on the toxicity of polyglutamine (polyQ) expansion proteins associated with Huntington's disease. The toxicity of polyQ fusion constructs is highly dependent on the sequences flanking the polyQ repeats. Thus, they represent a powerful tool to study the impact of fluorescent fusion partners. We observed significant differences on polyQ aggregation and toxicity between commonly used FPs. We generated a novel series of vectors with latest yeast-optimized FPs for investigation of Htt toxicity, including a newly optimized blue FP for expression in yeast. Our study highlights the importance of carefully choosing the optimal FPs when designing tagging strategies.

2.2 Introduction

The ability to visualize protein localization in living cells or whole organisms has been the driving force behind a myriad of new technological innovations in fluorescence microscopy that revolutionized our ability to address many biological problems (Betzig, 2015; Sengupta et al., 2014). The engineering of the green fluorescent protein (GFP) reporter (Chalfie et al., 1994) paved the way for the subsequent discovery and
optimization of other fluorescent genetically encoded reporters that can be employed to tag native proteins. Since the pioneer work on GFP, several other FPs have been characterized, covering the whole ensemble of the light spectrum, from the ultraviolet to the far red (Miyawaki et al., 2012; Morozova et al., 2010; Rizzo et al., 2009; Shaner et al., 2004; Shaner et al., 2007; Shaner et al., 2013). FPs vary extensively in their physical features beyond just their spectral properties. These properties can include brightness, photostability, maturation time, oligomeric state (monomers, dimers, multimers) and folding capacity (Suzuki et al., 2012). While these reporter proteins have been instrumental in several discoveries, the addition of a fluorescent tag can disrupt the normal function of the protein of interest (Han et al., 2015). First, not all FPs are equally suitable for every cellular compartment. For example, when using fluorescent fusion proteins that enter the oxidizing environment of the endoplasmic reticulum, one should avoid FP containing internal cysteines that may cause creation of aberrant, non-native disulfide bond resulting in formation of misfolded and non-fluorescent FP oligomers (Aronson et al., 2011; Costantini and Snapp, 2013; Costantini et al., 2013; Costantini et al., 2015). Accumulation of a significant pool of such dark misfolded oligomers can not only lead to a significant underestimation of protein concentrations, but also cause unwanted protein interactions, especially with the cellular protein quality control machinery (Aronson et al., 2011). Second, all FPs originally isolated from either corals or jellyfish exist as oligomers in their environment of origin. For example, GFP forms a complex with aequorin and the original DsRed is a tetramer. Original studies have shown that GFP can form dimers in crystals (Yang et al., 1996) and when present at high concentration in solution (Phillips, 1997). Therefore, efforts were directed into improving the monomeric status of FPs. Discovery of mutations that improve the monomeric nature of GFP (Zacharias et al., 2002) and DsRed (Shaner et al., 2004) paved the way for improvement of the fluorescent protein technology. The tendency of several FPs to oligomerize has been shown to artificially lead to cellular membrane reorganization (Costantini et al., 2012; Snapp et al., 2003). Moreover, several studies have shown that FPs originally characterized as monomers were prone to aberrant protein interactions (Costantini et al., 2012; Costantini et al., 2015; Lam et al., 2012; Shemiakina et al., 2012). It was recently shown that the choice of a specific FP can significantly alter the behavior
of tandem fluorescent protein timers used to measure protein turnover in living cells (Khmelinskii et al., 2016). The behavior of FP’s can also be potentially affected by several other factors such as pH and other physical properties (Patterson et al., 1997) and the size of the linker domain between the fusion partners (Chen et al., 2013). Thus, when designing strategies to produce FP-tagged proteins, the choice of a specific FP is crucial in order to minimize the effect on native protein and thus obtain optimal experimental conditions reflecting untampered functions and properties of the native proteins (Snapp, 2009).

Aberrant expansion of polyglutamine (polyQ) repeats within disease-causing proteins is at the root of several human neurodegenerative pathologies including Huntington’s disease (HD) (Macdonald, 1993). In HD, expansion of the polyQ region (typically over 36 repeats) within the N-terminal region of the first exon of the Huntingtin protein (Httex1) results in its misfolding and increased aggregation into detergent-insoluble, amyloid-like inclusion bodies (IBs) (Gövert and Schneider, 2013). Several studies showed that polyQ proteins assembly into toxic intermediate oligomers that preceded accumulation of IBs represent the most toxic species of the protein which may be associated to HD pathogenesis (Arrasate et al., 2004; Herrera et al., 2011; Lajoie and Snapp, 2010; Leitman et al., 2013; Takahashi et al., 2007). Thus, understanding the parameters regulating polyQ oligomer formation is critical to our understanding of HD. In order to decipher these mechanisms, a yeast model of HD was developed (Duennwald, 2013; Krobitsch and Lindquist, 2000; Mason and Giorgini, 2011; Meriin et al., 2002), allowing rapid and extensive dissection of the genetic mechanisms underlying polyQ aggregation and toxicity. Over the years, the yeast model of HD has led researchers to identify several determinants of toxicity in HD, from chaperone proteins that assist protein folding (Muchowski et al., 2000; Sakahira et al., 2002; Schaffar et al., 2004), to activation of endoplasmic reticulum stress pathways (Duennwald and Lindquist, 2008), that were subsequently validated in other models.

In most cases, assessment of polyQ aggregation and toxicity is performed using a fluorescent fusion protein consisting of Httex1 fused to fluorescent proteins at the C-terminus following the polyQ repeats (Duennwald, 2013; Duennwald et al., 2006a;
Duennwald et al., 2006b; Meriin et al., 2002). Based on the aforementioned ability of fluorescent proteins to affect the behavior of the protein of interest, we hypothesized that various fluorescent proteins could differentially affect the aggregation of toxic polyglutamine expansion (polyQ). For example, the tendency of certain FPs to oligomerize could lead to changes in the ability of expanded polyQ proteins to achieve or maintain their toxic conformation. This assumption was supported by previous studies showing that the sequences flanking polyQ proteins can alter their ability to oligomerize into the SDS-insoluble toxic IBs characteristic of HD (Duennwald et al., 2006a).

2.3 Materials and Methods

2.3.1 Yeast strains and culture conditions

All strains are derived from W303 Mat a (Thomas and Rothstein, 1989). In order to assess polyQ protein aggregation and toxicity, cells expressing the different plasmids were grown overnight in synthetic complete media using appropriate selection media to maintain plasmids. Cells were then washed twice with media containing 2% galactose and resuspended to OD$_{600nm}$ 0.2 and incubated at 30°C in a rotator drum for the indicated amounts of time to induce polyQ expression. Spot assays were performed by spotting 5X dilutions of OD$_{600nm}$ 0.2 on agar plates. Plates were photographed using a GelDoc system (Bio-Rad Laboratories) Liquid growth assays were performed using a Bioscreen C (Growth Curves USA, Piscataway, NJ) plate reader set at 30°C.

2.3.2 Plasmids

The original 25 and 72Q-CFP lacking the proline rich sequence (ΔPRO) vector were previously described (Duennwald et al., 2006b; Duennwald et al., 2006a). To generate the construct without the FP we introduced a stop codon downstream of the polyQ region. All new vectors were generated by cloning the GAL-polyQ-ΔPRO fragment into the SacI/SpeI sites of pRS415. The various new fluorescent proteins were subsequently cloned into the SpeI/SalI sites. All fragments were generated using standard PCR methods. The yemsfGFP vector was obtained from Erik Snapp (Albert Einstein College of Medicine) and a monomerizing mutation (V206K) was inserted using site directed mutagenesis. ymNeonGreen (Shaner et al., 2013) was obtained from Allelebiotech (San
Diego CA). yemRFP was from Neta Dean (Keppler-Ross et al., 2008). yomTagBFP2 (#44899) was obtained from Addgene and previously characterized (Lee et al., 2013). ymoxBFP was synthetized by Genscript (Piscataway, NJ) based on the moxBFP sequence previously published (Costantini et al., 2015). All constructs were validated by sequencing and are listed in Table 2.1. All primer sequences can be found in Table 2.2.
Table 2.1 Plasmids used in this study

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2.3.3 Fluorescence microscopy

Fluorescence microscopy was performed using a Zeiss 880 confocal microscope equipped with a 405 nm diode laser for yomTagBFP2 and ymoxBFP and CFP, a 488 diode laser for ymsfGFP and ymNeonGreen and a 561 diode laser for yemRFP. Upon polyQ expression, cells were transferred to LabTek imaging chambers (Thermo inc.) and imaged at room temperature using a 63x PlanAprochromat objective (1.4 NA). Images were analyzed using the ImageJ software (https://imagej.nih.gov/ij/).

2.3.4 Dot blot and SDD-AGE

Biochemical analysis of polyQ oligomers were performed as previously described (Duennwald, 2013). Briefly, cells were incubated in galactose-containing media to induce the various polyQ constructs lysed in lysis buffer (100mM Tris pH 7.5; 200mM NaCl; 1 mM EDTA; 5% glycerol, 1mM Dithiothreitol (DTT). Protease inhibitors were added directly before use: 4 mM phenylmethylsulfonyl fluoride (PSMF) and SigmaFast protease inhibitor cocktail (Sigma-Aldrich, St.Louis MO). Cells were lysed using glass beads. For dot blot, equally amount of proteins were spotted on nitrocellulose membranes using a vacuum manifold and processed for immunoblot detection using an anti-FLAG antibody (SIGMA-Aldrich, #F3040). For SDD-AGE or an anti-Pgk1 antibody, samples were loaded on a 1.5% agarose gel containing 0.1% SDS and transferred onto nitrocellulose membrane for immune detection as previously described (Halfmann and Lindquist, 2008).

2.3.5 Statistical analysis

In order to quantify changes in yeast cells growth, growth curves were generated using the BioscreenC and area under the curve was calculated using Graphpad Prism (La Jolla, CA). Statistical significance was calculated using a two-tailed Student’s t-test where indicated.
2.4 Results and Discussion

2.4.1 The presence of a fluorescent protein tag unmasks expanded Htt\textsuperscript{ex1} toxicity

In yeast, as in other model organisms, expression of Htt\textsuperscript{ex1} carrying disease-associated length of polyQ repeats results in oligomerization, aggregation into IBs and cellular toxicity that can be measured through reduced growth on agar plates (Duennwald, 2013). The original constructs systematically tested by the Lindquist group contains Htt\textsuperscript{ex1} tagged at the N-terminus with a FLAG epitope tag and a fluorescent CFP at the C-terminus (Duennwald et al., 2006b). In absence of the FLAG tag, the expanded HD-associated fluorescent Htt\textsuperscript{ex1} protein displayed a significant change in IBs morphology, resulting in soluble and non-toxic aggregates. These results highlight the fact that the nature of the sequences flanking the polyQ region is indeed regulating its ability to acquire the toxic conformation (Duennwald et al., 2006a). In our studies, we chose to use an expanded Htt\textsuperscript{ex1} construct containing 72Q rather than the original 103Q (Duennwald et al., 2006b). This length induces reduced toxicity therefore allowing us to test for increased sensitivity to different conditions. Consistent with the finding that flanking sequences modulate polyQ toxicity, we found that expression of FLAG-72Q protein without the C-terminal CFP fragment (72Q-Stop) was not toxic (Figure 2.1A and B) even when expressed at high levels from a multicopy plasmid (Figure S2.1). The CFP-tagged version of 72Q showed typical toxic aggregates observed by fluorescence microscopy (Figure 2.1C). Interestingly, both 72Q-CFP and 72Q-Stop constructs show similar ability to oligomerize when analyzed on Semi-Denaturing Detergent Agarose Gel Electrophoresis (SDD-AGE), a technique routinely employed to assess Htt\textsuperscript{ex1} aggregation and detergent solubility (Halfmann and Lindquist, 2008) (Figure 2.1D). A dot blot was performed using the same lysates to assess expression levels of the different constructs (Figure 2.1D and S2.2). Thus, our data argue that while both fusion proteins can oligomerize and aggregate, only the CFP-tagged polyQ appears to be able to acquire a toxic conformation. It is possible that addition of the fluorescent tag changes the biophysical properties of Htt\textsuperscript{ex1} oligomers, leading to formation of the toxic species similarly to what is observed with the addition of the FLAG tag at the amino terminus end of the protein (Duennwald et al., 2006a). Indeed, recent \textit{in vitro} data indicate that
untagged Htt\textsuperscript{ex1} can behave differently than the tagged versions (Vieweg et al., 2016). In addition, we found the CFP-tagged proteins produced a very weak fluorescent signal, especially for the soluble 25Q construct. This signal is so low that it is on par or even below that emanating from red vacuolar pigment due to the \textit{ade2-1} mutation in the W303 strain (Weisman et al., 1987) (Figure 2.1C). Given the variety of FPs available and their frequent use of them as interchangeable building blocks, we sought to further determine the impact of some of the most popular FPs on Htt\textsuperscript{ex1} aggregation and toxicity.
Figure 2.1 C-terminal tagging of Htt\textsuperscript{ex1} regulates toxicity in yeast.

(A) The different Htt\textsuperscript{ex1} constructs contain either non-toxic 25Q or HD-associated 72Q followed by either a stop codon or a CFP fluorescent protein. Growth assays on agar plate reveals increased toxicity of 72Q CFP –tagged Htt\textsuperscript{ex1} compared to the untagged version. (B) Growth curves generated by liquid growth assays show increased toxicity of the 72Q CFP –tagged Htt\textsuperscript{ex1}. Area under the curve (AUC) is shown. n=3 ±SEM, * p<0.001. (C) Representative fluorescent images of CFP-tagged Htt\textsuperscript{ex1} constructs following overnight induction in galactose media displays formation of IBs in 72Q expressing cells. (D) SDD-AGE and dot blots of cell lysates prepared for the indicated Htt\textsuperscript{ex1} constructs. For dot blots, three five-fold serial dilutions of protein lysates are shown. Htt\textsuperscript{ex1} proteins were detected using an anti-FLAG antibody.
2.4.2 Differential impact of fluorescent proteins on Htt\textsuperscript{ex1} aggregation and toxicity

We assessed the impact of different fluorescent proteins on the ability of expanded polyQ proteins to aggregate into toxic oligomers and IBs. We chose some of the brightest FPs available for expression in yeast. These include the yeast codon optimized and monomeric versions of superfolder GFP (Pédelacq et al., 2006) (ymsfGFP), mNeonGreen (Shaner et al., 2013) (ymNeonGreen), Cherry (Keppler-Ross et al., 2008) (yemRFP), and TagBFP2 (yomTagBFP2) (Lee et al., 2013). All polyQ fusion proteins were cloned into a low copy yeast vector under the control of the GAL1 promoter (Figure 2.2A). Expression of these constructs in yeast cells led to two unexpected observations: first, we observed that unlike its ymsfGFP, ymNeonGreen and yemRFP counterparts, 72Q-yomTagBFP2 showed no aggregation and displayed features similar to 25Q tagged with other FPs (Figure 2.2A). Second, we found that while both 72Q-ymsfGFP and ymNeonGreen displayed strong growth inhibition compared to their 25Q counterparts, 72Q-yomTagBFP2 showed no toxicity and grew as well as 25Q yom-TagBFP (Figure 2.2B and C). Interestingly, 72Q-yemRFP showed intermediate toxicity (Figure 2.2B and C). Since aggregation and toxicity of Htt\textsuperscript{ex1} can be traced back to its ability to oligomerize, we tested the ability of these proteins to oligomerize using SDD-AGE (Halfmann and Lindquist, 2008). Surprisingly, 72Q-yemRFP displayed lower levels of SDS-resistant oligomers compared to ymsfGFP or ymNeonGreen (Figure 2.2D). While 72Q-yemRFP IBs appear similar to ymsfGFP IBs by fluorescence microscopy, these appear to have different levels of solubility that could explain the milder growth defect. It is important to note that different disease-causing aggregating proteins can form different types of IBs. For example TDP43, a protein associated with amyotrophic lateral sclerosis (ALS), forms SDS-soluble oligomers that are distinct from Htt\textsuperscript{ex1} oligomers (Johnson et al., 2008). Moreover, TDP43 IBs formation is independent of the yeast prion protein Rnq1, which is required for formation of Htt\textsuperscript{ex1} IBs (Meriin et al., 2002). We found that expanded Htt\textsuperscript{ex1}-yemRFP proteins still required Rnq1 since their expression in \textit{rnq1}\textsuperscript{Δ} cells did not result in appearance of IBs (Figure S2.3). Therefore, yemRFP may only slightly mask polyQ protein toxicity. This is consistent with a previous report that red fluorescent proteins can impair protein behavior (Costantini et al., 2015; Shemiakina et al., 2012; Snaith et al.,...
One could envisage that robust folding of the FPs such as sfGFP and the bright mNeonGreen (Pédelaq et al., 2006; Shaner et al., 2013) favors formation of toxic species of polyQ oligomers by uncoupling the folding trajectory of the FP from the folding of Htt\textsuperscript{ex1}. By contrast, slower folding FPs are more prone to aberrant interactions that could in turn favor the formation of non-toxic oligomers similarly to what is observed in the construct that contain the proline rich region adjacent to the polyQ stretch in Htt\textsuperscript{ex1} (Duennwald et al., 2006a). However, we observed that robust β-barrel folders, such as ymsfGFP displays similar toxicity compared to the original, non-optimized, CFP tagged 72Q construct (Figure S2.4). These data argue against a role for an FP's folding capacity in Htt\textsuperscript{ex1} toxicity in yeast. Therefore, it appears that the phenotype observed for yemRFP is probably caused by the previously reported inherent ability of red FPs to oligomerize/aggregate (Costantini et al., 2012; Lam et al., 2012; Merzlyak et al., 2007; Shemiakina et al., 2012). We should also note that the original CFP construct is not monomerized but still induces toxicity, indicating that unlike what we observed for red FPs, for Aequorea-derived FPs (GFP and its derivatives), the weaker tendency to oligomerize does not appear to have a significant impact on Htt\textsuperscript{ex1}-fusion toxicity.
Figure 2.2 Differential impact of fluorescent proteins on Htt\textsuperscript{ex1} aggregation and toxicity.

(A) 25Q and 72Q FLAG-Htt\textsuperscript{ex1} were fused to indicated yeast-optimized fluorescent proteins. Representative fluorescent images of fluorescently tagged Htt\textsuperscript{ex1} constructs following overnight induction in galactose media. (B) Spot assay on agar plates reveals increased toxicity of 72Q-ymsfGFP/ymNeonGreen when compared to 72Q-yemRFP and yomTagBFP2. (C) Growth curves generated by liquid growth assays show increased toxicity of the 72Q-ymsfGFP/ymNeonGreen. Area under the curve (AUC) is shown. \( n=3, \pm\text{SEM} \) * \( p<0.001 \). (D) SDD-AGE and dot blots of cell lysates prepared for the indicated Htt\textsuperscript{ex1} constructs. For dot blots, three five-fold serial dilutions of protein lysates are shown. Htt\textsuperscript{ex1} proteins were detected using an anti-FLAG antibody.
2.4.3 Optimized blue Htt\textsuperscript{ex1} protein fusion

Curiously, 72Q-yomTagBFP2 proteins migrated faster on the SDD-AGE gel compared to the other 72Q constructs tagged with either ymsfGFP or ymNeonGreen (Figure 2.2D). This indicates the presence of smaller oligomers that are not incorporated into IBs when observed by fluorescence microscopy (Figure 2.2A). We originally chose yomTagBFP2 because it had been characterized as the optimal blue fluorescent protein for expression in yeast (Lee et al., 2013). Recently, a new generation of fluorescent proteins, the moxFPs have been described. These monomeric proteins lack internal cysteines and are therefore optimized for the secretory pathway (Costantini et al., 2015). We codon optimized moxBFP for expression in yeast and generated fusions with either 25 and 72Q Htt\textsuperscript{ex1} (Figure 2.3A). Interestingly, we found that unlike yomTagBFP2 tagged fusions, 72Q-ymoxBFP displayed robust aggregation and toxicity (Figure 2.3 A-C). When we compared the fluorescent signal of the diffuse 25Q constructs tagged with either of the blue FPs we observed that ymoxBFP was significantly brighter than yomTagBFP2 (Figure 2.3D). Interestingly, we observed that 72Q-yomTagBFP2 displayed lower fluorescent signal than the 25Q-yomTagBFP2 fusion (Figure 2.3E). We have previously observed a similar phenomenon with the expanded mHtt\textsuperscript{ex1} proteins in mammalian cells (Lajoie and Snapp, 2010). This may reflect increased degradation of the polyQ expansion proteins. Thus, the yomTagBFP2 vectors could help studying the behavior of expanded mHtt\textsuperscript{ex1} in absence of IBs that complicate both biochemical and fluorescent imaging experiments. Finally, increased aggregation and toxicity of the moxBFP tagged constructs correlated with slower migration of the ymoxBFP 72Q oligomers on SDD-AGE (Figure 2.3F).
Figure 2.3 New optimized ymoxBFP increases Htt\textsuperscript{ex1} toxicity.
Figure 2.3
(A) Htt\textsuperscript{ex1} constructs generated contain either non-toxic 25Q or HD-associated 72Q followed by either a yom-TagBFP2 or ymoxBFP fluorescent proteins. Growth assays on agar plates reveal increased toxicity of 72Q ymoxBFP –tagged Htt\textsuperscript{ex1} compared to the yomTagBFP2 tagged version. (B) Growth curves generated by liquid growth assays show increased toxicity of the 72Q ymoxBFP tagged Htt\textsuperscript{ex1}. Area under the curve (AUC) is shown. n=3, ±SEM * p<0.001. (C) Representative fluorescent images of yomTagBFP2/ymoxBFP-tagged Htt\textsuperscript{ex1} constructs following overnight induction in galactose media displays formation of IBs in 72Q-ymoxBFP expressing cells. (D) ymoxBFP significantly increases fluorescent intensity of 25Q-Htt\textsuperscript{ex1}. Representative fluorescent images of yomTagBFP2/ymoxBFP-tagged 25Q Htt\textsuperscript{ex1} constructs following overnight induction in galactose. Images were collected using the same acquisition parameters to allow direct comparison of fluorescent intensities. Inverted black and white images are shown for clarity. Plot of quantified individual cell mean fluorescent intensities is shown for comparison. * p<0.001 (E) 72Q- yom-TagBFP2 is significantly dimmer than its 25Q counterpart. Images were collected using the same acquisition parameters to allow direct comparison of fluorescent intensities. Inverted black and white images are shown for clarity. Plot of quantified individual cell mean fluorescent intensities is shown for comparison. * p<0.001 (F) SDD-AGE and dot blots of cell lysates prepared for the indicated Htt\textsuperscript{ex1} constructs. For dot blots, 3 different serial dilutions of protein lysates are shown. Htt\textsuperscript{ex1} proteins were detected using an anti-FLAG antibody.
Our imaging result contrasts with the original report showing that TagBFP is significantly brighter than EBFP2, at least in solution (Subach et al., 2008). However, optimal brightness will be possible only when FPs are expressed alone or when fused with well folded proteins (Pédelacq et al., 2002; Waldo et al., 1999). Indeed, the superfolder variant of GFP was created by screening for its ability to fluoresce when fused to poorly folded proteins (Pédelacq et al., 2006). Thus, fusion of yomTagBPF2 with misfolded Httex1 could be problematic. It is important to note that the new moxBFP generated by Costantini et al (Costantini et al., 2015) contains both superfolded mutations and additional cycle-3 GFP mutations (F99S and V163A) (Crameri et al., 1996) that would make it a robustly folded fusion partner. Indeed, we found that when expressed alone in the yeast cytosol under a constitutive promoter, yomTagBFP2 was slightly brighter than moxBFP (Figure 2.4). While the mean fluorescent intensity across the cell population was similar, the standard deviation of the fluorescence signal was significantly higher for yomTagBFP2. This result indicates that while yomTagBFP2 may be the brightest blue FP, ymoxBFP performs better in the context of fusion protein reporters. These results should be taken in consideration when designing new blue fluorescent protein fusions.
Figure 2.4 Cytoplasmic, untagged yomTagBFP2 is brighter than ymoxBFP. Cells constitutively expressing either yomTagBFP2 or ymoxBFP from the GPD promoter were cultured to early log phase. Images were collected using the same acquisition parameters to allow direct comparison of fluorescent intensities. Inverted black and white images are shown for clarity. Plot of quantified individual cell mean fluorescent intensities is shown for comparison.
TagBFP2 (Subach et al., 2011) is an optimized version of TagBFP (Subach et al., 2008), which was originally derived from TagRFP (Merzlyak et al., 2007), which, like most red FPs was derived from multimeric proteins isolated from Anthozoa corals and anemones. While these proteins are reported to be monomeric, several studies have found that they are actually strongly dimerizing (Costantini et al., 2012; Lam et al., 2012; Merzlyak et al., 2007; Shemiakina et al., 2012). Unlike mCherry, which is only weekly dimerizing (Costantini et al., 2015) and allows for the formation of IBs (Figure 2.2A), the TagRFP family of FPs are very prone to dimerization/oligomerization (Costantini et al., 2012; Costantini et al., 2015). Therefore, it is plausible that yomTagBFP2 strong tendency to oligomerize prevents expanded Httex1 interaction either with other Httex1 molecules or with other cellular components required for incorporation of polyQ proteins into IBs such as the prion protein Rnq1 or chaperone proteins (Duennwald et al., 2006b; Meriin et al., 2002).

