THE APPLICATION OF ANALYTICAL ULTRACENTRIFUGATION WITH FLUORESCENCE DETECTION SYSTEM TO THE STUDY OF MACROMOLECULAR COMPLEXES IN BIOLOGICAL SYSTEMS

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Abstract
Using the novel technique of analytical ultracentrifugation with fluorescent detection (AU-FDS), I have conducted the analysis of the properties of two types of intracellular macromolecular complexes: the translational mRNP complex and the intermediate soluble aggregates present in Huntington's disease. With AU-FDS it is possible to differentiate a broad size range of soluble molecules from complex mixtures and determine the size and abundance of each individual complex based on its sedimentation rate under a centrifugal field.

In the first part of my thesis, the characteristics of the translational repressor SBP1 were determined by analyzing the mRNP complexes it was associated with. SBP1, an RNA binding protein, plays a role in stress granules and P-body function which are involved in mRNA degradation processes. SBP1, whose role in translation is unknown, was found to co-immunoprecipitate the 77S monosomal translating mRNP complex. Two types of 77S complexes containing SBP1 were identified. The majority of the 77S complexes lacked other translation initiation factors, suggesting that this 77S is in the late elongation/early termination phase. SBP1 was also present in a 77S complex that contained other initiation factors but may not be active in translation, possibly as it is located in P-bodies or stress granules.

In the second part of this thesis, the ability of the huntingtin protein, HTT-103Q, to form soluble aggregates was analyzed. HTT-103Q is a glutamine rich protein fragment previously identified as a self-propagating protein capable of forming insoluble amyloids and to cause Huntington's disease. AU-FDS analysis identified soluble aggregates of HTT-103Q. A series of intermediate aggregates with sizes in between 30S to 180S were observed that dramatically changed in their abundance as a function of time. The deletion of chaperones HSP70 or HSP104 strongly suppressed the presence of these complexes and correspondingly suppressed amyloid formation and cell toxicity. In contrast, overexpression of the chaperones had less effect on the formation of these soluble aggregates. I also studied the effects on HTT-103Q aggregation of two aging factors known to be involved in amyloid aggregation. Overexpression of HSF1 and deletion of SIR2 significantly impaired the production of the HTT-103Q soluble aggregates. Overall, these results indicate that the 30-180S soluble aggregates detected for HTT-103Q may be critical to amyloid formation and cell toxicity.

These combined studies indicate that AU-FDS can identify and characterize novel macromolecular complexes in biological systems.

Keywords
Analytical Ultracentrifugation, Huntington's disease, mRNP complex, Poly-glutamine, SBP1, Biochemistry

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THE APPLICATION OF ANALYTICAL ULTRACENTRIFUGATION WITH
FLUORESCENCE DETECTION SYSTEM TO THE STUDY OF
MACROMOLECULAR COMPLEXES IN BIOLOGICAL SYSTEMS

BY

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Bachelor of Science, Wuhan University, 2006

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DISSERTATION

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in Partial Fulfillment of

the Requirements for the Degree of

Doctor of Philosophy

in

Biochemistry

December, 2015
This thesis/dissertation has been examined and approved in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry by:

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On August 25th 2015
Dedication

I dedicate my dissertation work to my family. To my parents, Yan Yi and Jingou Xi, far away in China, the distance doesn’t stop my receiving your steady love and support. To my parents-in-law, I will always appreciate your devotion to us. To my wonderful son Travis, be happy every day. Finally, special thanks to my wife Jingmin Chen, I am grateful to have you in my life, I love you!
ACKNOWLEDGMENTS

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ABSTRACT

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By

Wen Xi

University of New Hampshire, December, 2015

Using the novel technique of analytical ultracentrifugation with fluorescent detection (AU-FDS), I have conducted the analysis of the properties of two types of intracellular macromolecular complexes: the translational mRNP complex and the intermediate soluble aggregates present in Huntington’s disease. With AU-FDS it is possible to differentiate a broad size range of soluble molecules from complex mixtures and determine the size and abundance of each individual complex based on its sedimentation rate under a centrifugal field.

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These combined studies indicate that AU-FDS can identify and characterize novel macromolecular complexes in biological systems.
General Introduction

Analytical ultracentrifugation (AU) is a classical method to determine the molecular weight and size of protein and nucleic acids in biological researches. For decades, AU has been the standard method to determine the characterization of macromolecules in solution. Without an interaction with any matrix or surface, AU is able to monitor the sedimentation, under a centrifugal field, of different components in solution. From this AU data the sizes and quantities of these components can be calculated. AU can be used detect proteins and other macromolecules in solution using either a spectrophotometric system (AU-Abs) that detects absorbances ranging from 190 nm to 800 nm or by a newly developed fluorescence detection system (AU-FDS) that detects green fluorescent protein (GFP) (MacGregor I et al 2004).