It was previously shown that polyQ-expanded Httex1 proteins are recruited into preexisting IBs formed by non-toxic Httex1 expansion reporters containing the proline-rich domain adjacent to the polyQ repeats, irrespective of the flanking sequences (Duennwald et al., 2006b). Thus, we used the previously published construct encoding a non-toxic 103Q containing the Httex1 proline-rich domain tagged with eGFP (103QP-GFP) under the constitutively active GPD promoter (Duennwald et al., 2006b) to test whether yomTagBFP2 tagged constructs could be recruited into preexisting IBs induced by other Httex1 fusion constructs. We observed strong recruitment of both 25Q and 72Q-yomTagBFP2 into 103QP-GFP IBs upon induction in galactose (Figure 2.5A). Moreover, recruitment of 72Q-yomTagBFP2 in IBs results in increased toxicity compared to 25Q (Figure 2.5B). These observations were not solely the result of the increased total amount of Httex1 proteins since expression of 72Q-yomTagBFP2 from a multicopy plasmid failed to induce IBs or cause significant toxicity (Figure 2.6A and B). Another possibility would be that 103Q Httex1 has a greater affinity for 72Q and can override the yomTagBFP2 tendency to oligomerize. However, expression of a 103Q-yomTagBFP2 construct did not result in either IBs formation or toxicity (Figure 2.6C). Interestingly, IBs induced in the presence of 103QP-GFP were equally bright for both and ymoxBFP (Figure S2.5). This might be an indication that the combination of both a longer 103Q
Htt\textsuperscript{ex1} and the presence of GFP are required to alleviate the phenotype caused by yomTagBFP2.
Figure 2.5 Htt\textsuperscript{ex1}-yomTagBFP2 oligomers are recruited to preexisting 103Q-PRO-GFP IBs.  
Cells constitutively expressing 103Q-PRO-GFP were grown in galactose containing media overnight for induction of the various Htt\textsuperscript{ex1}-yomTagBFP2/moxBFP constructs. (A) Representative fluorescent images show incorporation of either Htt\textsuperscript{ex1}-yomTagBFP2 or ymoxBFP constructs into 103Q-PRO-GFP IBs. (B) Spot assays on agar plates reveal increased toxicity of 72Q- yomTagBFP2 when incorporated in 103Q-PRO-GFP IBs.
Figure 2.6 Effects of yomTagBFP2 on Htt\textsuperscript{ex1} toxicity and aggregation.

(A) High expression of 72Q Htt\textsuperscript{ex1}-yomTagBFP2 is not toxic. Representative fluorescent images of yomTagBFP2 and ymsfGFP tagged 25Q and 72Q Htt\textsuperscript{ex1} constructs expressed from high copy 2\mu vectors following overnight induction in galactose media.

(B) Growth assays on agar plates show that unlike for ymsfGFP, no significant toxicity of different 72Q-yomTagBFP2 Htt\textsuperscript{ex1} compared to the 25Q version.

(C) Growth assay on agar plate and fluorescent imaging shows that Htt\textsuperscript{ex1}-yomTagBFP2 is not toxic and remain diffused in the cytoplasm even in presence of 103Q.
These results indicate that while Htt$^{ex1}$-yomTagBFP2 constructs are unable to form IBs when expressed alone, they can be recruited by Htt$^{ex1}$ proteins tagged with other FPs (Figure 2.4C). Based on these data, it appears that the affinity of yomTagBFP2 is stronger than Htt$^{ex1}$. yomTagBFP2 oligomerization may prevent assembly of Htt$^{ex1}$ into toxic oligomers and IBs by either preventing interaction between different Htt$^{ex1}$ proteins or by potential sterically challenge recruitment of factors required for IBs formation such as Rnq1 (Figure 2.7). This strongly suggests that the inability of 72Q-yomTagBFP2 to form higher oligomers and incorporate into IBs is at the root of the decreased toxicity observed with this particular construct (Figure 2.2) Therefore, these results should be taken in considerations when using yomTagBFP2 and other red FPs and their derivatives for fusion proteins design.

Finally, it is important for scientists to appreciate the differences between the various properties of FPs when designing fusion proteins in experiments. In Figure 2.8, we compared side-by-side the complete panel of polyQ constructs used in our study. For each fluorescent fusion, imaging conditions were kept the same to acquire images of 25 and the 72Q variants (Figure 2.8). A negative control was also included to compare the fluorescent intensity of the different constructs over the autofluorescent background of W303 yeast cells. These experiments revealed interesting phenotypes regarding the 25Q Htt$^{ex1}$ fusions. While ymsfGFP, ymNeonGreen and yemRFP displayed robust signal, the CFP tagged version showed a much dimmer signal, closer to the background noise. Also, CFP tagged 25Q was much dimmer than the 72Q-CFP. An opposite phenotype was observed with the optimized FPs. These data highlight the advantages of using bright codon optimized FPs which greatly improved detection of the soluble 25Q Htt$^{ex1}$ fusions.
Figure 2.7 Hypothetical model of regulation of polyQ toxicity by fluorescent proteins.
While Htt^{ex1}-yomTagBFP2 cannot form IBs on their own, the can be recruited to preexisting IBs suggesting that the fluorescent proteins impairs Htt^{ex1} interaction with cellular component that regulate IBs formation.
Figure 2.8 Comparison of fluorescent intensities of the different Htt\textsuperscript{ex1} fusions. Htt\textsuperscript{ex1} constructs generated contain either non-toxic 25Q or HD-associated 72Q followed by either a CFP, ymsfGFP, yemRFP, ymNeonGreen, yom-TagBFP2 or ymoxBFP fluorescent proteins. For each fusion reporter, the imaging parameters were kept the same to acquire the images of W303 cells expressing either an empty vector, the 25Q- Htt\textsuperscript{ex1} or 72Q Htt\textsuperscript{ex1} fluorescent constructs.
2.4.4 Conclusion

Our study shows that Htt<sup>ex1</sup> polyQ toxicity assays in yeast can be used to detect variations among fluorescent protein fusions and could therefore be useful for development and optimization of current and future FPs. Our results using blue fluorescent proteins clearly show that there is no single FP that fits all the requirement for every experiments. Thus, we encourage scientists to use technical resources available to guide them though fluorescent proteins design (Costantini and Snapp, 2013; Costantini et al., 2012; Costantini et al., 2015; Cranfill et al., 2016; Lee et al., 2013; Snapp, 2009). In addition, our new palettes of optimized fluorescent Htt<sup>ex1</sup> constructs could be used for optimal imaging conditions to study Htt<sup>ex1</sup> aggregation in living cells. Finally, our study supports a role for sequences flanking the polyQ region in the toxicity observed in HD. Together with previous reports (Atwal et al., 2011; Caron et al., 2013; Duennwald et al., 2006a; Tam et al., 2009), our results suggest that targeting these regions may represent a suitable approach in modulating Htt toxicity in HD.
Figure S 2.1 High expression of 72Q Htt<sup>ex1</sup> is not toxic in absence of a fluorescent protein tag.
Cells expressing either 25Q or 72Q Htt<sup>ex1</sup>-stop from a 2µ plasmid were cultured on galactose overnight and spotted on agar plates.

Figure S 2.2 Specificity of the dot blot approach.
Whole lysates of yeast cells expressing either an empty vector W303 cells expressing an empty vector or the 25/72Q- Htt<sup>ex1</sup> either untagged or tagged with CFP constructs were processed for dot blotting using an anti-FLAG and anti PGK1 antibodies.
Figure S 2.3 *RNQ1* is required for aggregation and toxicity of the various fluorescent 72Q Htt\textsuperscript{ex1} constructs.

(A) Representative fluorescent images of fluorescently tagged 25Q and 72Q Htt\textsuperscript{ex1} constructs expressed in *rnq1Δ* cells following overnight induction in galactose media.

(B) Growth assay on agar plates shows no toxicity of different 72Q tagged Htt\textsuperscript{ex1} constructs compared to the 25Q version.
Figure S 2.4 CFP and ymsfGFP tagged Htt\textsuperscript{ex1} are equally toxic.
Growth assays on agar plates show no significant difference in toxicity between the CFP and ymsfGFP tagged 72Q Htt\textsuperscript{ex1}. Both are significantly more toxic than their 25Q counterpart.

Figure S 2.5 Fluorescent intensities of IBs induced by blue fluorescent proteins in cells expressing 103QP-GFP.
Plot of quantified individual cell mean fluorescent intensities is shown for comparison for cells expressing either 25Q or 72Q-yomTagBFP2 or ymoxBFP in presence of 103QP-GFP.
2.5 References


Costantini, L. M., Subach, O. M., Jaureguiberry-bravo, M., Verkhusha, V. V. and


Chapter 3

3 Polyglutamine toxicity assays highlight the advantages of mScarlet for imaging in Saccharomyces cerevisiae

The yeast polyQ model has been shown to be a good tool for assessing new fluorescent proteins. Although polyQ-yemRFP forms aggregates in yeast, it shows reduced toxicity. In this chapter, we will tag polyQ with newest red fluorescent proteins FusionRed and mScarlet to test their impact on fusion partners and on the other hand, generating an optimized yeast model of HD featured RFP.

3.1 Abstract

Development of fluorescent proteins (FPs) enabled researchers to visualize protein localization and trafficking in living cells and organisms. The extended palette of available FPs allows simultaneous detection of multiple fluorescent fusion proteins. Importantly, FPs are originally derived from different organisms from jelly fish to corals and each FP displays its own biophysical properties. Among these properties, the tendency of FPs to oligomerize inherently affects the behavior of its fusion partner. Here we employed the budding yeast Saccharomyces cerevisiae to determine the impact of the latest generation of red FPs on their binding partner. We used a yeast assay based on the aggregation and toxicity of misfolded polyQ expansion proteins linked to Huntington’s disease. Since polyQ aggregation and toxicity are highly dependent on the sequences flanking the polyQ region, polyQ expansions provide an ideal tool to assess the impact of FPs on their fusion partners. We found that unlike what is observed for green FP variants, yemRFP and yFusionRed-tagged polyQ expansions show reduced toxicity. However, polyQ expansions tagged with the bright synthetically engineered ymScarlet displayed severe polyQ toxicity. Our data indicate that ymScarlet might have significant advantages over the previous generation of red FPs for use in fluorescent fusions in yeast.

3.2 Introduction

Following the development of the green fluorescent protein (GFP) from the jellyfish Aequorea Victoria (Chalfie et al., 1994), several other FPs with various spectral
properties have been characterized (Thorn, 2017), allowing simultaneous detection of multiple fluorescent reporters. Among the most popular alternatives to GFP are the red fluorescent proteins (RFPs) isolated from Anthozoa coral and anemone species. One of the drawbacks of RFPs is that Anthozoa derived FPs are obligate tetramers (Baird et al., 2000; Verkhusha and Lukyanov, 2004). While development of RFPs into monomeric versions has been successful, it is often associated with reduced brightness of the fluorescent signal (Campbell et al., 2002) and therefore reduced overall performance of the resulting monomeric FPs. Moreover, RFPs such as TagRFP and mRuby2 reported as monomeric by passing purified proteins through sizing columns still display high tendency to oligomerize in living mammalian cells (Costantini et al., 2012; Costantini et al., 2015). Thus, under specific circumstances, FPs reported as monomeric can still be prone to oligomerization. Unwanted formation of oligomers could potentially significantly alter the function/localization of the protein of interest fused to the FP and render reporters unreliable (Costantini et al., 2015; Snapp et al., 2003; Zacharias et al., 2002). Indeed, various RFPs (mCherry, mKate2, mRuby, mKO2, mApple, TagRFP-T) have been shown to have differential effects on localization of cdc12 in yeast (Lee et al., 2013). Thus, being able to assess the behavior of fluorescent reporter in a given organism and/or cellular compartment is critical to help optimize fluorescent reporter design (Snapp, 2009).

We recently established a method to rapidly compare the behavior of FPs against a monomeric variant of superfolder GFP (msfGFP) in yeast (Jiang et al., 2017). The assays exploit the ability of polyglutamine expansions associated with Huntington’s disease (HD) to form toxic aggregates in yeast cells. The cause of HD can be traced back to abnormal expansion of a polyQ stretch within the first exon of the gene encoding the Huntingtin protein (Httex1) resulting in chorea and cognitive defects in patients (Gusella and MacDonald, 1995; Huntington, 2003; Penney et al., 1997). Expansion over 36 repeats is known to cause the Htt protein to misfold and aberrantly accumulate into detergent-insoluble amyloid-like aggregates in the cytoplasm of striatal neurons (Penney et al., 1997). Expression of expanded Httex1 in yeast results in severe polyQ aggregation and growth defect (Duennwald, 2013; Krobitsch and Lindquist, 2000; Mason and Giorgini, 2011; Meriin et al., 2002). Interestingly, the nature of the sequences flanking the polyQ
regions (in this case fluorescent or epitope tags) greatly affects the propensity of the polyQ expansions to aggregate and to display significant growth defects in yeast (Duennwald et al., 2006). Using polyQ toxicity assays in yeast, we previously showed that a yeast-optimized version of mCherry termed yemRFP (Keppler-Ross et al., 2008) displays only a mild growth defects compared to yeast-optimized msfGFP (ymsfGFP) (Jiang et al., 2017). These results lead us to exploit the polyQ toxicity and aggregation assays to explore the effects of two of the most recently available RFPs. Here, we focused on FusionRed, a red monomeric fluorescent variant of mKate2 known for its low cytotoxicity in cells (Shemiakina et al., 2012) that displays low propensity to oligomerize in mammalian cells (Costantini et al., 2015). We also included mScarlet, a monomeric synthetic RFP that was recently shown to outperform other RFPs in terms of brightness of the fluorescent signal (Bindels et al., 2017). Both have yet to be characterized for expression in yeast.

3.3 Materials and Methods

3.3.1 Yeast strains and culture conditions

All strains are derived from W303-1A (Thomas and Rothstein, 1989). All experiments were conducted in synthetic complete media (SC) at 30 °C.

3.3.2 DNA constructs

yemRFP (Keppler-Ross et al., 2008) was previously described. yFusionRed and ymScarlet were codon optimized for expression in yeast and synthesized by Genscript Inc. based on previously published sequences (Bindels et al., 2017; Shemiakina et al., 2012). RFPs were cloned into the SpeI/Sall site of p415 GPD. Alternatively, RFPs were cloned into the SpeI/SalI sites of p415 GAL1 25Q/68Q Httex1 lacking the proline rich domain, as previously described (Jiang et al., 2017). To generate 2µ vectors, the GAL1 25Q/68Q Httex1-ymsfGFP or GAL1 25Q/68Q Httex1-yFusionRed fragments were cloned into the SacI/SalI sites of pRS42N (Taxis and Knop, 2006). All Httex1 constructs lack the proline-rich domain since absence of this domain is required for Httex1 toxicity in yeast (Duennwald et al., 2006). We also noted that since the publication of our previous study
(Jiang et al., 2017), the original 72Q Htt<sup>ext</sup> plasmid has mutated into 68Q. We, therefore, used the latter in this study. See Table 3.1 for a list of plasmids used in this study.

### Table 3.1 Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Resistance marker</th>
<th>Source</th>
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<tbody>
<tr>
<td>P415 GPD</td>
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<td>(Mumberg et al., 1995)</td>
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<tr>
<td>P415 GPD-yemRFP</td>
<td></td>
<td>This study</td>
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<tr>
<td>P415 GPD-yFusionRed</td>
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<td>P415 GPD-ymScarlet</td>
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</tr>
<tr>
<td>P415 Gal1-FLAG-25Q-ymsfGFP</td>
<td></td>
<td>(Jiang et al., 2017)</td>
</tr>
<tr>
<td>P415 Gal1-FLAG-68Q-ymsfGFP</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>P415 Gal1-FLAG-25Q-yemRFP</td>
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<td>(Jiang et al., 2017)</td>
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<tr>
<td>P415 Gal1-FLAG-68Q-yemRFP</td>
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<td>P415 Gal1-FLAG-25Q-yFusionRed</td>
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<td>P415 Gal1-FLAG-68Q-yFusionRed</td>
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<td>PRS42N Gal1-FLAG-68Q-yFusionRed</td>
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#### 3.3.3 Growth assays

Yeast growth was measured by spotting assay on agar plates. Briefly, cells were cultured overnight to saturation in appropriate selection media. The next day, cells densities were equalized to OD<sub>600</sub> 0.2 and 5x serial dilutions were spotted on agar plates. Alternatively, cell densities were equalized to OD<sub>600</sub> 0.1 and 300 µl of cell suspensions were transferred into a 96 well plate and incubated at 30°C for 24h with constant shaking in a Biotek Epoch 2 microplate spectrophotometer and OD<sub>600</sub> was recorded every 15 minutes.
3.3.4 Dot blot

After induction in galactose media overnight, cells were lysed using glass beads in lysis buffer (100 mM Tris pH 7.5; 200 mM NaCl; 1 mM EDTA; 5% glycerol, 1 mM Dithiothreitol (DTT) 4 mM phenylmethanesulfonyl fluoride (PMSF) and protease inhibitor cocktail). Equal amount of proteins were spotted on a nitrocellulose membrane. Membranes were blocked for 30 min in PBS-0.05%Tween at room temperature and then processed for immunoblot. Membranes were probed with anti-FLAG primary antibody (Sigma F3040, 1:5000 dilution) overnight at 4 °C and subsequently with a secondary anti-mouse fluorescent antibody (Thermo Alexa 555 #A21424, 1:5000 dilution) for 1h at room temperature and imaged using a Bio-Rad ChemiDoc MP imaging system. Membranes were then stripped using the Gene Bio-Application stripping buffer and reprobed with an anti-Pgk1 primary antibody (Thermo 22C5D8) using the same secondary antibody. In Figure 3, for each individual antibody, both membranes were imaged simultaneously to allow direct comparison of fluorescent signal. Densitometric analysis was performed using Image J.

3.3.5 Fluorescence microscopy

Under the different experimental conditions, cells were diluted 10x in growth media and plated in Lab-tek (Thermo Inc.) imaging chambers and processed for fluorescence microscopy. Images in Figure 3.1 were acquired using a Zeiss AxioVert A1 wide field fluorescence microscope equipped with a 63X NA 1.4 Plan Apochromat objective, a 560 to 600 nm excitation/630 to 705 nm emission bandpass filter and Zeiss Axiocam 506 mono camera. Images presented in Figure 3.2 and Figure 3.4 were collected using a Zeiss 800 confocal microscope equipped with 488 nm and 561 nm diode lasers and a 63x PlanApochromat NA 1.4 objective.

3.3.6 Flow cytometry

Cell were cultured with appropriate media and processed for flow cytometry using a BD Bioscience FACS Celesta flow cytometer equipped with a 561 Yellow laser for imaging of RFPs. Data were analyzed using the BD FACS Diva software. All conditions were
performed in triplicates, 20,000 cells were analyzed and median fluorescence intensities were calculated. No gates were applied.

3.3.7 Statistical analysis

A one-way ANOVA followed by a multiple comparison test (Tukey’s or Dunnett’s according to figure legends) was used to determine statistical significance between the different experimental conditions in Figure 3.1D, 3.2B, 3.3 and 3.4A using GraphPad Prism v6.0h.

3.4 Results and Discussion

3.4.1 mScarlet has higher fluorescent intensity in yeast

To analyze the performance of the three different RFPs in yeast, we first generated codon optimized versions of both FusionRed and mScarlet (termed yFusionRed and ymScarlet, respectively) (Table 3.2). Centromeric plasmids encoding yFusionRed, yemRFP and ymScarlet under the control of the constitutive GPD promoter were transformed in yeast (Figure 3.1A). Fluorescence intensities were compared using wide-field fluorescence microscopy (Figure 3.1B). Median fluorescence intensity (MFI) was then quantified using flow cytometry. Quantification revealed that yFusionRed was significantly dimmer (~5x) than yemRFP (Figure 3.1C and D). This result was surprising given that previously published data reported a slightly increased brightness for FusionRed when compared to mCherry (Shemiakina et al., 2012). However, it is known that fluorescent brightness of FPs expressed in yeast can be different from the ones registered for pure purified proteins (Lee et al., 2013). As opposed to yFusionRed, ymScarlet displayed the strongest fluorescent signal (~2x brighter than yemRFP) (Figure 3.1C and D). These results are in agreement with previous studies reporting increased brightness of mScarlet compared to other RFPs variants (Bindels et al., 2017). Based on the intensity of the fluorescent signal, ymScarlet appears to be the optimal RFP for imaging in yeast.
### Table 3.2 Sequences of yeast optimized fluorescent proteins in this study

<table>
<thead>
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<th>Name</th>
<th>Sequences</th>
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<td>yFusionRed</td>
<td>ATGGTTTCTGAATTTGAAAAGAAACATGCCATGAAAGTTTGTAACA TGGAAAGTGTCATTTCCATATCTTTAAATGTACATCAAGAGG TGAAGGTTAAACCATACGAAATGTAACATTTAATGAAGTTGATAATCTTTGCAATTTTGGGCAACTT CTTTTATGTCAGTCTCAAAGAACCCTTTTATTTAAAGCACTCACCAGGATTATTCCAGATTTCTTTTTAAATTTTTTCATTTTGGGAAAGCTTCTTTCCAGAAGTACTTTTACGTTACAATCTACAGGTGGTGTGCTGAGG TGTTAATTTTAAAGAAAGCAGATGATGAAAGTTGCTGTTGCAAGATACTCTACTACAGGTGGTGTGCTGAGG TGTTAATTTTAAAGAAAGCAGATGATGAAAGTTGCTGTTGCAAGATACTCTACTACAGGTGGTGTGCTGAGG TGTTAATTTTAAAGAAAGCAGATGATGAAAGTTGCTGTTGCAAGATACTCTACTACAGGTGGTGTGCTGAGG</td>
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<tr>
<td>ymScarlet</td>
<td>ATGGTTTCTAAAGGTGAAAGCAGTTATTTAAAGGAAATTCATGAGATTCA AGGTACACATGGAAGGAGATGTAAACTTTAATGAAATGTCAGAACACATTTTGGGAAAGCTTCTTTCCAGAAGTACTTTTACGTTACAATCTACAGGTGGTGTGCTGAGG TGTTAATTTTAAAGAAAGCAGATGATGAAAGTTGCTGTTGCAAGATACTCTACTACAGGTGGTGTGCTGAGG TGTTAATTTTAAAGAAAGCAGATGATGAAAGTTGCTGTTGCAAGATACTCTACTACAGGTGGTGTGCTGAGG</td>
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Figure 3.1 Comparison of red fluorescent proteins (RFPs) fluorescent intensities in yeast.

(A) yemRFP, yFusionRed and ymScarlet were introduced into centromeric vectors under the control of a constitutive GPD promoter. (B) Representative images from 3 fields of yeast cells expressing different RFPs. Imaging conditions were kept constant between samples to allow direct comparison of fluorescent intensities. Inverted black and white images are shown for clarity. Bar: 5µm (C) Yeast cells expressing the different RFPs were analyzed by flow cytometry and compared to cells carrying an empty vector. (D) Median fluorescent intensities of the various RFPs were calculated from fluorescent data acquired using flow cytometry. *p<0.05.
3.4.2 PolyQ-mScarlet displays a toxic phenotype

Next, we sought to determine how the three different FPs affect their fusion partners in living yeast. To this end, we employed the polyQ toxicity assays. Each RFP was cloned in frame with a galactose inducible version of Htt\textsuperscript{ex1} carrying either 25Q (non-pathological length) or 68Q (HD-associated) (Figure 3.2A). 25Q constructs show no growth differences across the different FPs in both uninduced (glucose media) and polyQ-induced (galactose media) conditions indicating that expression of the different constructs results in similar growth phenotypes. When fused to 68Q Htt\textsuperscript{ex1}, yFusionRed displayed no significant toxicity when compared to the non-toxic 25Q fusion (Figure 3.2B). Interestingly, ymScarlet displayed severe toxicity, showing a slow growth phenotype comparable to what was observed for ymsfGFP (Figure 3.2B). Based on these observations, we then investigated the effects of the different FPs on polyQ aggregation using fluorescence microscopy. We found that yemRFP displayed robust 68Q aggregation similar to ymsfGFP as we previously described (Jiang et al., 2017). It is important to note that while prone to aggregation, yemRFP polyQ proteins were shown to form aggregates with different biophysical properties (increased detergent solubility) that can account for their moderately toxic nature (Jiang et al., 2017). In accordance with the absence of toxicity noted in the growth assay, 68Q-FusionRed did not form visible aggregates, while ymScarlet displayed strong aggregation propensity (Figure 3.2C).
Figure 3.2 ymScarlet displays a toxic polyQ phenotype similar to ymsfGFP.

(A) FPs were cloned in frame with FLAG-Httex1 into a centromeric vector carrying a GAL1 inducible promoter. (B) Yeast cells carrying a vector or 25/68Q Httex1 fused to either ymsfGFP, yemRFP, yFusionRed or ymScarlet were grown to saturation overnight in glucose (control) or galactose (polyQ induced) containing media. The next days, cell concentrations were equalized to OD$_{600}$ 0.2 and 5 fold serial dilutions of the cell suspension spotted on synthetic complete agar media plates containing either glucose or galactose. (C) Yeast cells carrying an empty vector or 25/68Q Httex1 fused to ymsfGFP, yemRFP, yFusionRed or ymScarlet or carrying an empty vector were induced overnight in galactose containing media and protein levels analyzed by dot blot using either an anti-FLAG (detection of fluorescent fusions) or anti-Pgk1 antibody (loading control). (D) Representative fluorescent images from 3 fields of yeast cells expressing 25/68Q Htt ex1 fused to ymsfGFP, yemRFP, yFusionRed or ymScarlet after overnight induction in galactose-containing media.
In addition, assessment of protein abundance for each construct using dot blot revealed that both 25 and 68Q yFusionRed fusions were present at lower levels compared to other fluorescent counterparts (Figure 3.3A). The cause of this phenotype is unclear and could result from increased turnover rate of the fusions. Interestingly, expression of 68Q-yFusionRed from a multicopy 2µ vector resulted in a growth defect, albeit toxicity was reduced compared to ymsfGFP-tagged polyQ (Figure 3.4A). Moreover, under these conditions, 68Q-yFusionRed displayed robust aggregation. This indicates that the lower expression levels observed for yFusionRed constructs can potentially explain, at least partially, the absence of polyQ toxicity when expressed at lower levels. Reduced toxicity of the 68Q-yFusionRed is also consistent with our previous observation showing that yomTagBFP2, a blue fluorescent proteins similarly does not form toxic aggregates (Jiang et al., 2017). In fact, both FusionRed and mTagBFP2 (Subach et al., 2011) are evolved versions of the wild-type RFP from sea anemone Entacmaea quadricolor (Merzlyak et al., 2007). In the case of mScarlet, the protein was evolved from a synthetic template design for generating a monomeric protein. Therefore, based on our data, mScarlet appears to be an attractive alternative to mCherry, which minimizes the effect of the FP on its fusion partner.
Figure 3.3 yFusionRed-tagged Htt<sup>ex1</sup> fusions are expressed at lower levels compared to other fluorescent fusions.

Yeast cells carrying an empty vector or 25/68Q Htt<sup>ex1</sup> fused to ymsfGFP, yemRFP, yFusionRed or ymScarlet or carrying an empty vector were induced overnight in galactose containing media and protein levels analyzed by dot blot using either an anti-FLAG (detection of fluorescent fusions) or anti-Pgk1 antibody (loading control). Quantification of the FLAG/Pgk1 ration is shown from 3 independent experiments. ***p<0.001 according to a one way ANOVA followed by a Tukey’s multiple comparison test comparing samples to ymsfGFP-tagged fusion of the same polyQ length.
Figure 3.4 Expanded Httex1-yFusionRed is toxic when expressed at high levels.  
(A) Images of yeast growth assays on agar plates. Yeast cells carrying 25/68Q Httex1 fused to either ymsfGFP or yFusionRed in a 2µ multicopy vector were grown to saturation overnight in glucose (control). The next days, cell concentrations were equalized to OD600 0.2, and 5-fold serial dilutions of the cell suspension spotted on synthetic complete agar media plates containing either glucose (control) or galactose (+polyQ). Alternatively, cells were cultured in liquid media and optical densities were recorded over time to generate growth curves. The area under the curve (AUC) was calculated from 3 experimental replicates. **p<0.01 and ****p<0.0001 according to a one way ANOVA followed by a Tuckey’s multiple comparison test comparing the 68Q sample to its 25Q counterpart unless indicated otherwise.  
(B) Representative fluorescent images from 3 fields of yeast cells expressing 25/68Q Htt Httex1 fused to ymsfGFP or yFusionRed from a multicopy 2µ vector after overnight induction in galactose-containing media. Under these conditions, 68Q-yFusionRed displays robust aggregation.
3.5 References


Chapter 4

Traffic, 2019 (4) cover image
4 Sfp1 links TORC1 and cell growth regulation to the yeast SAGA-complex component Tra1 in response to polyQ proteotoxicity

In the past two chapters, we introduced the optimized yeast model of HD by tagging mutant Htt<sup>ex1</sup> with different modern FPs. With the help of the yeast polyQ model, we are able to investigate the fundamental cellular mechanisms during HD. Proteotoxic stress disrupts trafficking and triggers transcriptional responses to cope with the accumulation of misfolded proteins. Transcriptional defects have been observed in different models of HD. The mechanisms involve dysregulation of transcription factors, coactivators, RNA polymerase II, and importantly, histones. In this chapter, we show that cells expressing toxic polyQ expansions display impaired assembly of the SAGA acetyltransferase complex that regulates chromatin remodeling and gene expression during stress. This culminates in the Sfp1/TORC1-dependent increased expression of the SAGA component Tra1. Our data identify a new nexus that links TORC1 to the regulation of SAGA activity during loss of proteostasis.

4.1 Abstract

Chromatin remodeling regulates gene expression in response to the accumulation of misfolded polyQ proteins associated with Huntington’s disease (HD). Tra1 is an essential component of both the SAGA/SLIK and NuA4 transcription co-activator complexes and is linked to multiple cellular processes, including protein trafficking and signaling pathways associated with misfolded protein stress. Cells with compromised Tra1 activity display phenotypes distinct from deletions encoding components of the SAGA and NuA4 complexes, indicating a potentially unique regulatory role of Tra1 in the cellular response to protein misfolding. Here, we employed a yeast model to define how the expression of toxic polyQ expansion proteins affects Tra1 expression and function. Expression of expanded polyQ proteins mimics deletion of SAGA/NuA4 components and results in growth defects under stress conditions. Moreover, deleting genes encoding SAGA and, to a lesser extent, NuA4 components exacerbates polyQ toxicity. Also, cells carrying a
mutant Tra1 allele displayed increased sensitivity to polyQ toxicity. Interestingly, expression of polyQ proteins upregulated the expression of \(TRA1\) and other genes encoding SAGA components, revealing a feedback mechanism aimed at maintaining Tra1 and SAGA functional integrity. Moreover, deleting the TORC1 effector \(SFP1\) abolished upregulation of \(TRA1\) upon expression of polyQ proteins. While Sfp1 is known to adjust ribosome biogenesis and cell size in response to stress, we identified a new role for Sfp1 in the control of \(TRA1\) expression, linking TORC1 and cell growth regulation to the SAGA acetyltransferase complex during misfolded protein stress.

4.2 Introduction

Eukaryotic cells need to correctly fold proteins to ensure their accurate function and avoid the aggregation of toxic misfolded intermediates, which form the basis of several human diseases (Balch et al., 2008; Henning and Brundel, 2017; Klaips et al., 2018; Ruegsegger and Saxena, 2016). In Huntington’s disease (HD), expansion of a polyglutamine region encoded by the first exon of the gene encoding the Huntingtin protein (Htt
\[ex1\]) leads to Htt misfolding and aggregation in detergent-insoluble, amyloid-like inclusion bodies (IBs) in the cytoplasm and nuclei of neuronal cells (Gusella et al., 2014; Huang et al., 1998; Telenius et al., 1994). Accumulation of toxic polyQ oligomers is linked to several cellular dysfunctions such as defects in protein trafficking, autophagy and endoplasmic reticulum stress (Ashkenazi et al., 2017; Duennwald and Lindquist, 2008; Jiang et al., 2016; Koga et al., 2011; Lajoie and Snapp, 2011; Ravikumar et al., 2004; Vidal et al., 2012). In response to the accumulation of misfolded proteins, including polyQ huntingtin, cells modify their gene expression profile to favor adaptive responses directed at restoring protein homeostasis (Becanovic et al., 2010; Neueder and Bates, 2014; Tauber et al., 2011). Well-characterized responses to proteotoxic stress, such as the unfolded protein response of the endoplasmic reticulum (Duennwald and Lindquist, 2008; Haynes et al., 2004; Lajoie and Snapp, 2011; Leitman et al., 2014; Vidal and Hetz, 2012; Vidal et al., 2012; Walter and Ron, 2011) and the heat shock response (Bersuker et al., 2013; Chafekar and Duennwald, 2012; Fujikake et al., 2008; Verghese et al., 2012) in the cytoplasm, increase the folding capacity of their respective compartments upon accumulation of misfolded polyQ expansions. These responses prevent the protein quality
control machinery from being overwhelmed by sudden changes in the misfolded protein burden.

It is now clear that multiple signaling pathways act in parallel to regulate gene expression during misfolded protein stress. Acetyltransferase complexes regulate chromatin remodeling, a process affected in HD (Dong et al., 2015; Guiretti et al., 2016; McFarland et al., 2012; Sharma and Taliyan, 2015; Steffan et al., 2001; Valor et al., 2013). The SAGA (Spt-Ada-Gcn5-Acetyltransferase) and NuA4 (Nucleosome acetyltransferase of H4) complexes were first identified in yeast as containing the lysine acetyltransferases Gcn5 and Esa1, respectively (Allard et al., 1999; Auger et al., 2008; Grant et al., 1997). Both complexes have homologues in mammalian cells, hSAGA and Tip60, respectively. The PIKK family member Tra1/TRRAP is an essential component of both SAGA and NuA4 complexes in yeast and mammalian cells (Ikura et al., 2000; McMahon et al., 1998). The group of PIKK proteins also includes mTOR, ATM and ATR, which are characterized by a C-terminal PI3K domain (Baretić and Williams, 2014). In SAGA and NuA4, Tra1 interacts with transcriptional activators thereby recruiting the complexes to target promoters (Bhaumik et al., 2004; Brown et al., 2001; Fishburn et al., 2005; Reeves and Hahn, 2005). Because of its presence in both SAGA and NuA4, reducing Tra1 function affects cells distinctly from deletions of components specific to either individual complex. For example, impaired Tra1 function causes generation-dependent telomere shortening, a phenotype that is not detected in cells carrying deletions of either SAGA or NuA4 components (Mutiu et al., 2007b).

Misfolded polyQ expansions specifically alter the composition of the SAGA complex and SAGA-regulated gene transcription in both yeast and mammalian models (Burke et al., 2013; Chen et al., 2012; Helmlinger et al., 2004; Lan et al., 2015; McMahon et al., 2005; Yang et al., 2015). These studies employed polyQ-expanded ataxin-7/Sca7, which causes the neurodegenerative disease spinocerebellar ataxia 7 (Holmberg et al., 1998). Sca7/ataxin-7 is a component of SAGA and SLIK (SAGA-like) acetyltransferase complexes, explaining the effect of polyQ expanded-Sca7 on SAGA function (McMahon et al., 2005; Yang et al., 2015). Targeting Htt\textsuperscript{ext} to the yeast nucleus also alters transcription similarly to cells carrying deletions in genes encoding SAGA components.
(Hughes et al., 2001); however, the specific molecular mechanism by which Htt\textsuperscript{ex1} polyQ expansions affect SAGA function remains unclear. Our previous genetic screen for synthetic interactions linked Tra1 to the regulation of several stress responses, including protein misfolding stress (Hoke et al., 2008). Tra1 is therefore a strong candidate target to regulate the transcriptional response to protein misfolding stress caused by polyQ proteins.

To study the effect of Htt\textsuperscript{ex1} polyQ expansion on yeast, we employed a well-characterized model that involves expressing fluorescently-tagged Htt\textsuperscript{ex1} (Albakri et al., 2018; Duennwald, 2013; Duennwald et al., 2006a; Duennwald et al., 2006b; Jiang et al., 2017; Meriin et al., 2002). We define the interplay between Tra1 and polyQ-induced stress and identify the TORC1 effector Sfp1 as a regulator of polyQ toxicity that regulates Tra1 expression during proteotoxic stress. Our study thus expands the understanding of its role beyond the regulation of cell growth and ribosome biogenesis (Fingerman et al., 2003; Jorgensen et al., 2004; Marion et al., 2004) and defines how TORC1, Sfp1 and Tra1 respond to toxic polyQ proteins.

4.3 Materials and Methods

4.3.1 Yeast genetic manipulation and growth assays

All strains are derivatives of either BY4741/4742 or W303a (see Table 4.1). Gene deletions were performed using standard yeast genetics procedures (Janke et al., 2004) and validated by sequencing. Plasmids were transformed using the lithium acetate method (Gietz and Woods, 2002). Cell growth was assessed by both spot assay on agar plates and growth in liquid culture as previously described (Jiang et al., 2018). Yeast cells were cultured overnight in selective synthetic complete media with 2% glucose as a sole carbon source. For spot assays, cultures were diluted to equal concentrations and then spotted in 4 five-fold dilutions using a pinning tool with the most concentrated spot equalized at OD\textsubscript{600} 0.2. Cells were grown on selective plates at 30 °C for 2 days and imaged using a GelDoc system (Bio-Rad). For liquid culture, cells were diluted to OD\textsubscript{600} 0.1 and incubated at 30°C. OD\textsubscript{600} was measured every 15 min using a BioscreenC plate reader (Growth curves USA) for 24 hours. Growth curves were generated and the area
under the curve calculated for each biological replicates and a two-tailed Student’s \( t \)-test was used to determine statistical significance between the different experimental conditions using Graphpad (Prism). To generate the \( sfp1\Delta \ tra1_{Q3} \) double mutant, the \( sfp1\Delta \) and \( tra1_{Q3} \) (CY6586) were mated and diploids were selected on media containing G418 and lacking leucine. The diploid strain (CY8479) was sporulated in 3 mL 1% potassium acetate and tetrads were dissected. The haploid double \( sfp1 \ tra1_{Q3} \) strain (CY8482) was selected based on its ability to grow on media containing G418 and media lacking leucine and histidine.

### 4.3.2 Drugs

Stock solutions of tunicamycin (5 \( \mu \)g/ml in DMSO; Amresco), Trichostatin A (10 mM in \( \text{H}_2\text{O} \); Biovision), calcofluor white (30 mg/ml in \( \text{H}_2\text{O} \); Sigma-Aldrich), rapamycin (1 mg/ml in DMSO, Fisher Scientific), \( \text{H}_2\text{O}_2 \) (9.79 M) cycloheximide (10 mg/ml in \( \text{H}_2\text{O} \); Fisher Scientific), and MMS (99%; Acros Organics) were prepared and used at the indicated concentrations.

### 4.3.3 DNA constructs

Plasmids encoding fluorescently tagged Htt\({ }^{ex1} \) and LacZ reporter constructs carrying \( \text{TRA1, PHO5, and NCW2} \) (Berg et al., 2018; Mutiu et al., 2007a) promoters in YCp87 (Brandl et al., 1993) were previously described (see Table 4.2). \( \text{SPT7} \) promoter sequences relative to the translational start, -633 to +68, \( \text{NGG1} \) promoter sequences -430 to +5 and \( \text{EAF1} \) promoter sequences -890 to +31 were engineered by PCR as BamHI/HindIII fragments using oligonucleotides listed in Table 3 and cloned into YCp87 (Brandl et al., 1993) to generate transcriptional reporters. Vectors encoding fluorescently tagged Tra1 with yemRFP (Keppler-Ross et al., 2008) were generated by replacing the eGFP coding sequence by the new codon-optimized fluorescent proteins using the BamHI/NotI sites in the previously described eGFP-Tra1 vector (Genereaux et al., 2012) using primers listed in Table 4.3.
Table 4.1 Strains used in this study

<table>
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<th>Strains</th>
<th>Genotype</th>
<th>Reference</th>
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<tr>
<td>W303</td>
<td>MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15</td>
<td>(Thomas and Rothstein, 1989)</td>
</tr>
<tr>
<td>BY4741</td>
<td>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
<td>(Brachmann et al., 1998)</td>
</tr>
<tr>
<td>BY4742</td>
<td>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</td>
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<tr>
<td>W303 GAL-FLAG-25Q ΔPRO-CFP</td>
<td>Isogenic to W303 + GAL-FLAG-25Q ΔPRO-CFP</td>
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<tr>
<td>W303 GAL-FLAG-46Q ΔPRO-CFP</td>
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</tr>
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<td>W303 GAL-FLAG-72Q ΔPRO-CFP</td>
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<td>(Duennwald et al., 2006)</td>
</tr>
<tr>
<td>TS161</td>
<td>MATα ura3-52</td>
<td>(Takahara and Maeda, 2012)</td>
</tr>
<tr>
<td>TS184</td>
<td>MATα ura3-52 TOR L2134M</td>
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<td>BY4742 sch9Δ</td>
<td>Isogenic to BY4742 sch9Δ:: KAN</td>
<td>Thermo/deletion collection</td>
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<td>BY4742 sfp1Δ</td>
<td>Isogenic to BY4742 sfp1Δ:: KAN</td>
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</tr>
<tr>
<td>W303 sfp1Δ</td>
<td>Isogenic to W303 sfp1Δ:: NAT</td>
<td>This study</td>
</tr>
<tr>
<td>BY4741 eaf1Δ</td>
<td>Isogenic to BY4741, eaf1Δ:: KAN</td>
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<tr>
<td>BY4741 ada2Δ</td>
<td>Isogenic to BY4741, ada2Δ:: KAN</td>
<td>Thermo/deletion collection</td>
</tr>
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<td>yMS928</td>
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<td>(Lopez et al., 2011)</td>
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<td>CY4350</td>
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<td>(Hoke et al., 2010)</td>
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<td>CY8479</td>
<td>MATα/a SFP1/sfp1::KanMX tra1/tra1Δ-HIS3 YCplac111-DEDIp-YHR100C</td>
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<td>CY8482</td>
<td>Mato sfp1::KanMX tra1Δ-HIS3 YCplac111-DedIp-YHR100C</td>
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<td>CY6808</td>
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<tr>
<td>YSC1178-202230938</td>
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### Table 4.2 Plasmids used in this study

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<th>Plasmids</th>
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<td>MET-25Q ΔPRO-CFP</td>
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<td>pRS426</td>
<td>URA</td>
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<td></td>
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<td>GAL-FLAG-25Q ΔPRO-ymsfGFP</td>
<td></td>
<td>pPL005</td>
<td></td>
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<tr>
<td>GAL-FLAG-72Q ΔPRO-ymsfGFP</td>
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<td>pPL006</td>
<td>LEU</td>
<td>(Jiang et al., 2017)</td>
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<tr>
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<td>pRS415</td>
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<tr>
<td>GAL-FLAG-103Q ΔPRO-CFP</td>
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<td>pRS416</td>
<td>URA</td>
<td>(Duennwald et al., 2006)</td>
</tr>
<tr>
<td>TRA1-ymsfGFP</td>
<td>pPL022</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRA1-yemRFP</td>
<td>pPL023</td>
<td>pTZ19</td>
<td>URA</td>
<td>This study</td>
</tr>
<tr>
<td>TRA1-LacZ</td>
<td></td>
<td>YCp87</td>
<td></td>
<td>(Berg et al., 2018)</td>
</tr>
<tr>
<td>SPT7-LacZ</td>
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<td></td>
</tr>
<tr>
<td>NGG1-LacZ</td>
<td></td>
<td></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>EAF1-LacZ</td>
<td></td>
<td></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>PHO5-LacZ</td>
<td></td>
<td></td>
<td></td>
<td>(Mutiu et al., 2007)</td>
</tr>
<tr>
<td>NCW2-LacZ</td>
<td></td>
<td></td>
<td>LEU</td>
<td>(Berg et al., 2018)</td>
</tr>
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### Table 4.3 Primers used in this study

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<th>Reverse Primer</th>
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<td>U3</td>
<td>CCCAGAGTGAGAAACCGAAA</td>
<td>AGGATGGGGTCAAGATCATCG</td>
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<td>RPL6</td>
<td>GCGTGCCCCTCTCTAGTTCCAG</td>
<td>CGTTGACACCTTCGACAGAA</td>
</tr>
<tr>
<td>RPL30</td>
<td>ATCATTGCCGCTAAACACTCC</td>
<td>CCGACAGCAGTACCCAAATT</td>
</tr>
<tr>
<td>RPL38</td>
<td>GACGTAAAGACGGCCACTGT</td>
<td>AAGTTGTGGGCAAAGATGG</td>
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<tr>
<td>TRA1</td>
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<td>SPT7 promoter</td>
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<td>AACCTTCGTCATTAAGTA AAGCTTTG</td>
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<tr>
<td>NGG1 promoter</td>
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<td>GGGGAAGCTTCAGACATTATTC CTGCTCAATACTTCTGT</td>
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<tr>
<td>EAF1 promoter</td>
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<td>AAAGGAATCCACCGGAAGTCG TTCCACCT</td>
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<tr>
<td>ymsfGFP</td>
<td>CGGGATCCATGGTATCCCAAAG GTGAAGAAT</td>
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</tr>
<tr>
<td>YemRFP</td>
<td>CGGGATCCATGGTTCAAAAG GTGAAGAAG</td>
<td>ATAAAGATGCGGCGGCGCTTTAT ACAATTCATCCATACC</td>
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</table>
4.3.4 Fluorescence microscopy

Cells were diluted 10X and transferred to LabTek imaging chambers (Thermo Inc.) and imaged at room temperature. Fluorescence microscopy was performed using a Zeiss 800 confocal microscope equipped with a 63× Plan Achromat objective (1.4 NA). Images were analyzed using the ImageJ software (Schindelin et al., 2015).

4.3.5 qRT-PCR

RNA extraction was performed using MasterPure Yeast RNA Purification Kit (Lucigen). cDNA synthesis was done by qScript Flex cDNA Synthesis Kit (Quanta Bioscience). The cDNA preparations were used as the template for amplification using PerfeCTa SYBR-Green Supermix (Quanta Bioscience). The primers used are listed in supplemental Table 3. The relative expression level was calculated using the comparative Ct method and U3 was used as a reference gene.

4.3.6 Western blot

Yeast cells were lysed using 0.1 M NaOH for 5 min at room temperature, resuspended in SDS sample buffer and boiled for 5 min (Kushnirov, 2000). Proteins were separated using SDS-PAGE and transferred to PVDF membrane. The membrane was blocked with 5% milk. Then the membrane was incubated with anti-Flag (M2, Sigma-Aldrich), anti-PGK1 (Invitrogen), anti-histone H3 and anti-acetyl-histone H3K14 (Abcam) overnight, followed by 1 h incubation with the appropriate fluorescent secondary antibody and imaged with an Odyssey infrared imager (Licor) to detect the signal.

4.3.7 β-galactosidase assay

Cells were harvested and resuspended in LacZ buffer. β-galactosidase activity from 50 µl of cell lysate was calculated using o-nitrophenol-β-D-galactosidase as substrate and values were normalized to cell densities as previously described (Berg et al., 2018; Mutiu et al., 2007b).
4.3.8 Pull-down assay

Pull down was performed as previously described (Berg et al., 2018). 4L of cells were harvested and washed in IPP150 buffer, expelled into liquid nitrogen and grinded to powder. Lysates were resuspended in 1ml IPP150 buffer and spun at 40k rpm for 1h at 4 °C. The middle layer was extracted and then transferred to a column and mixed with 400 µl IgG agarose resin. The column was then rotated at 4 °C for 2 h before transfer to a new column with 250 µl TEV cleavage buffer and 3 µl TEV. The column was inverted manually every 15 min for 3 h. Proteins were then collected in a new column with calmodulin resin and rotated 1 h at 4 °C. Samples were eluted with calmodulin elution buffer. Finally, protein was mixed with SDS sample buffer and processed for gel electrophoresis followed by staining with coomassie blue and imaging with a GelDoc system (Bio-RAD).

4.3.9 Statistical analysis

Unless indicated otherwise, a Student’s t-test was used to determine statistical significance between the different experimental conditions using GraphPad Prism v6.0h.

4.4 Results

4.4.1 PolyQ expansions compromise the SAGA histone acetyltransferase complex

In our experiments, Htt\textsuperscript{ex1} is placed under the control of the \textit{GAL1} promoter and induced by growth in galactose as sole carbon source. Under these conditions, expressing HD-associated polyQ lengths 46Q and 72Q result in a polyQ length-dependent growth defect compared to the non-HD associated 25Q (Duennwald and Lindquist, 2008; Duennwald et al., 2006b; Duennwald et al., 2006a) (Figure 4.1A). 25Q does not cause a growth defect when compared to the corresponding empty vector (Supplemental Figure 4.1). As opposed to other disease-causing misfolded proteins, such as α-synuclein, polyQ expression inhibits cell growth but does not cause significant cell death as measured by either regrowth assays or labeling of dead cells with propidium iodide (Supplemental Figure 4.2). The effect of a 103Q Htt\textsuperscript{ex1} polyQ expansion is also apparent when expressed at lower levels under the transcriptional control of the relatively weak \textit{MET25} promoter
(Duennwald and Lindquist, 2008) (Figure 4.1B). This model allows testing low polyQ toxicity without altering the carbon source. High expression of polyQ expanded Htt\textsuperscript{ext} results in polyQ length-dependent formation of cytoplasmic aggregates that can be observed using fluorescence microscopy (Figure 4.1C).
Figure 4.1 PolyQ expansions are toxic and aggregate in yeast.