As the centrifugation run begins, macromolecules start to deplete from the meniscus and form a concentration boundary moving towards the bottom of the centrifugation cell (Fig 1). The rate of the boundary movement of a spherical molecule can be defined as the sedimentation coefficient \( s \), which can be determined by the parameters given by the Svedburg equation:

\[
\frac{u}{\omega^2 r} = \frac{M(1 - \bar{v} \rho)}{N_A f} = \frac{MD(1 - \bar{v} \rho)}{RT}
\]

Eq.1 Svedburg equation. Where \( u \) is the observed radial velocity of the macromolecule, \( \omega^2 r \) is the centrifugal field, \( M \) is the molar mass, \( \bar{v} \), the partial specific volume, \( \rho \) is the density of the solvent, \( N_A \) is Avogadro’s number and \( f \) is the frictional coefficient, \( D \) is the diffusion coefficient, and \( R \) is the gas constant. The \( s \)-values are commonly reported in Svedberg (S) units, which correspond to 10^{-13} sec (Lebowitz J et al, 2002).
For all the samples we tested in the project, the S-values can be normalized as $S_{20,w}$ by the given condition at 20°C in water with the following equation:

$$S_{20,w} = S_{T,B} \left( \frac{\eta_{T,B}}{\eta_{20}} \right) \left( \frac{1 - \bar{v}\rho}{1 - \bar{v}\rho}_{T,B} \right)$$

Eq. 2 Standard correction equation, where $\eta$ is viscosity, $T, B$ are the experimental temperature and buffer condition (Laue T et al, 1992).

The molecular weight of a spherical macromolecule can be calculated by the following equation:

$$MW = \left( S_{20,w} \times 3.16 \right)^{3/2}$$

Eq. 3 Molecular Calculation

However, it is practically not possible to precisely calculate the MW with the $S_{20,w}$, as molecules tend not to be ideally spherical. A 2:1 length to width ratio subject has an S-value 1.4-fold smaller than an equal mass spherical subject due to a change in the frictional coefficient.
Fig. 1 Concentration boundary in centrifugal field
AU has several advantages compared with other methods of characterizing biological macromolecules. AU requires no chemical or physical contact to the samples. Samples are required only to be soluble and the composition of the solution is immaterial, which allows researchers to maximally retain the native state of the molecules by using an optimized buffer system. Moreover, AU provides a broader detection range and a better resolution than other methods as it monitors the sample movement in real time while sucrose gradient and chromatography only takes a snapshot at a particular end point. Furthermore, AU is able to distinguish different molecules/complexes in a mixture at one time, which requires no secondary purification of the samples. AU samples can be prepared in a remarkable short period of time, and even crude extract samples can be analyzed. Importantly, AU results can easily be optimized for different purposes by simply changing the parameters in the calculation program.

In this thesis, I used AU as the primarily method to conduct research on macromolecular protein complexes. The complexes that were studied were the translational mRNP complex and the intermediate amyloid propagation complexes in Huntington’s disease. The study of the translational mRNP conventionally has relied on indirect methods such as in vitro reconstitution of the mRNP, mass spectrometry, sucrose gradient and X-ray crystallographic studies (Strunk BS et al 2009; Zhang C et al 2014; Sampath P et al 2011; Melamed D et al 2007). None of these methods is perfect. In vitro reconstitution methods are good for studying the process of translation but only known factors can be tested. This makes the reconstituted translation complex a minimized model that contains only necessary factors of
translation, and, therefore, in vitro reconstitution is not capable of recapitulating the actual process of translation and all the non-essential regulatory factors of translation. The mass spectrometric technique helps identify several potential translational factors in the complex, but the analysis is based on fragmentized peptides not on the whole complex. The actual stoichiometric composition of the complex is difficult to determine from mass spectrometric procedures. Moreover, mass spectrometry usually can only analyze a single molecule or complex and cannot differentiate between different sized complexes in its analysis. The sucrose gradient method is similar to AU as both use ultracentrifugation to differentiate multiple complexes in solution. The difference is that sucrose gradient relies on the density equilibrium whereas AU uses sedimentation velocity. This allows the size of complexes to be quantified by S values from the AU results. In addition, the introduction of excessive amount of sucrose in solution may affect the integrity of the complexes. AU also has the option of FDS to detect GFP samples, a critical advantage since most yeast proteins have been tagged with GFP in vivo. Moreover, sucrose gradient analysis can only be monitored at one moment while AU analysis can be scanned over many time periods, providing better approximations of protein complex presence. Most importantly, the identification of particular proteins in sucrose gradient analysis is limited to taking only fifteen or so slices across the centrifugation cell, creating imprecision in the actual location of proteins in particular complexes. In contrast, AU analysis can take several hundred “slices” across the range of protein sizes, increasing by several orders of magnitude the precision for locating protein presence. Therefore, AU is a unique technique compared with these other methods. As described, we can use AU to analyze the target translation mRNP in its native state, obtain
size and stoichiometric information, all without losing the native state of the complexes.