(A) The yeast model of HD as previously developed (Duennwald, 2013; Duennwald and Lindquist, 2008; Duennwald et al., 2006a). Cell expressing high levels of CFP-tagged Htt<sup>ex1</sup> driven by the GAL1 promoter display polyQ length-dependent toxicity. 25Q serves as a control for non-pathological Htt<sup>ex1</sup>. Cell growth was assessed by serial dilutions on SC plates containing either glucose (control) or galactose (polyQ induced).

(B) Cells expressing low levels of CFP-tagged Htt<sup>ex1</sup> driven by the MET25 promoter display only modest growth defect even in presence of 103Q. Cell growth was assessed by serial dilutions on SC plates containing presence (control) or absence (polyQ induced) of methionine.

(C) Fluorescent images show accumulation of inclusion bodies in 46Q and 72Q-expressing cells as opposed to diffused cytosolic distribution of 25Q after induction in galactose.
Misfolded polyQ proteins associated with the polyglutamine disease spinocerebellar ataxia disrupt assembly of the SAGA complex and SAGA-dependent transcription (McMahon et al., 2005). The ensuing phenotype resembles those associated with deletions of SAGA complex components. Sca7, the protein responsible, is a subunit of the hSAGA complex. Thus, its impact on SAGA function is expected. To investigate the relationship between Htt<sup>ex1</sup>, a protein that is not part of the acetyltransferase complexes, we examined genetic interactions of Htt<sup>ex1</sup> with deletions of NuA4 (<i>eaf1Δ, eaf6Δ, yaf9Δ</i>) and SAGA (<i>ada2Δ, spt8Δ, ubp8Δ</i>) components (Figure 4.2A). Expanded polyQ expression displayed increased growth defects with deletion of the SAGA components Ada2 and Ubp8, but not Spt8. This could indicate that specific Spt8 activities such as interaction of SAGA with the TATA-box binding protein (TBP) are not targeted by polyQ expansions (Mohibullah and Hahn, 2008; Warfield et al., 2004). 72Q expression affected growth in one of the NuA4 related deletions, <i>eaf6Δ</i>. To look at the effect of polyQ on a Tra1 mutant, we employed a previously characterized loss-of-function mutant of <i>TRA1</i> (<i>tra1-F3744A</i>) that carries a mutation in the C-terminal FATC domain (Hoke et al., 2010). <i>tra1-F3744A</i> displayed growth defects in presence of expanded 46 Q in both plate and liquid growth assays (Figure 4.2B). Supporting these data, polyQ expression sensitized cells to stresses that result in slow growth of strains carrying <i>TRA1</i> mutations, i.e. calcofluor white, growth at 39 °C, 5% ethanol, MMS and caffeine (Berg et al., 2018) (Figure 4.2C). Expression of expanded polyQ proteins was also associated with decreased activity from SAGA regulated promoters (<i>HIS4</i> and <i>PHO5</i>) but not from the SAGA-independent <i>NCW2</i> promoter (Figure 4.3A). Similar to the results of expanded PolyQ on SAGA function described by Hughes et al., 72Q reduced global acetylation of the SAGA modified histone H3 approximately to 60% of wild-type (Figure 4.3B). Global acetylation of the NuA4-modified histone H4 was reduced to a lesser extent, approximately 80% of wild-type (Figure 4.3B).
Figure 4.2 PolyQ enhanced toxicity in strains deleted for the SAGA components $UBP8$ and $ADA2$ as well as a $tra1$-$F3744A$ mutant strain. 

(A) Wild-type, $ada2\Delta$, $spt8\Delta$, $ubp8\Delta$ (SAGA) and $eaf1\Delta$, $eaf6\Delta$, $yaf9\Delta$ (NuA4) cells expressing either $GAL1$-inducible 25Q or 46Q Htt$^{\text{ex1}}$ assessed by growth assays on SC media plates in presence of glucose (control) or galactose (polyQ induced). (B) Wild-type and $tra1$-$F3744A$ cells expressing either $GAL1$-inducible 25Q or 46Q Htt$^{\text{ex1}}$ were assessed by growth assay on SC media plates and in liquid growth assay in presence of glucose (control) or galactose (polyQ induced). The area under the curve was quantified for each replicates ($n=3$) **$p<0.005$. (C) Cells expressing either $MET25$-inducible 25Q or 103Q Htt$^{\text{ex1}}$ were spotted on plates containing various stressors (0.1% caffeine, 6µg/ml calcofluor white (CFW), 5% ethanol (ETOH), 0.1% caffeine, 0.03% methyl methanesulfonate (MMS) or incubated at 39°C) in presence or absence of methionine.
Figure 4.3 PolyQ expansions reduce Tra1-regulated gene expression.

(A) Expression of GAL1-inducible 72Q induced a significant decrease in the expression from the SAGA-regulated genes HIS4 and PHO5. NCW2 is shown as a control. Gene expression was analyzed using LacZ transcriptional reporters and was assessed after overnight induction of polyQ in galactose under conditions that induced each promoter (absence of histidine or low phosphate respectively). **p<0.005, n=3±SEM.

(B) Reduced histone acetylation in cells expressing expanded polyQ. Immunoblot of total (H3 and H4) and acetylated histone H3 (H3-AcK14) and H4 (H4-AcK16) with or without expression of GAL1-inducible 25 and 72Q. *p<0.05, **p<0.005, n=3±SEM.
4.4.2 Accumulation of misfolded polyQ proteins increase TRA1 expression

We previously observed that Tra1 bearing mutations in its PI3K domain (tra1Q3) decrease nuclear localization of the protein (Berg et al., 2018). We therefore assessed whether expressing polyQ expansion affects Tra1 localization using confocal microscopy. We found that expanded polyQ expression (72Q-ymsfGFP) did not change the nuclear localization of fluorescently tagged Tra1 (yemRFP-Tra1) (Figure 4.4A). Consequently, polyQ possibly alters SAGA function independently of Tra1 sequestration into cytoplasmic polyQ inclusions, possibly by altering composition of the SAGA complex. We tested this possibility by pulling down SAGA using a TAP-tagged Ada2 in cells expressing either 25 or 72Q and analyzing the associated proteins by SDS-PAGE. As shown in Figure 4.4B Tra1 was still assembled into SAGA in strains expressing 72Q. However other components such as Ngg1 and Spt7 displayed reduced incorporation. Cells also respond to the defective tra1Q3 allele by increasing the transcription of the TRA1 gene (Berg et al., 2018). We assayed expression from the TRA1 promoter to evaluate whether expanded polyQ results in increased TRA1 expression. As shown in Figure 4.4A, we found a ~2.5 fold increase in TRA1 expression of a TRA1-promoter LacZ fusion upon expression of 72Q compared to the non-toxic 25Q. Increased transcription was also observed from promoters of other components of SAGA and NuA4, i.e. NGG1, SPT7 and EAF1, as was the case for tra1Q3 (Berg et al., 2018) (Figure 4.5A). We also observed that the polyQ-induced increase in TRA1 expression was abolished when cells expressing 72Q were treated with the HDAC inhibitor trichostatin A (TSA) (Figure 4.5B), suggesting that changes in chromatin remodeling regulate TRA1 expression in the presence of polyQ expansion. As a consequence of increased mRNA levels (as measured by qRT-PCR (Figure 4.5C), Tra1 protein abundance increased upon expression of 72Q (Figure 4.5D). These results suggest that expanded polyQ impairs Tra1 function inducing a feedback mechanism to cope with disrupted SAGA/NuA4 function. As shown in Figure 4.5E, the increased TRA1 expression was specific to polyQ and was not observed with other stressors that result in protein misfolding, such as induction of endoplasmic reticulum stress by tunicamycin, heat shock, oxidative stress caused by H2O2, or perturbation of cell wall integrity by calcofluor white (Figure 4.5E). Moreover,
expression of α-synuclein, another disease causing misfolded protein, reduced TRA1 promoter activity, indicating that various misfolded proteins differentially affect TRA1 transcriptional regulation (Figure 4.5F). This is in agreement with previous data showing that α-synuclein and Httex1 expression in yeast display a different subset of genetic interactions and differential induction of stress responses (Cooper et al., 2006; Willingham et al., 2003).
Figure 4.4 Tra1 is not sequestered into polyQ inclusion bodies.
(A) Tra1 remains localized to the nucleus in the presence of polyQ aggregates. Fluorescent images showing localization of GAL1-inducible polyQ-ymsfGFP (25 and 72Q) and yemRFP-Tra1 after overnight culture in glucose (control) or galactose (polyQ induced). (B) Pull-down assay using Ada2TAP shows that Tra1 still incorporates into SAGA in presence of GAL1-inducible 72Q-ymsfGFP. However, levels of Ngg1 and Spt7 are reduced upon expression of 72Q-ymsfGFP.
Figure 4.5 TRA1 expression is increased in presence of toxic polyQ expansions.

(A) Increased expression of TRA1 and other SAGA (NGG1, SPT7) and NuA4 (EAF1) components as well as the control NCW2 after overnight induction of GAL1-inducible 72Q-CFP compared to 25Q-CFP. Expression was analyzed using promoter-LacZ reporters. **p<0.005, ***p<0.001 n=3±SEM

(B) Treatment with the HDAC inhibitor trichostatin A (TSA) abolished TRA1 upregulation caused by polyQ expansions. GAL1-inducible 25 and 72Q-CFP were induced overnight in galactose media in presence or absence of 80 µM TSA.

(C) Overnight expression of GAL1-inducible 72Q-CFP leads to increased expression of TRA1 as measured by qRT-PCR.

(D) Expanded polyQ increases Tra1 protein abundance. Cells expressing either GAL1-inducible 25 or 72Q-CFP and a chromosomally integrated Tra15x-Flag were cultured in glucose or induced overnight in galactose and processed for immunoblot.

(E) Effects of other stressors on TRA1 expression. Cells expressing the TRA1-LacZ reporter were treated with tunicamycin (Tm; 5 µg/ml), heat shocked at 42 °C, treated with calcofluor white (CFW; 300 µg/ml) or H2O2 (300 µM) for 2 hr. n=3 ±SEM.

(F) Expression of α-synuclein decreased TRA1 expression. Cells expressing a galactose inducible version of α-synuclein-GFP were cultured in either glucose or galactose overnight and TRA1 expression was measured using a LacZ reporter. n=3.
4.4.3 Both inhibition and hyperactivation of TORC1 exacerbate polyQ toxicity

Our previous genetic screen highlighted a potential role for Tra1 in stress responses (Hoke et al., 2008), including the control of cellular homeostasis by the TORC1 (Target of Rapamycin Complex 1) regulated signaling cascades that link nutrient availability to cell growth and division. In this screen, a tra1 mutant displayed a synthetic slow growth phenotype with a deletion of tor1 (Hoke et al., 2008). Studies in mammalian cells report both a protective and adverse role for TORC1 in HD (Ferrarelli, 2015; Lee et al., 2015; Pryor et al., 2014; Ravikumar et al., 2002; Ravikumar et al., 2004; Renna et al., 2010). We therefore tested the effect of modulating TORC1 activity on polyQ toxicity in yeast. First, we determined the effects of TORC1 inhibition using rapamycin (Figure 4.6A and B). We found that rapamycin treatment significantly reduced growth of cells expressing expanded 103Q protein in both solid and liquid media assays when compared to the untreated condition. Interestingly, rapamycin treatment did not significantly alter polyQ aggregation (Figure 4.6C). This result argues against a protective role for TORC1 inhibition by rapamycin through stimulating polyQ aggregates removal through autophagy (Ravikumar et al., 2002). Similar to rapamycin, a hyperactive allele of TOR1 (TOR1L2134M) (Takahara and Maeda, 2012) also exacerbated polyQ toxicity (Figure 4.6D and E). The TOR1 mutant had only a modest effect on the toxicity of TDP-43 (Supplemental Figure 4.4), a protein linked to amyotrophic lateral sclerosis (ALS) (Johnson et al., 2008), indicating that the role of TORC1 may diverge in different diseases. TORC1 hyperactivation did not prevent the formation of polyQ aggregates (Figure 4.6F). These data indicate that precise regulation of TORC1 signaling is crucial for cells to cope with polyQ expansion. Interestingly, the same hyperactive TOR1 mutant decreases levels of glutathione (Oku et al., 2013), which is important to counteract ROS accumulation in HD models (Mason et al., 2013). TORC1 regulates translation and ribosome biogenesis in both yeast and mammals and decreased translation has been proposed as a contributor to polyQ toxicity in mammals (Leitman et al., 2014). Incidentally, cells expressing 72Q displayed increased sensitivity to the translational inhibitor cycloheximide (Figure 4.7A and B) and decreased expression of ribosomal protein genes (Figure 4.7C) when compared to 25Q, further supporting a role for TORC1
signaling in polyQ toxicity. Therefore, we next investigated the role of downstream TORC1 effectors in polyQ toxicity.
Figure 4.6 Both inhibition and hyperactivation of TORC1 exacerbate polyQ toxicity.
Figure 4.6

(A) TORC1 inhibition by rapamycin exacerbates polyQ toxicity. Wild-type cells expressing either MET25-inducible 25Q or 103Q Httex1 assessed by growth assay on SC containing media plates untreated or supplemented with 2 ng/ml rapamycin in presence (control) or absence (polyQ induced) of methionine. (B) Wild-type cells expressing either MET25-inducible 25Q or 103Q Httex1 were assessed by liquid growth assay in presence (control) or absence (polyQ induced) of methionine ± 2 ng/ml rapamycin. The area under the curve was quantified for each replicates (n=3) ***p<0.001. (C) Representative fluorescent images of wild-type cells expressing GAL1-inducible 25Q or 72Q Httex1-ymsfGFP after overnight induction in galactose containing media untreated or supplemented with 100 ng/ml rapamycin. (D) Wild-type and TOR1L2134M cells expressing either MET25-inducible 25Q or 103Q Httex1 assessed by growth assay on SC containing media plates in presence (control) or absence (polyQ induced) of methionine. (E) Wild-type and TOR1L2134M cells expressing either MET25-inducible 25Q or 103Q Httex1 were assessed by liquid growth assay in presence (control) or absence (polyQ induced) of methionine ± 100 ng/ml rapamycin. The area under the curve was quantified for each replicates (n=3) ***p<0.001. (F) Representative fluorescent images of wild-type and TOR1L2134M cells expressing GAL1-inducible 25Q or 72Q Httex1-ymsfGFP after overnight induction in galactose containing media.
Figure 4.7 PolyQ expansions sensitize cells to translational inhibition. 
(A) PolyQ increased cell sensitivity to cycloheximide. Wild-type cells expressing either MET25-inducible 25Q or 103Q Htt\textsuperscript{ex1} assessed by growth assay on SC containing media plates untreated or supplemented with 0.1 µg/ml cycloheximide in presence (control) or absence (polyQ induced) of methionine. (B) Wild-type cells expressing either MET25-inducible 25Q or 103Q Htt\textsuperscript{ex1} were assessed by liquid growth assay in presence (control) or absence (polyQ induced) of methionine ± 0.1 µg/ml cycloheximide. The area under the curve was quantified for each replicate (n=3) ***p<0.001. (C) PolyQ expression decreased expression of ribosomal protein genes. RNA was isolated from wild-type cells expressing GAL1-inducible 25Q or 72Q Htt\textsuperscript{ex1}-CFP after overnight induction in galactose containing media and processed for RT-qPCR to assess transcript levels of RPL6, RPL30 and RPL38. *p<0.05, **p<0.01. (n=3)
4.4.4 Sfp1/TORC1 regulates TRA1 expression

In yeast, TORC1 controls gene expression via two main downstream effectors, the mammalian S6 kinase homologue Sch9 and the transcription factor Sfp1 (Loewith and Hall, 2011). Sch9 localizes to the vacuolar membrane and mediates TORC1 signaling that regulates ribosomal protein gene expression and cell cycle progression (Urban et al., 2007). Tor1 also interacts with the transcription factor Sfp1, which regulates expression of ribosomal proteins (Jorgensen et al., 2004; Lempiäinen et al., 2009; Marion et al., 2004; Singh and Tyers, 2009). During exponential growth, Sfp1 localizes to the nucleus, where it drives transcription of ribosomal protein genes. During various types of stress, including protein misfolding, oxidative stress, and nutrient deprivation, Sfp1 translocates to the cytoplasm (Jorgensen et al., 2004; Lempiäinen et al., 2009; Marion et al., 2004; Singh and Tyers, 2009) where it is degraded by the proteasome (Lopez et al., 2011). Here, we found that deleting SFP1, but not SCH9 exacerbated polyQ toxicity (Figure 4.8A). This agrees with Sfp1 and Sch9 having non-overlapping functions (Jorgensen et al., 2004). Indeed, the two deletion strains display specific phenotypes. Relevant to our study, sfp1Δ increases rapamycin sensitivity as compared to sch9Δ (Lempiäinen et al., 2009). Increased polyQ toxicity in sfp1Δ cells was not associated with significant changes in formation of inclusion bodies (Figure 4.8B). Expression of ribosomal protein genes decreased in the presence of misfolded polyQ (Figure 4.7C) consistent with a loss of Sfp1-mediated TORC1 signaling. This may include the global effects on translation due to targeting the ribosomal protein genes, since loss of protein translation due to cycloheximide treatment exacerbates polyQ toxicity. In the same way, reduced translation is linked to polyQ toxicity in mammalian cells (Leitman et al., 2014). Surprisingly, sfp1Δ cells crossed with the tra1Q3 mutant allele are viable with the mutations displaying epistasis, suggesting that both genes function in the same pathway (Figure 4.8C). Indeed, tra1Q3 displayed reduced ribosomal protein gene expression (Figure 4.8D). In the same way, nuclear depletion of Tra1 using the anchor-away approach decreases recruitment of Esa1 and Gcn5 to ribosomal protein gene promoters (Bruzzone et al., 2018).
Figure 4.8 Deletion of SFP1 exacerbates polyQ toxicity.

(A) Deletion of SFP1 but not SCH9 exacerbates polyQ toxicity. Wild-type, sfp1Δ and sch9Δ cells expressing either MET25-inducible 25Q or 103Q Htt[ex1] assessed by growth assay on SC media plates in presence (control) or absence (polyQ induced) of methionine. (B) Deletion of SFP1 does not affect formation of polyQ IBs. Representative fluorescent images of wild-type and sfp1Δ cells expressing GAL1-inducible 25Q or 72Q Htt[ex1]-ymsfGFP after overnight induction in galactose containing media. (C) The tra1Q3 allele displays an epistatic phenotype with sfp1Δ. Cells carrying the SFP1 deletion were crossed with cells carrying the tra1Q3 allele. Growth of the double mutant and the parental strains were assessed by spotting assays on agar plates. (D) The tra1Q3 allele displays reduced levels of ribosomal protein gene expression. Wild-type cells and cells carrying the tra1Q3 allele were processed for RT-qPCR to assess transcript levels of RPL6, RPL30 and RPL38. ***p<0.005. (n=3)
In agreement with reduction of ribosome protein gene levels, Sfp1-GFP relocalized to the cytoplasm upon 103Q expression, in contrast to its nuclear localization in cells expressing non-toxic 25Q (Figure 4.9A). Interestingly, cytoplasmic Sfp1 is recruited to polyQ aggregates (Figure 4.9A). To quantify recruitment or exclusion of Sfp1 in polyQ aggregates we adapted a size-normalized, averaging based analysis method developed by Li et al. for studying Htt\textsuperscript{ex1} aggregates in mammalian cells (Li et al., 2016). Briefly, we analyzed several 1.75 x 1.75 µm polyQ-aggregates containing regions of interest (Figure 4.9B). Then, we generated an intensity map by averaging multiple regions for 20 different cells (Figure 4.9C). We next calculated the fluorescent intensity of 103Q-RFP and Sfp1-GFP along a line across the region to generate an intensity curve for both channels. Similar shaped curves revealed incorporation of Sfp1-GFP into polyQ aggregates (Figure 4.9D).

Interestingly, Lempiäinen et al. demonstrated that Sfp1 physically interacts with Tra1, suggesting that Tra1 may be regulated by Sfp1 in the presence of polyQ proteins (Lempiäinen et al., 2009). Tra1 protein levels (Figure 4.10A) but not mRNA (Figure 4.10B) were modestly decreased in sfp1\textsuperscript{Δ} cells, potentially reflecting reduced global translation due to decreased ribosome production (Fingerman et al., 2003; Jorgensen et al., 2004; Lempiäinen et al., 2009; Marion et al., 2004). Thus, it appears that Tra1 function rather than protein level is affected by Sfp1. Indeed, sfp1\textsuperscript{Δ} cells are sensitive to high temperature, ethanol, MMS and calcofluor white treatment, hallmarks of impaired Tra1 function (Figure 4.10C). Reduced Tra1 activity in sfp1\textsuperscript{Δ} cells was further supported by the reduced expression of PHO5, a Tra1/SAGA regulated gene (Barbaric et al., 2003; Gregory et al., 1998). This phenotype was reversed by the HDAC inhibitor TSA (Figure 4.11A), indicating that deleting SFP1 may affect SAGA and possibly NuA4-mediated chromatin modifications that regulate PHO5 transcription (Barbaric et al., 2003; Gregory et al., 1998). We next tested the effect of deleting SFP1 on the polyQ-induced upregulation of SAGA and NuA4 components observed in Figure 4.5A. Whereas the transcription from the SPT7, NGG1 and EAF1 promoters were still increased upon expression of misfolded 72Q, SFP1 deletion abolished TRA1 upregulation (Figure 4.11B). These results suggest that Sfp1 is required for proper control of TRA1 expression upon polyQ expression. Sfp1 is a known TORC1 effector and TORC1 inhibition with
rapamycin downregulates *TRA1* expression (Marion et al., 2004) (Figure 4.11C) highlighting a role for TORC1 in the control of *TRA1* transcriptional regulation. Since deleting *SFP1* causes hyperactivation of TORC1 as a compensatory mechanism (Lempiäinen et al., 2009; Rousseau and Bertolotti, 2016), we hypothesized that hyperactivation of TORC1 may prevent upregulation of *TRA1* upon accumulation of misfolded polyQ expansion. Indeed, *TRA1* was not significantly upregulated after expression of expanded 103Q in the hyperactive *TOR1L2134M* strain (Figure 4.11D). Coupled to the observed decrease in *TRA1* expression upon TORC1 inhibition by rapamycin (Figure 4.11C), our results establish that TORC1 signaling regulates *TRA1* expression.
Figure 4.9 SFPI accumulates in cytoplasmic aggregates in response to expanded polyQ expression.

(A) Representative fluorescent images of wild-type cells expressing GAL1-inducible 25Q or 103Q Httex1-RFP and Sfp1-GFP after 2hr induction in galactose containing media. Red arrows highlight localization of Sfp1 into polyQ aggregates. Bar = 5µm.

(B) Intensity maps for 20 polyQ aggregates containing regions. Panels displaying fluorescent signal for both 103Q-RFP and Sfp1-GFP are shown. (C) Average of each panel presented in (B). An intensity trend line is calculated across the region. (D) Intensity plots as a function of the distance across the region are presented for both channels. N= 20 regions.
Figure 4.10 Loss of SFP1 does not significantly affect Tra1 expression but sensitizes cells to stress.

(A) Deletion of SFP1 has minimal effect on Tra1 protein abundance. Immunoblot performed with cell lysates of from wild-type and sfp1Δ cells expressing a chromosomally integrated Tra15x-Flag. Blot was probed with anti-Flag. Anti-Pgk1 was used as loading control. Densitometric analysis from 3 independent samples is shown. (B) Deletion of SFP1 has minimal effect on TRA1 mRNA levels. RNA was isolated from wild-type and sfp1Δ cells and processed for RT-qPCR to assess transcript levels of TRA1. (C) Wild-type and sfp1Δ cells were spotted on YPD agar plates untreated, supplemented with 6µg/ml calcofluor white (CFW), 5% ethanol (ETOH), 0.03% methyl methanesulfonate (MMS) or incubated at 39°C.
Figure 4.11 Sfp1 and TORC1 regulate TRA1 expression in response to misfolded polyQ.

(A) Sfp1 regulates PHO5 expression. Cells expressing the PHO5-LacZ reporter were incubated in absence of phosphate overnight and treated with trichostatin A (TSA) at the indicated concentration. Expression from the NCW2 promoter is shown as control. β-galactosidase activity is shown as the average of 3 replicates with the SEM indicated by the error bars. ***p<0.001 using a one-way anova followed by a Tukey’s multi comparison test. (B) Deletion of SFP1 specifically abolishes TRA1 upregulation by polyQ. Gene expression from TRA1 and other SAGA (NGG1, SPT7) and NuA4 (EAF1) promoters after overnight induction of GAL1-inducible 72Q-CFP compared to 25Q-CFP in wild-type and sfp1Δ cells. Gene expression was analyzed using LacZ transcriptional reporters. **p<0.005, ***p<0.001 n=3±SEM. (C) Rapamycin treatment decreases transcription from the TRA1 promoter in both wild-type and sfp1Δ cells. Gene expression was analyzed using the TRA1-LacZ transcriptional reporter. **p<0.005, ***p<0.001 n=3±SEM. (D) Hyperactive TORC1 signaling prevents upregulation of TRA1 by polyQ. RNA was isolated from wild-type and TOR1L213M cells after overnight induction of GAL1-inducible 25 and 103Q Httex1-CFP in galactose media and processed for RT-qPCR to assess transcript levels of TRA1.
4.5 Discussion

4.5.1 Tra1/SAGA and polyQ toxicity

The ability of cytoplasmic polyQ expanded Htt\textsuperscript{ex1} to disrupt Tra1 functions echoes previous reports showing that expressing nuclear-targeted Htt\textsuperscript{ex1} results in transcriptional changes similar to deleting components of the SAGA complex (Hughes et al., 2001). Other reports show a similar phenotype using a different disease-associated polyQ protein, the SAGA-associated spinocerebellar ataxia 7 (SCA7) (Hughes et al., 2001; McMahon et al., 2005). We do not detect significant nuclear localization of Htt\textsuperscript{ex1} in our model (Figure 4.1). Since Tra1 remains localized to the nucleus upon expression of expanded polyQ, it is unlikely that the two directly interact, thus favoring indirect regulation of Tra1 function by polyQ. Our previous genetic screen using a \textit{tra1} mutant allele revealed that Tra1 is linked to cellular responses of proteotoxic stress, such as the heat shock response, mitochondria homeostasis and TORC1 signaling (Hoke et al., 2008). Interestingly, all these processes are linked to polyQ toxicity (Chafekar et al., 2012; Costa et al., 2010; Ferrarelli, 2015; King et al., 2008; Lee et al., 2015; Oliveira et al., 2007; Pryor et al., 2014; Ravikumar et al., 2004; Sakahira et al., 2002; Shirendeb et al., 2011; Squitieri et al., 2006; Wyttenbach et al., 2002). It is reasonable to deduce that impaired Tra1-regulated transcription has an important role in the toxic phenotype observed in HD models. Indeed, reduced histone acetylation is closely associated with HD and histone deacetylase inhibitors improve the HD phenotype in animal models (Guiretti et al., 2016; Jia et al., 2016; McFarland et al., 2012; Sharma and Taliyan, 2015; Steffan et al., 2001; Yeh et al., 2013). Thus, further analysis of the role of the major regulators of chromatin remodeling, gene expression and their specific targets may provide avenues for modulating the toxicity of polyQ expansion in HD.

4.5.2 Sfp1/TORC1 regulates \textit{TRA1} expression in presence of toxic polyQ proteins

Sfp1 is well characterized for its role in adjusting cell size and ribosome production during stress (Fingerman et al., 2003; Jorgensen et al., 2004; Lempiäinen et al., 2009; Marion et al., 2004; Singh and Tyers, 2009), yet its functions outside the control of ribosome biogenesis are understudied. Recently, Matthew \textit{et al.} found that sequestration
of the splicing factor Hsh155 during genotoxic stress was regulated via TORC1 signaling through Sfp1 (Mathew et al., 2017). Together with our data showing that Sfp1 regulates TRAI expression, this study supports an expanded role for Sfp1 in stress response, beyond regulating ribosome biogenesis. The mechanism by which Sfp1 contributes to TRAI expression during proteotoxic stress is unclear, but the results mimic our previous findings showing that compromising Tra1 function by mutating its PI3-kinase domain increases transcription of TRAI (Berg et al., 2018). It appears that the cells use a transcriptional feedback mechanism to increase the expression of TRAI and other components of the SAGA and NuA4 complexes to compensate for the loss of Tra1 function. Whether a common transcription factor is involved remains unknown.

PolyQ expansions and expression of the traiQ3 mutant allele (Berg et al., 2018) are the only experimental conditions identified to date that increase TRAI expression. Deletion of other SAGA/NuA4 components does not upregulate TRAI, indicating that disruption of HAT complexes cannot solely explain the phenotype (Berg et al., 2018). Moreover, polyQ expansions also upregulated TRAI in the absence of ADA2 (Supplemental Figure 4.5). Thus, an intact SAGA complex does not appear to be required for TRAI upregulation by polyQ. Interestingly, polyQ-induced TRAI expression requires Sfp1 (Figure 4.8). Microarray analyses revealed that overexpressing SFP1 does not upregulate TRAI, indicating that Sfp1 might regulate Tra1 indirectly (Jorgensen et al., 2004). Similar to what we observe with TRAI, deleting SFP1 abolishes upregulation of the proteasome regulator ADC17 during tunicamycin-induced endoplasmic reticulum stress (Rousseau and Bertolotti, 2016). Since in both cases, stress leads to Sfp1 relocalization to the cytoplasm, it appears that Sfp1 regulates these targets indirectly, potentially via its regulation of TORC1. In our case, relocalization of Sfp1 to the cytoplasm may disrupt the previously reported Sfp1 interaction (Lempiäinen et al., 2009) with Tra1 (which remains in the nucleus). Moreover, recruitment of Sfp1 to polyQ aggregates could contribute to reduced Sfp1 function and signaling in these cells, resulting in impaired Tra1/SAGA function. Sfp1 is known for its ability to acquire a prion form termed [ISP+] (Matveenko et al., 2016; Rogoza et al., 2010; Volkov et al., 2002). At the same time, polyQ toxicity relies on certain prions such as [RNQ+] (Manogaran et al., 2011; Meriin et al., 2002) and polyQ expansions both stimulate formation (Derkatch et al., 2004) and interact with
prions (Douglas et al., 2009). Thus, one could postulate that recruitment of Sfp1 and/or its prion form [ISP+] could play a role in restricting Tra1 function. That could explain why TRA1 upregulation observed with polyQ is not seen with α-synuclein, which toxicity is largely independent of the prion status (Derkatch et al., 2004). It is also possible that Sfp1/[ISP+] interacts with polyQ oligomers outside of the visible aggregates. Several studies in mammalian cells have implicated intermediate oligomers as the cause of toxicity in HD (Arrasate et al., 2004; Lajoie and Snapp, 2010; Moily et al., 2017; Takahashi et al., 2007).

The physiological relevance of the Tra1-Sfp1 interaction will require further investigation. A possible explanation for TRA1 transcriptional regulation by Sfp1/TORC1 is that TRA1 is regulated at multiple levels, one that involves TORC1 but also other pathways modulated by toxic polyQ expansions. Interestingly, recent data show that Sfp1 can negatively regulate a subset of genes, in particular the Swi4 regulon, which are upregulated when Sfp1 is depleted from the nucleus (Albert et al., 2018). This offers an attractive model where Sfp1 could act as a negative regulator of TRA1 expression. Finally, though there is no homologue of SFP1 in mammals, Sfp1 shares key functions with the mammalian proto-oncogene MYC. These functions include regulating ribosomal protein gene expression and cell growth (Dang et al., 2006). Importantly, mammalian TRRAP interacts with and is required for the activity of Myc (Bhattacharya and Ghosh, 2015; Kenneth et al., 2007; Liu et al., 2003; Zhang et al., 2014). These results delineate a link between Myc and TRRAP that is reminiscent of the Sfp1/Tra1 connection observed in yeast.

4.5.3 TORC1, SAGA and polyQ toxicity

Our data show that TORC1 has to be finely tuned for yeast cells to compensate for polyQ toxicity. This is not surprising considering the multitude of cellular processes regulated by TORC1 (Betz and Hall, 2013; González and Hall, 2017; Liko and Hall, 2015). Interestingly, both activation and inhibition of TORC1 are protective in rodent models of HD (Ferrarelli, 2015; Lee et al., 2015; Pryor et al., 2014; Ravikumar et al., 2004). Our results suggest that downstream targets of TORC1 function in mammalian cells, similar to Sfp1 in yeast, to regulate polyQ toxicity. The inter-relationship of these targets is
important for understanding the therapeutic potential of TORC1 for HD. A TRA1 mutant allele is hypersensitive to rapamycin and results in a synthetic slow growth phenotype with tor1 deletion (Hoke et al., 2008). In fission yeast, SAGA control of cell proliferation and differentiation in response to starvation requires the differential TORC1/TORC2-dependent phosphorylation of the SAGA component Taf12 (Laboucarié et al., 2017). TORC1 also regulates yeast histone acetylation by regulating the sirtuin deacetylases Hst3 and Hst4 (Workman et al., 2016) and integrates signals through the INO80 chromatin remodeling complex (Beckwith et al., 2018). Because of the breadth of cellular functions affected by TORC1 and transcriptional regulators like SAGA, a comprehensive understanding of how these pathways converge to regulate homeostasis is crucial. Inhibiting histone deacetylase is protective in various disease models, including HD (Jia et al., 2016; Valor, 2015; Valor and Guiretti, 2014; Yeh et al., 2013). Defining how disease-associated proteins affect the global acetylome and identifying specific targets for acetylation, including non-histone targets, could lay the basis for the development of new small molecule/compounds for combinational therapy.
Figure S 4.1 The non-HD associated 25Q Htt<sup>ex1</sup> does not induce a growth defect. Cell expressing high levels of GAL1-inducible CFP-tagged 25Q-Htt<sup>ex1</sup> display no growth defect when compared to the corresponding empty vector. Cell growth was assessed by serial dilutions on SC plates containing either glucose (control) or galactose (polyQ induced).
Figure S 4.2 PolyQ expansions do not cause cell death. 

(A) Representative fluorescent images of wild-type cells expressing \textit{GAL1}-inducible 25Q or 72Q Htt\textsuperscript{ex1}-CFP after overnight induction in galactose containing media and stained with propidium iodide (PI). Boiled cells are shown as a positive control. 

(B) \(\alpha\)-synuclein expression results in yeast cell death. Growth of cells expressing an empty vector or \(\alpha\)-synuclein-GFP was assessed by serial dilutions on SC plates containing either glucose (control) or galactose (induced). Staining with PI assessed cell viability.
Figure S 4.3 Hyperactive $TOR1^{L2134M}$ strain is resistant to rapamycin.  
(A) Growth of wild-type and $TOR1^{L2134M}$ cells on agar plate containing 10 ng/ml rapamycin.
Figure S 4.4 Hyperactive TORC1 signaling has minimal effect on TDP-43 toxicity.

(A) Growth of wild-type and $TOR1^{L2134M}$ cells expressing a control empty vector (+Vector) or TDP-43 (+TDP-43) was assessed by serial dilutions on SC plates containing either glucose (control) or galactose (TDP-43 induced). Cells were incubated either in glucose (top) or galactose (bottom) prior to spotting on agar plate.

(B) Growth of wild-type and $TOR1^{L2134M}$ cells expressing an empty vector or TDP-43 was assessed by growth in either glucose (control) or galactose (TDP-43 induced) liquid media. Cells were incubated either in glucose (top) or galactose (bottom) prior to spotting on agar plate.

(C) Quantification of the liquid growth assays. The area under the curve was quantified for each replicates ($n=3$) ***(p<0.001 when compared to the control empty vector under the same conditions unless noted otherwise.***
Figure S 4.5 *ADA2* deletion does not abolish *Tra1* upregulation by polyQ expansions.

Increased expression of *TRA1* after overnight induction of *GAL1*-inducible 103Q-CFP compared to 25Q-CFP in wild-type and *ada2Δ* cells. Expression was analyzed using promoter-LacZ reporters. ***p<0.001 n=3±SEM.
4.6 References


Berg, M. D., Genereaux, J., Karagiannis, J. and Brandl, C. J. (2018). The Pseudokinase Domain of Saccharomyces cerevisiae Tra1 Is Required for Nuclear Localization and Incorporation into the SAGA and NuA4 Complexes. G3 (Bethesda).


Lee, J. H., Tecedor, L., Chen, Y. H., Monteys, A. M., Sowada, M. J., Thompson, L.


Meriin, A. B., Zhang, X., He, X., Newnam, G. P., Chernoff, Y. O. and Sherman, M.


Chapter 5

5  Impaired Tra1-dependent transcription during heat shock in the presence of polyQ expansions

In the last chapter, we demonstrated that HD-associated polyQ expansions lead to abnormal upregulation of TRA1 and dysregulate the SAGA HAT complex assembly. As reviewed in chapter one, impaired activation of the HSR is one of the hallmarks of HD. How expanded polyQ expression dysregulates the HSR remains unclear. Given that Tra1/SAGA is involved in the activation of the HSR, we next focused on how polyQ–dependent impairment of Tra1/SAGA affects the HSR. Studying Tra1-dependent transcription during heat shock in polyQ cells will provide new insights into the mechanisms underlying the loss of proteostasis in HD.

5.1 Abstract

Dysregulation of proteostasis is a significant contributor of polyglutamine (polyQ) toxicity in Huntington’s Disease (HD). The heat shock response (HSR) is a vital pathway activated under multiple stress conditions to maintain protein homeostasis. The activation of HSR allows transcription of genes encoding the heat shock proteins (HSPs) to improve the cellular folding environment. Impaired HSR activation has been observed in HD and correlates with the reduced cellular capacity to fix damaged protein under stresses such as exposure to high temperature and oxidative stress. To understand how polyQ expansions impair the HSR, we used a yeast model of HD to study the transcriptional regulation under heat shock in the presence of polyQ expansions. The yeast cell wall protects cell survival during stresses associated with protein misfolding, including heat shock. We found that polyQ impairs the cell wall integrity and exacerbates cell sensitivity to heat shock. The transcription co-activator Tra1 has been found to be involved in cellular stress response that regulate polyQ toxicity. Our data showed that cells with a mutant Tra1 allele fail to activate the HSR. These cells were also more sensitive to cell wall stress. Our findings suggest that polyQ impairs cell growth under heat shock stress through a non-canonical HSR that allows the yeast cells to remodel their cell wall. Thus, our data
reveals a new mechanism by which polyQ impairs cellular stress responses and proteostasis in the yeast model of HD.

5.2 Introduction

Protein homeostasis is crucial for cell viability. Accumulation of misfolded proteins is at the root of many neurodegenerative disorders (Parakh and Atkin, 2016; Tan et al., 2009; Tayubi et al., 2015). One example is Huntington’s disease (HD), which is linked to expanded polyglutamine (polyQ) repeats within the exon 1 of Huntingtin (Htt) protein (Gusella and MacDonald, 2009). Misfolded Htt oligomerizes into insoluble aggregates in the cytosol and nucleus of the neuronal cells, causing a series of cellular dysfunctions such as transcriptional dysregulation, increased endoplasmic reticulum stress, mitochondria defects, and impaired HSR (Chafekar and Duennwald, 2012; Duennwald and Lindquist, 2008; Kumar et al., 2014; Quintanilla and Johnson, 2009). PolyQ expansion leads to neurodegeneration resulting in chorea, cognitive decline, and muscle contracture (Roos, 2010).

Cellular stress responses are signaling pathways induced under stress conditions, which are crucial to increase cellular capacity to handle misfolded protein accumulation and maintain proteostasis. The HSR is an evolutionarily conserved signaling pathway to regulate protein folding and degradation during cell stress (Morimoto and Westerheide, 2010). Reduced activation of heat shock factor 1 (HSF1), the major transcription regulator of the HSR, as well as decreased expression of heat shock proteins (HSPs) during heat shock has been observed in cells expressing mutant Htt and in HD knock-in mice (Chafekar and Duennwald, 2012). However, the mechanism of how polyQ impairs HSR is not clear. A chromatin immunoprecipitation sequencing study reported that most genes lost HSF1 binding during polyQ expansion were not directly related to proteostasis, but involved in cytoskeletal binding, focal adhesion, and GTPase activity (Riva et al., 2012). Furthermore, overexpressing Hsp26, Hsp42, and Hsp104 have been shown to attenuate polyQ toxicity in a yeast HD model (Cashikar et al., 2005). Interestingly, knocking out HSP26 or HSP42 causes cell dehydration, cytoskeleton and cell wall damage (Haslbeck et al., 2004). These results suggest that the impaired HSR in cells
expressing expanded polyQ is linked to multiple cellular processes not necessarily linked to protein folding.

Yeast cells have a cell wall structure which is vital in protecting cell integrity and survival under stress conditions (Lesage and Bussey, 2006). The cell wall integrity (CWI) pathway is activated in response to cellular stresses which regulates cell wall biosynthesis and actin cytoskeleton polarization (Levin, 2005). Previous studies demonstrated a clear link between the CWI pathway and the HSR in yeast (Dunayevich et al., 2018; Lamoth et al., 2012; Levin, 2011). For example, Truman et al. suggested the crosstalk between the two stress response pathways by showing that the osmotic stabilizer sorbitol rescued the temperature sensitivity of cells carrying a mutant HSF1 lacking the C terminal domain, and that cells with an impaired CWI displayed a growth defect during heat shock (Truman et al., 2007).

Transcriptional regulation of stress-induced genes is critical for the activation of cellular stress responses (Boy-Marcotte et al., 1999; Godon et al., 1998; Rep et al., 2000). Many stress-related gene transcriptions require the recruitment of the SAGA histone acetyltransferase (HAT) complex (Bonnet et al., 2014; Huisinga and Pugh, 2004; Koutelou et al., 2010; Sanz et al., 2016). Tra1, an essential component of the SAGA complex, has been shown to be involved in cellular stress responses (Berg et al., 2018; Hoke et al., 2008; Hoke et al., 2010). Our previous study found that polyQ dysregulated Tra1 expression and SAGA assembly (Jiang et al., 2019). Thus, the impaired cellular stress responses during polyQ expansion may be traced back to Tra1-dependent transcriptional defects.

We found that cells expressing expanded polyQ displayed impaired cell wall homeostasis, which contributes to the previously reported high-temperature sensitivity. Furthermore, cells carrying a Tra1 PI3K mutant (tra1Q3) allele failed to activate the HSR during heat shock. These cells also showed a cell wall defect. Our results raise the possibility that polyQ impairs cellular stress responses through the dysregulation of Tra1-dependent transcription.
5.3 Materials and Methods

5.3.1 Yeast strains

All yeast strains used in this study were derived from BY4741/4742. The Tra1 mutant strain was described in Berg’s study (Berg et al., 2018). The polyQ strains were described in our previous paper (Jiang et al., 2019).

5.3.2 Monitoring yeast growth

Yeast growth assays were performed in both agar plate spot and liquid growth curve following previous protocols (Jiang et al., 2017). Briefly, cells were cultured in selective synthetic complete media to stationary phase. For cells expressing 25/103Q, cells were washed twice and re-incubated in media without methionine overnight. For spot assay, each yeast culture was diluted to OD<sub>600</sub> 0.2, 0.04, 0.008, 0.0016 and spotted on selective plates. After incubating for two days, images were taken with a GelDoc system (Bio-Rad). Yeast cultures were also diluted to OD<sub>600</sub> 0.1 into a 96 well plate and incubated in a plate reader (Biotek). Concentration was measured every 15 min for 24 h. Growth curves were generated and quantified by Student's t-test using GraphPad (Prism).

5.3.3 Chemicals

Caffeine (1% in H<sub>2</sub>O; ScholAR Chemistry), Trichostatin A (10 mM in H<sub>2</sub>O; Biovision) and calcofluor white (30 mg/mL in H<sub>2</sub>O; Sigma-Aldrich) were diluted to indicated concentration for each experiment.

5.3.4 Western blot

Cells were lysed using standard protocol. The concentrations of each sample were measured using BCA assays and diluted equally. Samples were separated using SDS-PAGE and transferred to PVDF membranes by semi-dry blotting. Membrane was blocked with 5% milk, washed with PBST and then incubated with indicated antibodies overnight. The anti-Hsp104 antibody was from the Linquist lab (Whitehead Institute, MIT, USA). The anti-Hsp26 antibody was from the Buchner’s lab (TU Munich, Germany). Next, the membrane was washed and incubated with secondary antibody for 1h. Membrane was washed before images taken using a Odyssey infrared imager (Licor).
5.3.5 Real-time qPCR

Cells expressing 2X MET25-polyQ were heat shock at 42 °C for 1 hour. RNA was extracted using the MasterPure Yeast RNA Purification Kit. The reverse transcription was performed using Quanta Bioscience qScript Flex cDNA Synthesis Kit. Then, 1 µl cDNA was used as the template for qPCR reaction and detected by PerfeCTa SYBR-Green Supermix (Quanta Bioscience). U3 was used as a reference control. Primers used are listed below.

<table>
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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>HSP104</td>
<td>AGCAGGCTCGTCAAGGTAAA</td>
<td>TTACCGATACCTGGCTCACC</td>
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5.3.6 Fluorescence microscopy

20 µl of overnight culture was loaded to LabTek imaging chambers (Thermo Inc.) and mixed with 180 µl of growth media. Images were acquired using a Zeiss 800 confocal (63X) and then analyzed using the free ImageJ software.

5.3.7 Statistical analysis

Student’s t-test was performed using the GraphPad Prism 7 to analyze the statistical significance between the different experimental groups in Figure 5.1B, 5.3A, and 5.3B.
5.4 Results and Discussion

5.4.1 PolyQ impairs the heat shock response in yeast

Impaired HSR has been observed in a striatal cell line expressing full-length Htt with polyQ expansion and HD knock-in mouse model (Chafekar and Duennwald, 2012). However, the underlying mechanisms remain elusive. Studying the highly conserved and well-characterized HSR in yeast will help us better determine how polyQ affects stress response thus further understand the basic pathology of HD. In our model, Htt^ex1 expressing expanded polyQ was designed under a MET25 promoter. The expression was induced by culturing cells in growth media without methionine. To investigate the effects of polyQ on the HSR, we incubated cells expressing Htt carrying non-pathological (25Q) and HD-associated polyQ (103Q) at 39 °C. Cells expressing toxic 103Q showed a growth defect at high temperature compared to cells growth at 30 °C (Figure 5.1A). Consistent with the previous findings in mammals, there was a substantial decrease in the expression of Hsp26, Hsp42, and Hsp104 in 103Q cells upon heat shock at both mRNA and protein level (Figure 5.1B), indicating reduced activation of the HSR.
Figure 5.1 PolyQ impairs the heat shock response in yeast.

(A) Cells expressing polyQ showed a growth defect at high temperature. Cells were induced in growth media without methionine overnight and spotted on selective plates incubated at 30 or 39 °C. (B) Expression of Hsp104, Hsp26 were reduced in polyQ cells during heat shock. Cells were heat shock at 42 °C or incubated at room temperature for 1 hour before lysed. Protein expression was measured by western blotting. Pkg1 was used as a control. The mRNA level of HSP104, HSP42, and HSP26 was also quantified in cells expressing 2X polyQ after heat shock for 1 hour.
5.4.2 Dysregulation of the cell wall integrity pathway during polyQ expansion

Previous studies found that the osmotic stabilizer sorbitol can rescue the growth of cells expressing truncated HSF1 at high temperature (Truman et al., 2007). Thus, we tested whether sorbitol attenuates growth defect caused by polyQ at 39 °C. Indeed, the growth defect of polyQ cells incubated at high temperature was improved by the addition of 1M sorbitol (Figure 5.2A). Thus, the growth defect of cells expressing 103Q at high temperature can be traced back to cell wall defects. To test whether polyQ expansion leads to cell wall stress, cells carrying wild-type or expanded polyQ were treated with cell wall stressor caffeine. 103Q cells showed increased sensitivity to caffeine, suggesting these cells had cell wall defects (Figure 5.2B). The CWI pathway activation has been shown to have a protective effect under conditions that affect cell wall integrity (Levin, 2005). Thus, we examined the impact of polyQ expansion in cells carrying a deletion of SLT2, a gene encoding the MAP kinase essential for the proper execution of the CWI transcription program. The slt2Δ cells displayed exacerbated polyQ toxicity (Figure 5.2C). This result further supports that polyQ impairs the CWI pathway, rendering to multiple stresses such as heat shock.

Interestingly, Truman et al. showed that overexpressing Hsp90 rescues Slt2 activity during heat shock in cells expressing a defective HSF1 (Truman et al., 2007). However, we did not observe the same effect in polyQ cells (Figure S5.1). It therefore appears that the phenotype associated with polyQ expansion is somewhat different from the HSF1 mutant. The CWI pathway is functionally conserved with the MAP kinase pathways in mammalian cells, which plays an important role in regulating cell proliferation and differentiation (Wang et al., 2006). Especially, ERK5, the human homolog of Slt2, is vital in cardiovascular development and neural differentiation (Truman et al., 2006; Wang et al., 2006). Interaction between ERK5 and HSPs such as Hsp70 and Hsp90 have also been studied (Sohn et al., 2002; Zheng and Studzinski, 2017). Furthermore, polyQ expansions have been found to dysregulates ERK signaling and activating the ERK pathways showed protective effects against HD (Apostol et al., 2006; Maher et al., 2011; Varma et al., 2007). Even though human cells do not have a cell wall, investigating the crosstalk
between the CWI pathway and the HSR in the yeast model will help to understand the role of ERK in polyQ toxicity and identify potential therapeutic targets for HD.
Figure 5.2 PolyQ impairs the cell wall integrity pathway.

(A) Sorbitol rescues sensitivity to high temperature of polyQ cells. Cells were induced in growth media without methionine overnight and spotted on selective plates incubated at 39 °C ± 1M sorbitol. (B) Cells expressing toxic polyQ were sensitive to cell wall stressor. Cells were induced in growth media without methionine overnight and spotted on selective plates in the presence or absence of 0.05% caffeine and incubated at 30 °C for 2 days ± 1M sorbitol. (C) Deletion of SLT2 exacerbates polyQ toxicity. Wild type and Slt2 deletion cells expressing polyQ were induced in growth media without methionine overnight and spotted on selective plates and incubated at 30 °C for 2 days.
5.4.3 Tra1 is involved in cellular stress response

Previous studies have shown that Tra1, and its mammalian homolog TRRAP, play a crucial role in the activation of HSR (Breitkreutz et al., 2010; Herceg et al., 2003). However, the mechanism needs further investigation. The PI3K domain is vital for Tra1 function (Berg et al., 2018). Thus, I tested the HSR activation in cells carrying a tra1Q3 allele in which the three arginine residues within the PI3K domain are mutated to glutamine. When incubating the tra1Q3 cells at 39 °C, they displayed a strong growth defect at high temperature (Figure 5.3A).

Moreover, treating the cells with a histone deacetylase inhibitor trichostatin A (TSA) attenuated the phenotype (Figure 5.3A). The data indicated that Tra1 regulates the HSR through its role in histone acetylation. To further study how Tra1 regulates the HSR, a GFP reporter fused to the heat shock element (HSE) was expressed in wild-type and tra1Q3 cells. As shown by fluorescence microscopy, GFP intensity was significantly lower in tra1Q3 cells compared to wild-type after heat shock for 2 hours, suggesting a failure to activate HSPs transcription in the tra1Q3 strain (Figure 5.3B). The tra1Q3 cells were also shown to be more sensitive to cell wall stress (Berg et al., 2018). I was able to reproduce the experiment by treating these cells with a cell wall stressor calcofluor white. Cells carrying the tra1Q3 mutation displayed a growth defect under cell wall stress. Furthermore, the defect can be rescued by adding sorbitol, indicating that Tra1 is vital for cell wall stress responses. Moreover, the growth defect of tra1Q3 strain at 37 °C was also alleviated by sorbitol treatment (Figure 5.4). Overall, these data suggested that Tra1 regulation is crucial in cellular stress responses.
Figure 5.3 Tra1 plays an important role in the heat shock response.

(A) Tra1 mutant cells displayed growth defect at high temperature. Cells were induced in growth media without methionine overnight and spotted on selective plates incubated at 30 or 39 °C for 2 days. WT and tra1Q3 cells treated with TSA were assessed by liquid growth assay at 37 °C. The area under the curve was quantified for each replicate (n = 3), ***P < 0.001. (B) Reduced HSE transcription during heat shock in Tra1 mutant cells. Representative fluorescent image of WT and tra1Q3 cells incubated at 42 °C for 2 hours. Fluorescent intensity was quantified by imageJ. ***P < 0.001.
Figure 5.4 Tra1 is crucial in regulating cell wall stress.
Cells expressing \(tra1_{Q3}\) mutation are more sensitive to heat shock and cell wall stress. The defect was rescued by sorbitol. Cells were spotted on selective plates with calcofluor white and incubated at 30 or 37 °C for 2 days ±1M sorbitol.
The connection between impaired cellular stress response during polyQ expansion and the regulation of Tra1 needs further investigation. The Triple T complex which contains Tel2, Tti1, and Tti2 is required for the stabilization of PIKK family proteins (Hurov et al., 2010). Tti2 has been shown to be involved in stress responses via its role in maintaining Tra1 folding and nuclear localization (Genereaux et al., 2012; Hoffman et al., 2016). Therefore, polyQ may affect Tra1 dependent cellular stress responses through the dysregulation of Tti2. Our data showed that Tti2 was sequestered into polyQ aggregates, indicating that polyQ impairs Tti2 function (Figure 5.5). However, overexpressing Tti2 was not able to rescue the sensitivity to heat shock and cell wall stress in cells expressing toxic polyQ (Figure S5.2). Thus, increased sensitivity to high temperature could be, at least in part, linked to loss of function of Tti2.
Figure 5.5 Tti2 is sequestered into polyQ aggregates.
Cells expressing GAL1-25/68Q-mScarlet were induced in galactose overnight and then monitored by confocal microscope. Representative fluorescent images showed co-localization between Tti2-GFP and 68Q aggregates.
5.4.4 Perspective

I have shown that cell wall stress and defect in the CWI signaling pathway is involved in cell sensitivity to heat shock during polyQ expansions. Tra1-dependent transcriptional regulation plays a critical role in the HSR activation and cell wall stress response. Thus, Tra1 dysregulation caused by expanded polyQ expression may explain the impaired cellular stress responses in HD (Figure 5.6). Future studies need to determine the recruitment of Tra1 to the promoters of HSPs and CWI targets using Chromatin immunoprecipitation during polyQ toxicity to better understand the mechanism. Defining how polyQ impairs cellular stress responses and the transcriptional regulation will guide future studies in developing target specific compounds for HD therapy.
Figure 5.6 Hypothetical model of heat shock sensitivity in a yeast model of HD.
The sensitivity to high temperature in cells expressing expanded polyQ is due to
dysregulation of HSR and a cell wall defect. The impaired HSR and CWI during
polyQ expansions may be caused by dysregulation of Tra1-dependent stress-induced
gene transcriptions.
Figure S 5.1 Overexpressing Hsp90 did not rescue growth defect at high temperature in polyQ cells. 

*MET25* induced 25/103Q cells were induced in -methionine media overnight before spotted on selective plates and incubated at 30 or 37 °C for 2 days. 103Q cells overexpressing Hsc82 still displayed a growth defect at 37 °C.
Figure S 5.2 Overexpressing Tti2 did not rescue sensitivity to cell stress in polyQ cells.
Cells carrying *MET25* induced 25/103Q and *GAL1* induced Tti2 were induced in -methionine media overnight before spotted on selective plates containing 10 μg/ml Calcofluor white. 103Q cells overexpressing Tti2 were still more sensitive to high temperature and cell wall stress compared to 25Q.
5.5 References


**Sohn, S. J., Sarvis, B. K., Cado, D. and Winoto, A.** (2002). ERK5 MAPK regulates embryonic angiogenesis and acts as a hypoxia-sensitive repressor of vascular


Chapter 6

6 Tra1 is a novel potential target for antifungal therapy

The emergence of drug-resistant strains among fungal pathogens represents a major public health challenge. Thus, it is essential to identify new therapeutic targets to fight pathological yeast infections. The previous chapters demonstrated that Tra1 plays a vital role in promoting yeast survival during stress conditions (including cell wall perturbation; a major mode of action of antifungal compounds), suggesting that it could represent a rational target for antifungal treatment. In this chapter, I will investigate the role of Tra1 in antifungal drug resistance, especially through the regulation of the calcineurin pathway.

6.1 Abstract

Fungal infections have become a growing threat to human health. Current treatment is very limited and often insufficient. Furthermore, several pathological yeast strains have acquired significant multidrug resistance. Thus, it is urgent to understand the mechanisms of fungal stress resistance to identify new therapeutic targets for antifungal drug development. The conserved Tra1 protein is involved in the yeast cellular stress responses critical for cell viability and survival under stress conditions. Our new data showed that Tra1 is involved in the calcineurin signaling pathway that regulates calcium homeostasis, which has been shown to be crucial for fungi virulence and drug resistance. Cells expressing a mutant Tra1 allele displayed impaired activation of the calcineurin pathway and were more sensitive to antifungal drugs. Furthermore, Tra1 plays a critical role in the cell wall stress response. Synthesis of the cell wall is the target of a major class of antifungal drugs such as echinocandins. Our study provides new insights into the mechanisms of cellular stress responses in yeast and identifies Tra1 as a novel target for antifungal drug development.

6.2 Introduction

Fungal infections are affecting 25% of the population worldwide (Havlickova et al., 2008). Most common fungal pathogens include Cryptococcus, Candida, and Aspergillus,
which can cause serious chronic diseases and are potentially fatal if not diagnosed and treated in time (Brown et al., 2012). There are only a limited number of antifungal agents available. Azaoles, such as fluconazole and miconazole, target ergosterol synthesis by inhibiting lanosterol 14α-demethylase that converts lanosterol to ergosterol, a major component of fungal plasma membranes. Membrane sterol is also targeted by polyenes through direct binding which causes changes in membrane permeability and leakage of cytoplasmic material. Another class of antifungal drugs is the echinocandins (caspofungin and anidulafungin), which inhibit fungal β(1,3)-d-glucan biosynthesis and lead to severe cell wall defect (Ghannoum and Rice, 1999; Groll et al., 1998; Kathiravan et al., 2012). These drugs usually have strong side effects and they speed the fungal evolution to gain antifungal resistance. Multidrug resistance of fungal pathogens has been observed in many cases, specifically among Candida species (Cowen, 2008; Cowen et al., 2014). Therefore, developing new antifungal drugs which are more specific and efficient is challenging but urgent.

Cellular stress responses including the heat shock response (HSR), the cell wall integrity (CWI) pathway and the calcium-calcineurin pathway are activated under stress conditions to regulate cell viability and survival by assisting with protein folding, cell wall synthesis, and maintaining calcium homeostasis (Cyert, 2003; Levin, 2005; Morimoto and Westerheide, 2010). Therefore, key proteins in the cellular stress response pathways can be targets for antifungal drug development. For example, a major heat shock protein Hsp90 has been found to be involved in antifungal resistance (Lamoth et al., 2016). Hsp90 inhibitors significantly enhance the efficacy of antifungal drugs (Cowen et al., 2009). The combination of Candida albicans Hsp90 antibody and an antifungal agent amphotericin B has shown beneficial effects in a clinical trial (Pachl et al., 2006). The regulation of cell stress responses requires the transcription of stress-induced genes. The transcriptional regulation involves the recruitment of specific activators and co-activators such as histone acetyltransferase (HAT) complexes (Bonnet et al., 2014). An essential component of SAGA and NuA4 complexes, Tra1, has been shown to be involved in cellular stress responses (Hoke et al., 2008; Hoke et al., 2010). Literature showed that yeast cells expressing mutant Tra1 were more sensitive to cell stressors MMS, ethanol, and caffeine (Berg et al., 2018; Mutiu et al., 2007). Therefore, Tra1 is critical for cell
survival and growth during stress and could be a target for antifungal therapy. To test the role of Tra1 in antifungal resistance, we assessed the effects of common antifungal agents on yeast growth in cells carrying a mutant Tra1 (tra1Q3) allele in the well-characterized *Saccharomyces cerevisiae*.

Calcineurin signaling is conserved among fungal species, critical for cell growth and stress responses (Cyert, 2003). The calcineurin pathway has been shown to be involved in pathogen virulence and drug resistance in different fungal pathogens through its role in the cell membrane and cell wall synthesis (Juvvadi et al., 2014; Liu et al., 2015; Yu et al., 2014). Furthermore, calcineurin inhibitors FK506 and cyclosporine A and their analogs have been studied for antifungal treatment (Lee et al., 2018; Pandit, 2003). However, due to the immunosuppressive effects, they cannot be directly used in the clinic. Tra1 mutant strain showed significant growth defect in presence of high calcium, suggesting that Tra1 is vital for calcium homeostasis (Berg et al., 2018). However, the mechanism of how Tra1 regulates the calcineurin pathway remains unclear. Understanding the vital role of Tra1 in the calcineurin pathway activation is crucial for developing target-specific drugs for antifungal treatment.

Using the well-studied yeast model, we found that Tra1 is essential for the calcineurin pathway activation. Cells expressing tra1Q3 failed to activate the calcineurin signaling, leading to increased sensitivity to antifungal drugs. Our results expand the understanding on the role of Tra1 in cellular stress responses and identify its potential for the development of novel antifungal therapy.

### 6.3 Materials and Methods

#### 6.3.1 Yeast strains and culturing

The *TRA1/tra1Q3* strains used in this study was described in Berg et al., 2018. Preparation of growth media was instructed by standard protocol from Amberg and Strathern, 2005. Cell growth was assessed by spot and liquid culture. After OD<sub>600</sub> of the overnight culture was measured, cells were diluted to OD<sub>600</sub> 0.2 and then spotted in three fivefold dilutions for spot assay. For liquid culture assay, yeast cells were diluted to OD<sub>600</sub> 0.1 from the
beginning. Three replicates for each group were cultured in a 96 well plate and concentration was measured per 15 minutes by a plate reader (Biotek) for 24 hours.

6.3.2 Chemicals and drugs

Trichostatin A (10 mM in H2O; Biovision), calcofluor white (30 mg/mL in H2O; Sigma-Aldrich), tunicamycin (5 μg/mL in DMSO; Amresco), fluconazole (50 mg/ml in DMSO; Alfa Aesar), miconazole (30 mg/ml in DMSO; Alfa Aesar), and caspofungin (1mg/ml in H2O; Sigma-Aldrich) were diluted to concentrations listed for each experiment.

6.3.3 qRT-PCR

Cells were treated with 200mM CaCl2 for four hours before RNA extracted using MasterPure Yeast RNA Purification Kit. Reverse transcription was done by qScript Flex cDNA Synthesis Kit (Quanta). The cDNA samples were diluted five times as the qPCR reaction templates. PerfeCTa SYBR-Green Supermix (Quanta) was employed for amplified DNA measurement. The primers used were listed in Table 6.1. The relative expression level was calculated by the ΔΔCT method using U3 as a housekeeping control.

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<td>PMC1</td>
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6.3.4 Beta-galactosidase assay

Overnight culture was washed and resuspended by lacZ buffer. To measure beta-galactosidase activity, 50 μl of cells were diluted in 950 μl lacZ buffer with 2.7μl β-mercaptoethanol, one drop 0.1% SDS, two drops chloroform and incubated 15 minutes at 30 °C. 100 μl ONPG (4mg/ml) was added to each sample to start the reaction. Then the cells were put back to 30 °C until the color changed to yellow. Time was recorded. To
stop the reaction, 300 μl 1M Na₂CO₃ was added. The beta-galactosidase activity was determined by measuring 420 nm absorbance using a plate reader (Biotek), standardizing to cell density.

6.3.5 Statistical analysis

Growth curves were generated to quantify the differences in yeast growth under different conditions. The area under the curve was measured using the Graphpad Prism 7 software. Statistical significance was calculated using the Student’s t-test.

6.4 Results and Discussion

6.4.1 Tra1 is a regulator of the calcium-calcineurin pathway

The calcineurin pathway is crucial for fungal survival and resistance to antifungal drugs (Juvvadi et al., 2014). Literature showed that the activation of the signaling pathway requires an intact SAGA complex. Deletion of the gene encoding the histone acetyltransferase Gcn5 leads to a significant downregulation of the calcineurin A subunit in fungal pathogen Cryptococcus neoformans (O’Meara et al., 2010). To comprehensively determine the effects of SAGA and NuA4 components on the regulation of calcium homeostasis in S. cerevisiae, we assessed the sensitivity to high calcium stress in strains carrying deletions of the major components of the SAGA or NuA4 HAT complexes (Han et al., 2014; Searle et al., 2017). We found that knocking out the SAGA components GCN5, ADA2, and SPT20 or NuA4 component EAF1 resulted in a severe growth defect in presence of 200 mM CaCl₂ (Figure 6.1). The data suggest that both SAGA and NuA4 are required for calcium homeostasis.
Figure 6.1 SAGA and NuA4 complexes are essential for calcium homeostasis.
Deletion of SAGA/NuA4 components increases cell sensitivity to high Ca\(^{2+}\). Cells were incubated in YPD media containing 0.05M succinic acid overnight before spotted on YPD plus 0.05M succinic acid plates in the presence or absence (Control) of 200mM CaCl\(_2\) and incubated for 2 days.
Tra1 is the only essential subunit within both SAGA and NuA4 complexes and has been found to be involved in stress responses (Hoke et al., 2008). Therefore, we further investigated whether Tra1 played a role in regulating cell growth towards high calcium. The Tra1 PI3K domain is essential for maintaining protein stability and nuclear localization (Berg et al., 2018). My study involved a tra1Q3 allele, where three arginine residues within the PI3K domain were mutated to glutamine (Berg et al., 2018). As shown in Figure 6.2, the mutant tra1Q3 stain had a significant growth defect when cultured in media containing 200mM CaCl2.
Figure 6.2 A functional Tra1 is required for growth in presence of high calcium levels.

Tra1 mutation strain displayed a growth defect at high calcium concentration. WT and tra1Q3 cells were incubated in YPD media containing 0.05M succinic acid overnight before spotted on YPD plus 0.05M succinic acid plates in the presence or absence (Control) of 200mM CaCl₂ and incubated for 2 days. Growth of WT and tra1Q3 cells were also assessed by liquid growth assay. The area under the curve was quantified for each replicate (n = 3), ***P < 0.001.
Previous synthetic genetic array analysis showed a negative genetic interaction between Tra1 and the calcineurin regulatory subunit, Cnb1 (Hoke et al., 2008). Indeed, the \( \text{tra1}_{Q3} \) mutant displays a synthetic slow growth phenotype when crossed with the \( \text{CNB1}\Delta \) strain (Figure 6.3A). The activation of the calcineurin pathway targets requires Crz1 binding to the calcineurin-dependent response element (CDRE) (Stathopoulos and Cyert, 1997). To further explore the mechanisms underlying the sensitivity to high calcium of the \( \text{tra1}_{Q3} \) strain, we measured the activation of the calcineurin pathway using a lacZ reporter under the control of a promoter carrying the CDRE motif (Stathopoulos and Cyert, 1997). Results in Figure 6.3B indicated that cells expressing \( \text{tra1}_{Q3} \) fail to activate the calcium-calcineurin pathway when treated with a high concentration of \( \text{Ca}^{2+} \). Moreover, unlike in wild-type cells, the expression of downstream calcineurin target genes \( \text{PMR1} \) and \( \text{PMC1} \) in \( \text{tra1}_{Q3} \) cells remained unchanged upon treatment with high calcium (Figure 6.3C). The data suggested that Tra1 is vital for activation of the calcineurin pathway to regulate calcium homeostasis. Furthermore, addition of TSA, a histone deacetylase inhibitor significantly alleviated the growth defect in Tra1 mutant cells at high \( \text{CaCl}_2 \) concentration, indicating the critical role of histone acetylation in regulating the calcineurin pathway (Figure 6.3D). How Tra1 is involved in the calcineurin pathway activation remains unclear. Expression of a truncated CNA1 without the autoinhibitory domain that leads to constitutive activation of calcineurin (Chaudhuri et al., 1995), or overexpressing the transcriptional regulator \( \text{CRZ1} \) did not rescue the sensitivity to high \( \text{Ca}^{2+} \) treatment of cells expressing the \( \text{tra1}_{Q3} \) allele (Figure S6.1). The data suggested that Tra1 is required for the activation of Crz1 downstream target gene transcriptions, potentially by targeting the transcription factor to its targeted promoters.
Figure 6.3 Tra1 regulation is required for the calcineurin pathway.

(A) Genetic interaction between Tra1 and Cnb1. CNB1 deletion strain carrying Tra1 mutation cause slow cell growth. (B) Cells carrying tra1Q3 allele cannot activate the calcineurin pathway. Cells expressing the CDRE-LacZ reporter were incubated in SC media containing 0.05M succinic acid in the presence or absence of 200mM CaCl2. Gene expression was analyzed using LacZ transcriptional reporters. ***P < 0.001, n = 3 ± SEM. (C) Reduced PMRI and PMC1 expression during high calcium stress in cells expressing mutant Tra1. WT and cells carrying the Tra1Q3 allele were processed for RT-qPCR to assess transcript levels of PMRI AND PMC1. **P < 0.005, ***P < 0.001 (n = 3). (D) Inhibiting histone deacetylation partially attenuates sensitivity to high Ca2+ in Tra1 mutant cells. The growth of WT and tra1Q3 cells were assessed by liquid growth assay. Experimental groups were treated with 40 μM trichostatin A (TSA). The area under the curve was quantified for each replicate (n = 3), ***P < 0.001.
6.4.2 Tra1 is involved in yeast tolerance of antifungal agents

Since Tra1 is vital for the activation of the calcineurin pathway, we next investigated the role of Tra1 in cell sensitivity to antifungal reagents. As shown in Figure 6.4A, the \( \text{tra1}^{-3} \) strain was significantly more sensitive to the antifungal agents fluconazole, miconazole, and caspofungin. To test the activation of calcineurin signaling, the expression of CDRE was determined by a lacZ reporter. We found that cells carrying mutant Tra1 were unable to activate the calcineurin pathway when treated with azoles (Figure 6.4B).

Previous studies and our results show that Tra1 plays an essential role in cellular stress responses such as the HSR and cell wall stress response. As shown in Figure 6.5A, \( \text{tra1}^{-3} \) cells displayed a growth defect when treated with a cell wall stressor calcofluor white as well as incubated at a higher temperature. In addition, the \( \text{tra1}^{-3} \) mutant shows no growth defect when treated with tunicamycin, suggesting that the requirement for Tra1 function (or at least its PI3K domain) is specific to several stresses. Furthermore, our data show that osmotic stabilizer sorbitol attenuated the growth defect of the \( \text{tra1}^{-3} \) mutant cells when treated with antifungal drugs (Figure 6.5B), reinforcing the crucial role of Tra1 in cell wall homeostasis. The stress responses are vital in regulating cell survival when exposed to antifungals. Thus, Tra1 emerges as a new potential therapeutic target for fungal infections. The Tra1 PI3K domain is highly conserved from \( S. \text{cerevisiae} \) to fungal pathogen \( C. \text{albicans} \) (Figure 6.6). Therefore, the results found in \( S. \text{cerevisiae} \) can direct future investigations on the role of Tra1 in \( C. \text{albicans} \) and antifungal drug development.
Figure 6.4 Tra1-dependent activation of calcineurin pathway is critical for antifungal resistance.

(A) Cells expressing \( \text{tra1Q3} \) mutation are more sensitive to antifungals. WT and \( \text{tra1Q3} \) cells were spotted on selective plates containing fluconazole, miconazole, and caspofungin at indicated concentrations and incubated for 2 days. Cell growth was assessed by liquid growth assay.

(B) Tra1 mutant cells fail to upregulate the calcium-calcineurin pathway treated with antifungals. Cells expressing the CDRE-LacZ reporter were incubated in SC media in the presence or absence of fluconazole for 6 hours or miconazole for 4 hours. Gene expression was analyzed using LacZ transcriptional reporters. **P < 0.005, n = 3 ± SEM.
Figure 6.5 Tra1 plays an important role in cellular stress responses. 
(A) Cells expressing mutant Tra1 are more sensitive to heat shock and cell wall stress. Cells were spotted on selective plates with calcofluor white (CFW) or tunicamycin (TM) and incubated at 30 or 39 °C for 2 days. (B) The sensitivity to antifungals of tra1Q3 cells is linked to cell wall stress. WT and Tra1 mutant cells were spotted on selective plates containing fluconazole, miconazole, or caspofungin and incubated at 30 °C for 2 days ±1M sorbitol.
Figure 6.6 Sequence alignment of homologous Tra1 PI3K domain.

The PI3K domain of Tra1 in *S. cerevisiae*, *C. albicans*, and TRRAP in human was aligned using the online MUSCLE tool (http://www.ebi.ac.uk/Tools/msa/muscle/).

The three arginine (R3389, R3390, R3456) to make the tra1 Q3 strain was marked with the red box.
6.4.3 Perspective

My study investigated the role of Tra1 in the activation of the calcineurin pathway and antifungal resistance. Future studies including growth assays, protein expression quantification, ChIP, and genome-wide sequencing assays comparing tra1Q3 to wild-type strain will be performed in the fungal pathogen C. albicans in collaboration with Dr. Rebecca Shapiro at the University of Guelph to investigate whether the phenotypes observed in S. cerevisiae are recapitulated in pathogenic fungi. These experiments will help gain a better understanding on the mechanisms of Tra1 regulated stress responses during antifungal drug treatment.

The PI3K domain is vital for Tra1 stability and function. Although lacking kinase motifs, structural studies suggested that the PI3K domain of Tra1/TRRAP is still able to catalyze the transfer of a modifying unit other than phosphate moieties (Mutiu et al., 2007). Many PI3K inhibitors and small molecules have been designed targeting the PI3K domain of the PIKK family proteins for treatment of cancer and several other diseases (Fruman et al., 2017), this domain could be an excellent binding target for target-specific antifungal drug development. Sequence alignment has identified the differences between fungal Tra1 and human TRRAP within the PI3K domain (Berg et al., 2018), further studies should investigate the structure and function of these specific targets in regulating Tra1 and cell survival.
Figure S 6.1 Hyperactivation of the calcineurin pathway did not rescue sensitivity to high Ca\(^{2+}\) concentration in cells expressing mutant \(tra_{1Q3}\).

(A) Constitutively activated calcineurin did not rescue the growth defect of \(tra_{1Q3}\) cells at high Ca\(^{2+}\) treatment. Truncated Cna1 was transformed into wild-type and Tra1 mutant cells. Cells were incubated in YPD media containing 0.05M succinic acid overnight before spotted on YPD plus 0.05M succinic acid plates in the presence or absence (Untreated) of 200mM CaCl\(_2\) and incubated for 2 days. (B) Overexpressing Crz1 showed little effects on cells carrying the \(tra_{1Q3}\) allele. \(GAL1\) induced Crz1 was expressed in WT and \(tra_{1Q3}\) mutant cells. Cells were incubated with 0.05M succinic acid overnight before spotting on selective plates containing 200mM CaCl\(_2\).
6.5 References


Chapter 7

7 Discussion

In this last chapter, I will summarize the main findings and discuss the overall implications and significance of my thesis project. First, I will introduce the methods available to assess new fluorescent proteins including the advantage of using the yeast assay I have developed. Then, I will address the link between protein aggregation and toxicity in neurodegenerative disorders. Furthermore, I will discuss the controversial role of the TORC1 signaling in HD and the importance of combining different experimental models in HD research. Finally, I will evaluate the therapeutic potential of Tra1/TRRAP for human disease therapy.

7.1 Summary of major results

The main goal of my thesis is to investigate the function of Tra1 in regulating cellular stress responses and the implications for stress-related diseases. To accomplish that, I employed the well-characterized yeast model of HD to investigate Tra1-dependent regulation on polyQ toxicity. First, I developed an optimized yeast model to study polyQ toxicity and aggregation in yeast by tagging mutant Htt exon1 with newest yeast codon optimized FPs. I found that different FPs can alter polyQ toxicity and aggregation formation. Following this, I assessed the effects of two latest generation of RFP, FusionRed and mScarlet, using the yeast polyQ model. I found that tagging polyQ with FusionRed attenuated its toxicity in yeast. With the help of the optimized HD model in yeast, I revealed how Tra1 and SAGA complex regulates polyQ toxicity. My data suggested that polyQ expansion impairs SAGA assembly and leads to upregulation of Tra1 expression dependent on Sfp1, a downstream target of the TORC1 signaling pathway regulating ribosome biogenesis. Impaired HSR has been found in HD before; however, the precise mechanism remains unclear. Functional Tra1 has been linked to cellular stress responses. Therefore, I studied how Tra1 regulates heat shock tolerance in the presence of expanded polyQ. I showed that the heat shock sensitivity of cells expressing polyQ is linked to a defect in cell wall integrity, which requires Tra1
regulation. Since Tra1 is crucial for cellular stress responses and yeast survival, I then investigated its role in antifungal resistance. My results indicated that a functional Tra1 is vital for yeast resistance to antifungal agents, through the activation of the calcineurin pathway. My studies therefore identify Tra1 as a novel potential therapeutic target for fungal infections.

7.2 Assessing fluorescent proteins and the impact on their fusion partners

The development of GFP (2008 Nobel Prize in Chemistry jointly to Osamu Shimomura, Martin Chalfie, and Roger Y. Tsien) and other FPs make a powerful tool in nowadays’ scientific field. In modern biological researches, a great number of findings were achieved with the help of FPs to visualize protein localization and trafficking, protein-protein interactions, and organelle metabolic functions (Kremers et al., 2011; Wiedenmann et al., 2009). There are now numerous FP options available. However, because of the different nature of FPs, whether a given FP is appropriate can vary from experiment to experiment. My study is a good example and showed that tagging expanded polyQ with different fluorescent proteins can alter its toxicity and aggregation (Jiang et al., 2017). Many factors can affect the expression of FPs and one needs to take them all into consideration when designing a protein fusion. These include N versus C-terminal fusion, FP maturation and photostability, environmental conditions, codon optimization, and oligomerization (Costantini et al., 2015; Huang et al., 2014; Palmer and Freeman, 2004; Snapp, 2009). Due to the lack of rapid and quantitative methods to evaluate FPs, especially their impact on fusion partners, testing newly synthesized FPs and choosing the correct FPs for specific experiments has always been challenging.

Most quantitative comparisons between different FPs have been made in vitro, which do not necessarily reflect their behavior in living cells. Cranfill et al. characterized more than 40 FPs based on their visible spectrum, photostability, and monomeric nature to look for the best performing FPs. The light absorbance and fluorescence quantum yield of purified FPs were measured to determine the brightness. Photobleaching rates were measured by embedding FPs in polyacrylamide or microdroplets using laser scanning microscopy (Patterson and Piston, 2000; Piston et al., 1999). The monomeric quality was quantified.
using the OSER assay (Costantini et al., 2012). Using the monomeric FP is critical for correct folding and maintaining the structure of fusion targets. The OSER assay is based on the ability of oligomerized membrane protein fluorescent fusions to transform the branching tubules network ER into a whorls-like organized smooth ER (OSER) structure (Snapp et al., 2003). Tagging FPs to ER-targeted transmembrane domain of cytochrome p450 to form the CytERM-FP and quantify the OSER structures can represent the FP oligomeric status in vivo. Compared to the traditional in vitro assays to assess the oligomeric tendency of FPs through ultracentrifugation or gel filtration methods, the OSER assay allows scientists to study FP behavior in living cells. Furthermore, another study expressed FPs in transgenic C.elegans embryos to assess their performance in a multicellular animal model (Heppert et al., 2016). However, both of the in vivo methods are not high throughput.

In Chapter 2 and 3 of my thesis, I demonstrated an ideal tool to study FP brightness, photostability and specifically the impact on their fusion partners using the yeast polyQ model. Different FPs alter the toxicity and aggregation of mutant Htt. Thus, this model can be used to characterize new FPs and evaluate the effects on their targeted proteins. With the advantage of using yeast as a model, this method provides researchers with a rapid and high throughput tool to screen for newly synthesized FPs or potential mutations of classic FPs that may improve their performance. Therefore, the polyQ assay is a good complement to existing methods to test the next generation FPs. Furthermore, the method can also be used to test other genetically encoded tags.

7.3 Correlation between aggregation and neurodegeneration

Aggregation is a hallmark of a series of neurodegenerative disorders including AD, PD, and HD; however, its role in toxicity is debatable. It is proposed that the non-soluble large aggregates directly contribute to disease pathology of neurodegeneration (Donaldson et al., 2003; McCampbell et al., 2000). However, more evidence is showing that these amyloid-like aggregates may actually be cytoprotective (Todd and Lim, 2013).
In HD, inclusion bodies (IBs) accumulate in the striatum, cerebral cortex, cerebellum, and the spinal cord. The formation of IBs is found before neuron cell death and correlates with severe behavioral symptoms in HD transgenic mice model (Davies et al., 1997). Although the function of normal Htt is not fully understood, it has been found to be critical for clathrin-mediated endocytosis, apoptosis, and vesicle transport (Harjes and Wanker, 2003). Therefore, misfolded Htt may disrupt essential cellular functions by impairing the functions of the wild-type allele. Due to the nature of the polyQ oligomers/IBs, expanded polyQ may sequester other proteins. A transcriptional regulator CBP was found to be incorporated into the IBs in an HD cell line (McCampbell et al., 2000). Furthermore, sequestration of essential ubiquitin binding proteins into the aggregates was also found in other polyQ diseases, e.g. Spinocerebellar Ataxia (Donaldson et al., 2003). Thus, the recruitment of other proteins may cause cellular dysfunctions and lead to neurotoxicity. However, this sequestration is not observed in all different HD models (Yu et al., 2002). Moreover, the impairment of the proteasome can occur before IBs formation (Mitra et al., 2009). Several studies showed that there is no correlation between aggregations and toxicity. An HD mouse model expressing human Htt 120Q displayed no significant neuronal degeneration, although IBs were observed in the striatum and cortex (Slow et al., 2005). Interestingly, a longitudinal survival analysis by following long-time survival of the same living neurons in HD demonstrated that neuronal cell death highly correlates with diffuse Htt (Arrasate and Finkbeiner, 2012; Miller et al., 2010). The accumulation of IBs reduced diffuse Htt expression and the risk of striatal neuronal death. The results suggested that the aggregation may be actually protective against neuron cell death. In addition, Takahashi et al. also reported that the cytotoxicity is caused by soluble oligomers but not IBs (Takahashi et al., 2008).

The toxicity also seems dependent on the localization of mutant Htt. Tissue culture cells have been found to take up chemically synthesized polyQ peptides. IBs were observed mainly in the cytoplasm and do not affect cell viability. Adding a nucleolus localization signal on the other hand, significantly induce cell death in the PC-12 cell line (Yang et al., 2002). Indeed, nuclear aggregation is quite rare in HD patient striatal neurons (Gutekunst et al., 1999; Kuemmerle et al., 1999), further suggesting that polyQ aggregates may not be a predictor of neuronal cell death. The kinetics of aggregation formation may also
affect its toxicity. A time-lapse microscopy analysis showed that cells that formed delayed IBs formation survived longer than those forming IBs quickly (Gong et al., 2008).

Several studies also supported the idea that aggregations are epiphenomena or protective in PD and AD, representing a compensating mechanism to cellular stress to promote cell survival (Espay et al., 2019; Wan and Chung, 2012). Accepting the hypothesis that aggregation is protective, new therapeutic targets other than preventing aggregation and activating misfolded protein degradation pathways are investigated for HD and other misfolded protein diseases. A chemical compound library screen identified a compound B2 to be protective against HD and PD in mammalian cell line by promoting IBs formation however reduce cytotoxicity (Baldo et al., 2012).

The yeast model of HD provides researchers with a practical tool to study the connection between aggregation and toxicity. Even though polyQ affects Tra1/SAGA functions, it did not sequester Tra1 into the aggregates, indicating that sequestration into aggregates is not the only mechanism that contributes to the cellular toxicity. Furthermore, we observed that compared to other FPs, cells expressing 72Q-moxBFP displayed the strongest growth defect (Figure 2.5). Interestingly, these cells seem to form fewer aggregates although further quantitation is required. Therefore, further studies focusing on the relationship between aggregation and toxicity as well as other specific factors that may coordinate polyQ toxicity and aggregation morphology, for example flanking sequences outside of the polyQ region (Dehay and Bertolotti, 2006; Duennwald et al., 2006), is crucial for identifying new targets for HD therapy.

### 7.4 TORC1 signaling in Huntington’s disease

The highly conserved TORC1 signaling controls many cellular processes that regulate cell growth and proliferation, including transcription, stress responses, cell cycle, and importantly, autophagy (Kamada et al., 2010). TORC1 is a negative regulator of autophagy, which is critical for degrading protein aggregates (Ravikumar et al., 2002). Thus, one could quickly think that TORC1 should be inhibited in HD therapy. By contrast, conflicting views argue that TORC1 activation can be beneficial in HD because
it promotes cellular functions that have been found to be impaired by polyQ expansion (Ferrarelli, 2015).

The clearance of misfolded protein aggregates is crucial for HD therapy. Multiple studies showed that autophagy plays a vital role in degrading aggregate-prone proteins (Ravikumar et al., 2002; Roscic et al., 2011; Webb et al., 2003). Ravikumar et al. (2004) reported that inhibiting autophagy leads to increased aggregation and cell death in the COS-7 cell line expressing mutant Htt. Treatment of mTOR inhibitor rapamycin, which activates autophagy, had opposite effects. Furthermore, another study demonstrated that mTORC1 is sequestered into mutant Htt aggregates in an HD cell model, transgenic mice, and patient brain samples (Ravikumar et al., 2004). The recruitment impairs mTOR kinase activity and induces autophagy. They observed increased aggregation formation and cell death in COS-7 cell line carrying polyQ expansion when mTOR signaling is enhanced by overexpressing the upstream regulator Rheb (Manning and Cantley, 2003). The authors hypothesized that inhibiting mTOR has protective effects in HD. Using a fly model of HD, they showed slower neurodegeneration when treated with rapamycin. Furthermore, when treated with a rapamycin analog CCI-799, Ross/Borchelt mice expressing Htt N-terminal fragment with 82Q showed fewer aggregates in the striatum and performed better in behavioral tests (Ravikumar et al., 2004). However, rapamycin treatment leads to significant weight loss and reduced brain mass. It also does not improve the lifespan of HD mice, which makes the potential of rapamycin analog in HD treatment questionable.

Lee and colleagues hold the opposite opinion (Lee et al., 2015). When they activated mTORC1 through overexpression of Rheb (Ras homolog enriched in the brain) or Rhes (Ras homolog enriched in the striatum) in HD mice striatal neurons, they observed protective effects. mTORC1 activation promoted mitochondrial function, increased cholesterol synthesis, attenuated striatal atrophy, and improved HD mice disease phenotypes. Notably, they observed increased levels of autophagy markers LC3-II and Beclin-1 when mTORC1 is activated. Several studies have shown that autophagy can happen through mTORC1-independent pathways (Gordon et al., 1993; Petiot et al., 2000; Sarkar, 2013). Furthermore, mTOR reactivation can regulate lysosome formation under
starvation (Yu et al., 2010). Therefore, mTOR-independent or mTOR-promoted autophagy may be contributing to proteostasis during polyQ expansion. Targeting mTOR-independent autophagy pathways have shown benefits to HD in cell lines, fly, and zebrafish models (Sarkar et al., 2009; Williams et al., 2008).

The role of mTOR in HD can also be tissue specific. mTOR activation is higher in skeletal muscles than in the brain in the transgenic R6/2 mice model of HD (She et al., 2011) and is believed to contribute to muscle atrophy. These results may explain the behavior improvement seen in the animal following treatment of rapamycin. Furthermore, inhibiting mTORC1 has shown protective effects against other neurodegenerative diseases such as Fragile X syndrome and ALS (Bhattacharya et al., 2012; Saxena et al., 2013).

The regulation of TORC1 in mammals is complicated. Suppressing or hyperactivating TORC1 can cause strong side effects (Li et al., 2014). The well-studied *S. cerevisiae* allows researchers to investigate the role of TORC1 in polyQ toxicity and identify downstream proteins that can be therapeutic targets for HD. Our data suggested that functional TORC1 is critical in HD. TORC1 inhibition and activation both lead to increased sensitivity to polyQ toxicity (Figure 4.6). TORC1 hyperactivation suppressed the upregulation of *TRA1* during polyQ expansion, which may partly explain the growth defect. It is vital to investigate downstream mechanisms of the TORC1 signaling pathway that is crucial for HD and identify specific therapeutic targets.

### 7.5 HD model organisms

Other than the yeast model of HD used in my thesis, there are different model organisms developed to study HD pathology including worms, fruit flies, rodents, and larger mammals such as monkeys and pigs (Pouladi et al., 2013). Each model has its advantages and limitations. Thus, the combination of diverse HD models is meaningful for the research field to investigate fundamental cellular mechanisms together with neuronal functions and behavioral phenotypes resulting from polyQ expansions. The following section is extracted from Jiang, Chadwick, and Lajoie (2016).
Researchers have studied HD in worms; they are a useful invertebrate model of neuronal dysfunction and death because of their conserved basic neuronal functions compared to vertebrates and their simple neuroanatomy. The Hart’s group developed a C. elegans HD model specifically expressing Q150 in sensory neurons induced by an osm-10 promoter (Faber et al., 1999). There are other worm models mimicking HD, as well. One uses a mec-3 promoter expressing 57-residue fragment of Htt followed by GFP (Parker et al., 2001) and another study expressed expanded polyQ in the C. elegans body wall muscle cells (Satyal et al., 2000). Despite lacking a homolog to the human Htt, worms provide researchers a good tool to study cellular events in HD, especially in neuronal cells. Fruit flies are also an excellent system to study HD because the Htt gene is conserved with mammals (Sipione and Cattaneo, 2001). Expressing Htt exon1 in photoreceptor neurons in the compound eye in Drosophila was achieved via the UAS-GAL4 system (Jackson et al., 1998; Kim et al., 2004). This small model organism allows rapid genetic manipulation and, as it is the case with the Huntingtin protein, it expresses a large number of genes with a homologue to the human proteome (Wangler et al., 2015). As with many other human diseases, rodents are commonly-used models in investigating the basis of HD. The R6/1 and R6/2 transgenic mouse model expressing Htt exon1 designed by Mangiarini and colleagues shows many HD symptoms, including chorea, involuntary stereotypic movements, tremor, and loss of muscle strength (Mangiarini et al., 1996). Another model expresses a cDNA encoding the N-terminal fragment of the Htt protein with 82Q, which also show severe Huntington’s disease phenotypes (Schilling et al., 1999). The Hayden laboratory also created a yeast artificial chromosome (YAC) transgenic mice expressing the full-length HTT gene with 18Q, 46Q or 72Q. YAC mice expressing the 72Q HTT gene (YAC72) show a selective degeneration of medium spiny neurons associated with N-terminal Htt translation to the nucleus (Hodgson et al., 1999). Another way to express mHtt in mice is through knocking in a human HTT exon1 into the endogenous mouse Huntington’s disease gene homolog (Hdh) (Trettel et al., 2000; Wheeler et al., 2000). These mice always show a milder phenotype compared to transgenic models. For example, in the mutant HdhQ50 and HdhQ111, where mutant Htt is expressed under the endogenous mouse HTT gene promoter, an age-dependent Htt nuclear relocalization and inclusion bodies were observed after six months. However,
these mice did not show a severe movement disorder (White et al., 1997). Although over 20 different rodent HD models have been created over the years, each has its own advantages and disadvantages. The transgenic models may just mimic the disease symptoms rather than the age-dependent onset of HD, but the knock-in models do not mimic the human symptoms because the Hdh gene in mice differs from HTT in humans. Thus, the choice of a specific model should be made with caution (Jiang et al., 2016; Pouladi et al., 2013).

The major advantage of the yeast model is the ease of genome editing and large-scale screening which allows researchers to focus on specific signaling pathways in cells expressing mutant Htt expansion and identify potential modifiers and suppressors of polyQ toxicity. The findings in yeast can be verified in higher eukaryotic models and guide the future therapeutic studies in clinic. The majority of HD yeast studies focus on rescuing growth defect during fermentative metabolism. A genome-wide overexpression screen identified that suppressors for polyQ toxicity are mainly glutamine- and asparagine-rich prion-like proteins (Kayatekin et al., 2014). However, there are a lot of questions left to be answered. Future studies can investigate the mechanisms by which polyQ affects mitochondria respiration in yeast, how cells cope with polyQ toxicity at early time points, and the role of chromatin remodeling and transcriptional regulation during polyQ expansions.

7.6 Tra1/ TRRAP as a novel target for human diseases

In my thesis, I demonstrated that Tra1 is a major regulator of the cellular stress responses to protein misfolding, which indicates the possibility to target Tra1 or its mammalian ortholog TRRAP in the treatment of HD and fungal infections. Dysregulation of Tra1/TRRAP and dysfunctions in histone acetylation has been found in other stress-related human diseases, especially in cancer.

The protein TRRAP was initially identified through its interaction with the oncoprotein c-Myc by affinity purification (McMahon et al., 1998). In addition, the group also identified the binding of TRRAP to E2F transcription factors in controlling the cell cycle. The constitutive activation of E2F is considered an important event in oncogenesis.
(Johnson and Schneider-Broussard, 1998). More studies have implicated the role of TRRAP in cancer development in recent years. TRRAP is a coactivator of an important tumor suppressor p53 (Barlev et al., 2001). However, it has also been found to promote mutant p53 accumulation in cancer cells by regulating p53 degradation machinery (Jethwa et al., 2018). Furthermore, TRRAP is the target of viral oncoprotein E1A. The adenovirus E1A directly binds to TRRAP/GCN5 and dysregulates transcription activation (Lang and Hearing, 2003). Additionally, TRRAP Ser722Phe mutation is frequently observed in melanoma (Wei et al., 2011). Patients with high expression of TRRAP exhibit lower survival rates in ovarian cancer according to cBio Cancer Genomics Portal (Cerami et al., 2012). Importantly, TRRAP is essential for cell survival and TRRAP knockout causes embryonic lethality (Herceg et al., 2003). Conditional knock out or knock down techniques allow researchers to study the role of TRRAP during cancer development. TRRAP knockdown was found to attenuate brain tumor formation in a mouse model (Wurdak et al., 2010). Another study using a high throughput shRNA library screen also reported that TRRAP was required for glioblastoma stem-like cells and TRRAP expression was increased in the gliomas (Wurdak et al., 2010). However, a microarray analysis of breast cancer tissue showed a negative correlation between TRRAP and patient survival (Wang et al., 2016). The evidence suggests that its role may vary in different types of cancer. Moreover, histone deacetylase inhibitors (HDACi) have been studied as potential cancer therapeutics due to their potential to compensate for the attenuation of histone acetylation observed in cancer development (Li and Seto, 2016). While some HDACi have been approved for clinical trials, these therapies still have serious limitations including side effects and low efficacy (Dell’Aversana et al., 2012). Therefore, targeting specific regulators of histone acetylation could be a better solution for cancer treatment.

All the evidence suggests that TRRAP is an essential modifier of various diseases and could be a therapeutic target. The yeast models allow scientists to investigate the basic functions of the highly conserved homolog Tra1 and reveal the mechanisms of Tra1 regulation during diseases. Previous studies and ours have implicated Tra1 in cellular stress responses. Future studies should focus on understanding the mechanisms by which
Tra1 regulates stress response pathways and how to control Tra1 activity in stress-related disease therapy.

7.7 The potential of Tra1/TRRAP in drug development

Tra1 and its mammalian homolog TRRAP is essential for cell survival during stresses. We have explored the critical role of Tra1 in HD and antifungal resistance. Thus, drugs and small molecules that regulate Tra1 function can be investigated in disease therapies. Although missing the catalytic residues, this PIKK family member has the similar substrate binding cleft between the N- and C-lobes comparing to TOR, ATM, and ATR (Díaz-Santín et al., 2017; Sharov et al., 2017), which makes it an ideal druggable target. Currently, there is no drug targeting Tra1/TRRAP available.

Many PI3K inhibitors and small molecules have been designed to target the PI3K domain of PIKK proteins, which benefits various disease in model organisms and even clinical trials. As introduced previously, mTOR inhibitors showed protective effects against HD (Ravikumar et al., 2004). A PI3K inhibitor LY294002 inhibits PI3K activity by competitive inhibition of ATP binding, attenuated molecular hallmarks of AD (Aoyagi et al., 2010). PI3K inhibitors have also been used in cancer therapy. Through suppression of PI3K p110α catalytic subunit, BYL719 inhibited cancer cell proliferation both in vitro and in vivo (Keam et al., 2015). It has shown beneficial effects in breast cancer patients (Mayer et al., 2017). Furthermore, our data suggested that yeast cells carrying \( \text{tra1}_{Q3} \) mutation were more sensitive to antifungal drugs, indicating that yeast Tra1 can be a potential therapeutic target for fungal infections. Sequence alignment data showed that the PI3K domain of Tra1 homologs in different species is not 100% identical (Berg et al., 2018). Thus, it is possible to target specific amino acids in fungal Tra1 without interfering the host cells and thus significantly reduce side effects.

The specific function of Tra1 PI3K has not been fully described. Studies showed that the PI3K domain is crucial for Tra1 stability and nuclear localization (Berg et al., 2018). The authors also observed reduced incorporation with the HAT complexes and increased sensitivity to cell stress when three arginine residues proximal to the PI3K binding cleft are mutated (\( \text{tra1}_{Q3} \)). These results suggested an important role of the PI3K domain in regulating Tra1 function. Even though lacking ATP binding motifs, structural studies
suggested that the substrate-binding pocket of Tra1 PI3K is still able to interact with small molecules (Mutiu et al., 2007). The function of Tra1/TRRAP, primarily its PI3K domain needs further investigations. No small molecule ligands or RNAs have been identified to interact with the Tra1 PI3K domain. Our data showed that \textit{tra1}Q3 cells fail to activate the HSR and the calcineurin pathway, indicating that the PI3K domain may be implicated in its binding to other transcription factors and target gene promoters. Understanding the critical role of the Tra1 PI3K domain will provide insights into the transcriptional regulation of cellular stress responses and guide the development of drugs and small molecules that target Tra1 for the treatment of human diseases.

7.8 Limitations of the research

There are some limitations to this study. The yeast model of HD we use expresses only the Htt\textsuperscript{ex1} fragment instead of the full length Htt, which may not fully represent the cellular functions and mechanisms of the disease-causing protein. Previous studies in mice models of HD have shown that, although disease progressions happen earlier, mice expressing mutant Htt\textsuperscript{ex1} develop similar phenotypes compared to full length Htt knock in mice (Woodman et al., 2007). The evidence supports the continued employment of the more efficient Htt\textsubscript{ex1} to investigate the basic cellular mechanisms of HD in different model organisms. Moreover, the proline-rich region and protein tags can affect polyQ toxicity and aggregation morphology in yeast, which limits the use of the model to understand the aggregation formation in neurons (Dehay and Bertolotti, 2006; Duennwald et al., 2006; Jiang et al., 2017). However, this result develops ideas that flanking sequences outside of the polyQ region can affect polyQ toxicity thus could be potential therapeutic targets for HD. In addition, the polyQ toxicity in yeast depends on cellular prion status that may result from yeast specific mechanisms (Jiang et al., 2017; Meriin et al., 2002; Ripaud et al., 2014). Overall, the findings in this thesis can be tested in mammalian cell models.

In chapter 6, we demonstrate the role of Tra1 in antifungal resistance. The preliminary experiments were performed in the non-pathological yeast \textit{S. cerevisiae}. Therefore, the results need to be verified in infectious fungi for further studies. We will evaluate the
functions of Tra1 and its potential as a target for antifungal treatment in the pathogenic yeast *C. albicans* in collaboration with Dr. Rebecca Shapiro (University of Guelph).

### 7.9 Significance of the research

Dysregulation of cellular stress responses is a hallmark of several human diseases, such as neurodegenerative disorders and fungal infections and antifungal resistance. An ideal model to study misfolded protein caused cellular stress is HD. By optimizing the yeast model of HD with modern FPs, my research supports the view that flanking sequences can alter polyQ toxicity and aggregation formation. We also provided a high throughput platform to rapidly assess the impact of FPs on their fusion partners. Moreover, the active stress responses in fungi promote cell survival and contribute to antifungal resistance. A key process of cellular stress responses is the transcriptional activation of stress-induced genes, which is regulated by transcription factors as well as co-activators. The highly conserved transcription co-activator Tra1 within the SAGA and NuA4 HAT complexes is linked to cellular stress responses. However, the roles of Tra1 and mechanisms on how it regulates cellular stress response pathways remains obscure. Therefore, it is crucial to investigate the specific regulatory roles of Tra1 and connections to disease pathology. Using *S. cerevisiae*, my studies target the regulation of Tra1 on the HSR, the CWI pathway, and the calcineurin signaling during polyQ expansions and antifungal treatment.

First, we identified a novel connection between TORC1/Sfp1 pathway regulated cell growth and histone acetylation controlled by Tra1/SAGA during misfolded protein stress. Next, we showed that impaired HSR in HD can be traced back to a cell wall defect, which may all regulated by Tra1. By showing the critical role in the calcineurin pathway, we reported that Tra1 is vital for yeast survival against antifungal drugs.

My thesis will provide new insights into the novel transcriptional mechanisms of cellular stress responses that promote cell survival as well as the underlying cellular mechanisms of HD and antifungal resistance. Understanding the specific functions of Tra1 within the HAT complexes will help uncover the secrets of transcriptional defects and dysregulation of cellular stress responses observed in HD and other human diseases. Furthermore, my study reveals a novel potential therapeutic target for the research of stress-related disorders generally.
7.10 References


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Appendices

Appendix A: Effect of Fluorescent Proteins on Fusion Partners Using Polyglutamine Toxicity Assays in Yeast


Abstract

For the investigation of protein localization and trafficking using live cell imaging, researchers often rely on fusing their protein of interest to a fluorescent reporter. The constantly evolving list of genetically encoded fluorescent proteins (FPs) presents users with several alternatives when it comes to fluorescent fusion design. Each FP has specific optical and biophysical properties that can affect the biochemical, cellular, and functional properties of the resulting fluorescent fusions. For instance, several FPs tend to form nonspecific oligomers that are susceptible to impede on the function of the fusion partner. Unfortunately, only a few methods exist to test the impact of FPs on the behavior of the fluorescent reporter. Here, we describe a simple method that enables the rapid assessment of the impact of FPs using polyglutamine (polyQ) toxicity assays in the budding yeast Saccharomyces cerevisiae. PolyQ-expanded huntingtin proteins are associated with the onset of Huntington’s disease (HD), where the expanded huntingtin aggregates into toxic oligomers and inclusion bodies. The aggregation and toxicity of polyQ expansions in yeast are highly dependent on the sequences flanking the polyQ region, including the presence of fluorescent tags, thus providing an ideal experimental platform to study the impact of FPs on the behavior of their fusion partner.
Introduction

Since the initial characterization of the green fluorescent protein (GFP) from *Aequorea Victoria* (Chalfie et al., 1994), a wide palette of genetically encoded FPs have been developed, allowing cell biologists to simultaneously localize and track multiple cellular events/proteins in living cells (Shcherbakova et al., 2012; Thorn, 2017). FPs are derived from multiple organisms, from jellyfish to coral, and therefore, display specific biophysical properties that divert extensively beyond their respective fluorescent spectrum. These properties include brightness, photostability, and a tendency to oligomerize among others (Snapp, 2009; Thorn, 2017). Selecting monomeric FPs is an important aspect in the selection of a suitable tag when designing a fluorescent reporter, in order to minimize inappropriate interactions and alterations of the fusion partner’s function and maximize the reporter efficiency for a given cellular compartment (Costantini and Snapp, 2013; Costantini et al., 2015; Snapp, 2009). While GFP has, over time, been evolved to minimize the effect of adding the fluorescent tag to the fusion partner (Costantini et al., 2015; Yang et al., 1996; Zacharias et al., 2002), how new FP variants perform compared to GFP remains difficult to assess.

Few methods exist to characterize the behavior of FPs. Most of them involve testing biophysical properties of FPs using biochemical approaches, such as ultracentrifugation and gel filtration protocols (Baird et al., 2000; Laue and Stafford, 1999; Pédelacq et al., 2002; Pédelacq et al., 2006). Such methods have the caveat of using purified FPs in solution, offering little insight into their behavior in intact cells. The development of the organized smooth endoplasmic reticulum (OSER) assay offers a quantifiable assessment of FPs’ tendency to oligomerize in living cells (Costantini et al., 2012) by testing the ability of overexpressed FPs to reorganize endoplasmic reticulum tubules into OSER whorls (Snapp et al., 2003). This technique can successfully detect changes between monomeric and oligomeric variants of GFP and other FPs. However, it relies mostly on overexpression in transiently transfected cells, and the quantitation and image analysis can be time-consuming unless the technique is adopted as an automated data collection and analysis workflow.
In order to complement these approaches, we established an assay that takes advantage of the effect of fluorescent tags on the toxicity and aggregation of polyQ expansions in yeast (Duennwald et al., 2006a; Jiang et al., 2017). The expansion of the polyQ stretch with more than 36 repeats within the first exon of the gene encoding the huntingtin protein (Htt) is associated with Huntington’s disease (Gusella and MacDonald, 2006; Penney et al., 1997). The expression of expanded Htt\textsuperscript{ex1} in yeast results in a strong aggregation of the misfolded Htt protein coupled to a severe growth defect. Interestingly, these phenotypes are strongly influenced by the sequences flanking the polyQ stretch, including FPs (Duennwald et al., 2006a; Jiang et al., 2017). It was rationalized that the different properties of FPs can differentially affect polyQ toxicity in yeast. Indeed, compared to GFP-like FPs, red fluorescent proteins and their evolved forms have shown a reduced toxicity and aggregation (Jiang et al., 2017). This manuscript provides a detailed protocol to assess the effect of the next generation of FPs on polyQ toxicity and aggregation in yeast. This assay allows for a rapid and potentially high-content analysis of FP variants that can be used in parallel with previously characterized techniques for the optimal characterization of new FPs and can assess how they perform compared to GFP.

**Protocol**

**1. Generation of New Fluorescently Tagged Htt\textsuperscript{ex1} Reporters for an Expression in Yeast**

Note: This section has been modified from the protocol by Jiang et al. and Albakri et al. (Albakri et al., 2018; Jiang et al., 2017).

1.1. Design primers to amplify the sequence encoding the fluorescent protein or interest by PCR. The forward primer should include a leader sequence to assist the restriction enzyme during digestion (GATC), followed by a SpeI restriction site (ACTAGT) and 20 bases downstream of the ATG (excluding ATG) of the fluorescent protein gene of interest. The reverse primer should include the leader sequence (GATC), followed by a SalI restriction site (GTCGAC) and the reverse
complement of 20 bases upstream of the stop codon of the FP sequence (including stop).

1.2. Using the primers designed in step 1.1, perform the PCR reaction using a thermocycler with the following settings: heat to 95 °C for 1 min and cycle at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min per kB of PCR product. Performing 18 cycles is ample.

1.3. Run the PCR reaction on an agarose gel (0.5% in Tris-acetate-EDTA). There should a single band corresponding to the expected product size. Isolate the fragment using a gel purification kit.

1.4. The protocol employs an Httex1 vector carrying 25 (nontoxic) and 72 (HD-associated, displaying a strong aggregation) polyQ repeats. Digest both the PCR fragments and the vector with SpeI and SalI restriction enzymes for 3 h at 37 °C.

1.5. Purify the digested vector by running it on an agarose gel as in step 1.3.

1.6. Purify the digested PCR fragment using a PCR purification kit.

1.7. Ligate the resulting digested PCR fragment and p415-GAL1-FLAG-25/72QpolyQ plasmids1 using T4 ligase (1 h at room temperature). Use a 10 µL reaction (1 µL of T4 enzyme, 1 µL of 10X buffer, 6 µL of PCR fragment, and 2 µL of vector).

1.8. Transform 2 µL of the ligation reaction into 50 µL of Escherichia coli-competent cells and incubate them on ice for 30 min. Then, heat-shock the cells at 42 °C for 30 s. Add 1 mL of SOC outgrowth media and incubate at 37 °C for 1 h in a shaker. Plate 200 µL of the reaction on an LB-agar plate containing 100 µg/mL ampicillin. Incubate the plate at 37 °C overnight.

1.9. Select three individual bacterial colonies, grow them overnight in 3 mL of LB-broth containing 100 µg/mL ampicillin at 37 °C in a shaker and extract the plasmid DNA using a plasmid purification kit.

1.10. Check the plasmid by digesting 500 ng of DNA using SpeI and SalI restriction enzymes for 1 h at 37 °C and run the reaction on an agarose gel (0.5% in Tris-acetate-EDTA). There should be two bands at the right sizes of the vector (~7 kb) and the insert (size varies according to the gene of interest). Then, verify the plasmid by sequencing.
1.11. Transform the p415-GAL1-FLAG-polyQ-FP plasmids into the yeast strain W303 following a standard yeast transformation protocol.

2. Spotting Assay

2.1. Streak the yeast clones carrying 25Q/72Q tagged with the FP of interest on an agar plate containing yeast selection media (synthetic complete-SC without leucine) with glucose as the carbon source. At the same time, also streak 25Q/72Q-ymsfGFP to serve as a positive control.

Note: 25Q/72Q constructs that do not contain a fluorescent tag are not toxic and can serve as negative control.

2.2. Incubate the plates at 30 °C for 2 - 3 d.

2.3. Select up to three single colonies from the plate.

2.4. Inoculate 5 mL of SC supplemented with 2% glucose as the carbon source.

2.5. Pellet 200 µL of each overnight culture and wash it 3x with sterile distilled water.

2.6. Resuspend the cells in SC media containing 2% galactose as the carbon source to induce the expression of polyQ fusions. Incubate the galactose media overnight at 30 °C in a tube rotator. As a control, repeat this step by using glucose-containing media.

2.7. The next morning, equalize the cell densities to optical density at 600 nm (OD$_{600}$) of 0.2 in 100 µL of SC media in a sterile 96-well plate.

2.8. Prepare four fivefold dilutions of each sample with sterile water by pipetting 20 µL of the sample from the previous well into 80 µL of media in the next well.

2.9. Use a yeast pinning tool to spot the cells onto selective plates (containing glucose or galactose) and incubate at 30 °C for 2 d.

2.10. Image the plates with an image documentation device.
3. Quantification of Cell Growth in Liquid Culture

3.1. Prepare the cell cultures, following steps 2.1 - 2.5 of this protocol.

3.2. Measure the OD\(_{600}\) using a spectrophotometer.

3.3. Dilute the cells to an OD\(_{600}\) of 0.1 in 300 µL of media in a 96-well plate.

3.4. Run each sample in triplicate.

3.5. Incubate the plate in a plate reader/incubator with shaking capabilities. Set the number of samples, the temperature at 30 °C, the absorbance at 600 nm, the length of the experiments to 24 h, and the measurement intervals to 15 min, and select the continuous shaking mode.

3.6. Create the growth curve and quantify the area under the curve using scientific graphing software. The GraphPad Prism 7 is recommended. Paste the data into an XY table with three replicate values. The growth curve will be shown under the Graphs folder at the left side. To quantify the area under the curve, select Analyze at the top left and click Area under curve in XY analyses.

4. Fluorescence Microscopy

4.1. Prepare the cell cultures, following steps 2.1 - 2.5 of this protocol.

4.2. Dilute the cells 10x in growth media and transfer 200 µL of each sample to 8-well imaging chambers.

4.3. Image the cells using a confocal microscope equipped with a 63X Plan Aprochroomat objective (1.4 NA) at room temperature.

Note: The usage of a confocal microscope is optional. A standard wide-field fluorescent microscope can also be employed.

4.4. Adjust the pinhole and laser power for optimal image acquisition. Since the 72Q aggregates are much brighter than the diffuse 25Q signal, it is often required to use a different acquisition setting between the different plasmids in order to avoid saturation of the fluorescent signal.

4.5. Process the images using ImageJ (Schindelin et al., 2015) or another image-processing software. At this step, the percentage of cells that display aggregate can be calculated manually is desired.
5. Dot Blot

Note: In this protocol, dot blot is used to examine the protein expression levels. Prepare the cell cultures, following steps 2.1 - 2.5 of this protocol.

5.1. Generate protein lysates using glass beads in lysis buffer (100 mM Tris, pH 7.5; 200 mM NaCl; 1 mM EDTA; 5% glycerol, 1 mM dithiothreitol [DTT]). Add protease inhibitors, 4 mM phenylmethylsulfonyl fluoride (PSMF) and protease inhibitor cocktail, directly before use. Pellet 5 mL of the overnight culture and resuspend it in 200 µL of glass beads and 200 µL of lysis buffer. Vortex 30 s for 12 rounds. Centrifuge at 12,000 x g at 4 °C for 10 min and collect the supernatant.

5.2. Spot an equal amount of total proteins on a nitrocellulose membrane using a microfiltration apparatus. Prewet the membrane with PBS and assemble the apparatus. Connect to a vacuum source and make sure the screws are tightened. Turn on the vacuum and let the sample filter through the membrane by gravity.

5.3. Block the membrane in PBS- 0.05% Tween/5% fat-free milk.

5.4. Incubate the membrane with primary anti-FLAG antibody overnight at 4 °C. The monoclonal anti-FLAG M1 is recommended.

5.5. Wash the membrane 3x for 10 min each with PSB- 0.05% Tween.

5.6. Incubate the membrane with a fluorescently labeled secondary antibody (anti-mouse IgG) for 1 h at room temperature in PBS- 0.05% Tween/5% fat-free milk.

5.7. Wash membrane 3x 10 min with PSB- 0.05% Tween.

5.8. Image-blot using an immunoblot documentation system.

Representative results

FPs have different biophysical properties, including their tendency to oligomerize, that can affect the behavior of their fusion partners in the context of fluorescent reporters. This protocol describes a simple method where multiple FPs can be fused to toxic polyQ expansions. Since polyQ toxicity is highly dependent on the sequences flanking the polyQ stretch (Duennwald et al., 2006a), this assay allows a rapid and direct comparison of fluorescent polyQ fusion reporters (Figure 1). A non-HD-associated polyQ length
(25Q) is used as a negative control and does not display significant toxicity or aggregation (Duennwald, 2013; Duennwald et al., 2006a; Duennwald et al., 2006b; Jiang et al., 2017). 72Q is employed to obtain the HD-like phenotypes, including strong growth inhibition and polyQ aggregation. Importantly, the Htt$^{ex1}$ coding sequence employed lack the proline-rich domain that follows the polyQ stretch. In the presence of the proline-rich domain, Htt$^{ex1}$ is not toxic in yeast (Duennwald et al., 2006a). In this assay, an Htt$^{ex1}$ fused to a yeast-optimized monomeric variant of superfolder GFP (Pédelacq et al., 2006) (ymsfGFP) (Jiang et al., 2017) is used as a positive control as previously described (Jiang et al., 2017). The constructs also contain a FLAG epitope tag at the N-terminus of Htt$^{ex1}$. This allows detection of the different fusions with the same antibody (anti-FLAG) for biochemical analysis. As a proof-of-principle, 72Q Htt$^{ex1}$ fused to yeast-optimized TagBFP (yomTagBFP) (Lee et al., 2013) does not result in slow growth measured by either spot assays on agar plates or growth in liquid media (Figure 2), indicating that the nature of the fluorescent tag can indeed impede polyQ expansion behavior in cells.
Figure 1: Workflow diagram for the analysis of the effect of fluorescent protein tag on the aggregation and toxicity of polyQ expansion proteins in yeast.

First, FPs are cloned into yeast expression vectors encoding a galactose-inducible version of FLAG-tagged Htt\textsuperscript{ex1} harboring either 25Q (nontoxic) or 72Q (HD-associated, aggregating and toxic) repeats. Clones are selected and verified by sequencing and, subsequently, transformed in yeast. Following the induction of polyQ fusion expression by incubation in galactose-containing media, either spotting assays on agar plates or growth liquid media can assess the polyQ toxicity. PolyQ aggregation is analyzed by fluorescence microscopy. A relative expression of the different constructs is assessed using dot blot.
Figure 2: Representative growth assay results following the expression of Htt$^{ex1}$ fluorescent fusions in yeast.
Yeast expressing either 25Q or 72Q Htt$^{ex1}$ fused to ymsfGFP or yomTagBFP was cultured in glucose (control) or galactose media (polyQ-induced) overnight and either (A) spotted on agar plates or (B) incubated further in liquid media to assess growth under the different conditions. While 72Q-ylmsfGFP induces a significant growth defect, 72Q-yomTagBFP displays a growth phenotype similar to the nontoxic 25Q counterparts.
Aggregation of the fluorescent polyQ fusions can be assessed using fluorescence microscopy. 72Q-ymsGFP displays significant aggregation compared to 25Q. However, the 72-yomTagBFP fluorescent signal remains diffused throughout the cytoplasm (Figure 3). In most of the cases, it is not recommended to use the same image acquisition settings (laser power, exposure time) to acquire both 25Q and 72Q images. The aggregates in the 72Q-expressing cells are much brighter than the diffused 25Q signal. Therefore, under imaging conditions used to acquire 72Q images, the diffused 25Q signal may appear very weak or not be visible at all. Appropriate acquisition settings should also be applied to minimize the saturation during the imaging of the 72Q-expressing cells.
Figure 3: Representative fluorescent images of Httex1 fluorescent fusions in yeast.
Yeast expressing either 25Q or 72Q Httex1 fused to ymsfGFP or yomTagBFP was cultured in glucose (control) or galactose media (polyQ-induced) overnight and imaged with a confocal microscope. While the 72Q-ymsfGFP expression results in a strong polyQ protein aggregation, 72Q-yomTagBFP displays a diffused cytoplasmic signal similar to the nontoxic 25Q counterparts.
Expression levels of the various polyQ fusions could affect toxicity. Detergent-insoluble amyloids, such as polyQ aggregates, are notoriously difficult to study biochemically and are not suitable for an analysis by standard SDS-PAGE. Therefore, dot blots can be performed to assess protein levels. The inclusion of the FLAG tag at the amino terminus end of Httex1 allows detection of all the fluorescent fusions simultaneously, despite the presence of FPs (Figure 4). Alternatively, semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) can be performed to assess the formation of polyQ oligomers (Jiang et al., 2017). A detailed protocol and video are available in Halfmann and Lindquist (Halfmann and Lindquist, 2008).
Figure 4: Representative dot blot analysis of Htt\text{ex1} fluorescent fusion expression in yeast. Yeast expressing 25Q, 46Q, 72Q, or 103Q Htt\text{ex1} fused to CFP was cultured in galactose media (polyQ-induced) overnight and processed for dot blot analysis. Fivefold dilutions of the cell lysates are shown.
Discussion

In this article, various assays to measure the aggregation of Htt\textsuperscript{ex1} polyQ expansions and their effect on yeast growth were employed as a model to study how different fluorescent proteins alter their fusion partners in the context of fluorescent reporters. Using a GFP variant (ymsfGFP) as a positive control, we showed that this detects significant changes in polyQ toxicity and aggregation between different fluorescent tags and allows for a direct and rapid comparison of the polyQ-FP fusion performance against GFP-tagged constructs (Albakri et al., 2018; Jiang et al., 2017).

While the present protocol focuses on fluorescent proteins, various parts of the protocol could be readily adapted to test the effects of other protein tags. In addition, the present protocol employs low-copy yeast centromeric vectors that can vary in terms of copy numbers (generally one to two copies) present in cells (Sikorski and Hieter, 1989). Using integrative vectors to ensure a uniform expression across experimental conditions could circumvent this problem. While this protocol has been optimized for use in the W303 background, other S. cerevisiae strains can be employed. However, susceptibility to polyQ toxicity should be determined using the ymsfGFP-tagged vectors prior to designing new constructs. In certain cases, it may be appropriate to employ high-copy (2µ) vectors to generate a significant growth defect. It is also suggested to test multiple isolates following the yeast transformation with polyQ vectors to avoid selecting spontaneous suppressors showing a reduced polyQ toxicity. Of note, the W303 yeast strain (Thomas and Rothstein, 1989) is usually used as it is more sensitive to polyQ toxicity than other S288C derivatives, such as BY4741/BY4742 (Brachmann et al., 1998), thus allowing for a wider range of growth phenotypes. Importantly, strains employed for this assay need to carry the Rnq1 prion protein since rnq1\Delta cells do not display polyQ toxicity and aggregation (Meriin et al., 2002). It is also important to use Htt\textsuperscript{ex1} constructs carrying the amino-terminal FLAG tag and lacking the proline-rich domain. Other variations of the fusion design may alter toxic phenotypes (Duennwald et al., 2006a). Finally, the induction of the polyQ fusion expression in galactose-containing media is a critical step of the protocol (Duennwald, 2013). When transferring cells from glucose- to galactose-containing media, it is important to wash the cells at least three times with
sterile water to eliminate all traces of glucose that could contribute to repressing the induction of the Gal1 promoter (Mumberg et al., 1995). When performing spotting assays, culturing the cells overnight in galactose media to induce the expression of the fusion can exacerbate the toxic phenotype of the 72Q fusion and help discriminate changes in growth across different fusions (Jiang et al., 2017).

As a limitation, previous studies did not observe differential effects between a nonmonomeric version of CFP (a GFP derivative) and ymsfGFP (Jiang et al., 2017). Thus, at least for GFP variants, this assay may not be sensitive enough to discriminate between monomeric and oligomeric variants, highlighting the need to complement the polyQ toxicity assays with other standard methods, such as the OSER assay (Costantini et al., 2012) and biochemical analysis (Baird et al., 2000; Laue and Stafford, 1999; Pédelacq et al., 2002; Pédelacq et al., 2006) that can directly assess oligomerization. Also, it should be noted that FPs can behave differently in yeast compared to in vitro assays or their expression in other organisms (Lee et al., 2013).

Collectively, these methods allow researchers to rapidly characterize new FPs and measure their effect on their fusion partner. In the future, this protocol will enable the quick screening of new derivatives of previously characterized FPs to identify mutants that behave similarly to GFP variants, which are still the gold standard measure for FP reporters. While this protocol focuses on fluorescent proteins, it can easily be adapted to screen for the effects of other genetically encoded tags, such as SNAP-tag (Keppler et al., 2003) and SunTag (Tanenbaum et al., 2014).

In conclusion, this protocol provides a rapid and easily scalable assay to enable further characterization of the new generation of FPs and other genetically encoded tags to guide research in fusion protein design.
References


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Includes cover image.


**Selected Presentations:**

Presented research poster “Sfp1 links TORC1 and cell growth regulation to the yeast SAGA-complex component Tra1 in response to polyQ proteotoxicity” at PRinCE (Proteostasis Researchers in Canada, Eh) inaugural meeting, *Toronto, ON, Canada, Jun. 10, 2019.*

Presented research poster “Sfp1 regulates the SAGA component Tra1 during proteotoxic stress in *Saccharomyces cerevisiae*” at TREnd (Toronto RNA Enthusiasts Day) 2018, *Toronto, ON, Canada, Jul. 31, 2018.*
Presented research talk “Regulation of Polyglutamine toxicity in a yeast model of Huntington’s Disease” at Anatomy and Cell Biology seminar series, 
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Presented research poster “Impaired TORC1 signalling underlies Polyglutamine toxicity in a yeast model of Huntington’s Disease” at CSMB (Canadian Society for Molecular Biosciences) annual meeting 2017, 
*Ottawa, ON, Canada, May 17, 2017.*

Presented research poster “A MAP kinase pathway in a yeast model of Huntington's disease” at ASCB (The American Society for Cell Biology) annual meeting 2016, 
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Presented research poster “Regulation of endoplasmic reticulum stress and ribosome biogenesis in a yeast model of Huntington’s disease” at SFN (Society for Neuroscience) annual meeting 2015, 
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