The analysis of Huntington’s disease amyloid propagation with AU is also a first attempt at differentiating the actual aggregate sizes present in huntingtin aggregation. Previously, there has only been one attempt made with AU to analyze this process (Olshina MA et al 2010). However, only recombinant proteins and in vitro aggregation were analyzed, the speed of centrifugation they used was only good for monomeric protein detection, and the effects of chaperone abundance changes were not tested. Almost all the other previous research on the amyloid formation have been focused on the detectable phenotypes of the cells. These studies relied on canonical techniques like fluorescent microscopy and western blot analysis (Krobitsch S et al 2000; True HL et al 2000; Meriin AB et al 2002). The presence of protein aggregates was inferred from crude filter assays and SDS-PAGE analysis in which aggregated proteins migrated at best as smears (Kimura Y et al 2001). The use of AU on huntingtin aggregates makes it possible to observe ranges of aggregate sizes, how the aggregates change in size during amyloid formation, and the effect of a various factors on aggregate formation. These AU research analyses on aggregate formation may hence provide a potential method for future studies in this area.
Chapter I Study of translational mRNP complexes associated with SBP1 in *Saccharomyces cerevisiae*

**Introduction**

Protein translation is a complex and highly organized process requiring the cooperation of ribosomes and various protein factors. During the stages of translation, initiation, elongation, and termination, different protein factors associate and dissociate from the translating ribosome. These protein factors mediate the interactions between the ribosome and mRNA, and, in addition, serve as the regulatory targets of translational regulation. To thoroughly understand translational regulation, it is necessary to investigate what are the protein factors associating with ribosomes during different translational stages. In the first part of this thesis, I studied the role and presence of the SBP1 protein in translating ribosomal complexes. SBP1 is a single strand RNA-binding protein, which previously was found to be involved in mRNA degradation and translational repression (Segal SP *et al* 2006).
Eukaryotic Translation Initiation

In eukaryotes, translation initiation relies principally on the cap-dependent pathway, which requires the m7G cap on the 5’ end of the mRNA. The detailed mechanism of this pathway is described in the following sections.

Assembling the Pre-Initiation Complex (PIC)

In the first step of initiation, a 43S pre-initiation complex (PIC) is assembled. To create the 43S complex, the tRNA carrying the methionine (Met-tRNAi) binds to the eukaryotic initiation factor 2 (eIF2). eIF2, in turn, is activated by binding to one molecule of GTP, and consequently forms the ternary complex (eIF2-GTP-Met-tRNAi). This ternary complex, with other factors, eIFs 1, 1A, 3 and 5, then binds to a 40S ribosomal subunit, to form the 43S pre-initiation complex (PIC).

Closed-loop mRNP

The mRNA is activated by another set of protein factors, with eIF4E binding to the 5’-Cap of the mRNA and the poly(A) binding protein (PAB1) binding to the 3’ Poly (A) tail of the mRNA. eIF4G binds to both eIF4E and PAB1, forming a closed-loop complex (Mangus DA, et al, 2003; Amrani N, et al, 2008), in which the head and tail of the mRNA is connected by eIF4G. eIF4G also contains RNA binding sites, and facilitates as a scaffold the binding to eIF4A and eIF4B. eIF4A is a DEAD-box helicase. Together with eIF4B, eIF4A promotes conformational changes of the mRNA, thereby unwinding and activating the mRNA which promotes the binding of the PIC.
Ribosome Assembly

The 43S PIC loads onto the closed-loop mRNA complex at the 5’ proximal regions of the mRNA, thereby forming the 48S initiation complex. The 48S complex then moves linearly along the mRNA, base-by-base scanning for the first AUG codon. This process is promoted by eIF4A which removes the secondary structures on the 5’ untranslated region (5’UTR). Once the start codon has been recognized, eIF2-GDP and eIF5 dissociate from the complex and are replaced by the 60S subunit, which forms the translationally active mRNP. This monosomal translating complex has been identified as a 77S translation complex (Wang et al 2012). Additional ribosomes coming on the 5’ end of the mRNA create polysomal translating complexes that are much larger (116S and larger). Translation then processes through the elongation phase.
Fig. 1.1 Eukaryotic Translation Initiation
Poly (A) binding protein PAB1

PAB1 (PABP in human) is an evolutionarily conserved protein that has a high binding affinity to poly(A) sequences. During translational initiation, PAB1 is physically associated with the poly(A) tail of the mRNA. PAB1 binds 25-30 nucleotides (Sachs AB et al, 1987), and therefore for a typical yeast poly(A) tail of 75 A’s, one would expect three PAB1 molecules to be bound. PAB1 consists of 577 amino acids and contains four highly conserved RNA recognition motif (RRMs) domains. Binding to poly(A) stimulates PAB1 to bind eIF4G and form the closed-loop mRNP. As described above, these interactions are important for aiding the formation of the activated 43S complex, the forming of the 48S complex and the further assembly with the 60S complex to form the 77S translating complex (Wang X. et al 2012). Deletion of the PAB1 gene results in lethality to yeast (Wyers F et al 2000). Moreover, PAB1 is known to control the length of the poly(A) tail of mRNA during different stages of translation (Minvielle-Sebastia et al., 1997; Brown et al., 1998). There are also interactions between PAB1 and the mRNA deadenylase PAN2/PAN3, allowing the trimming of the poly(A) tail of newly transcribed mRNA from more than 200 nucleotides into its final length of about 50 to 90 nucleotides. Binding of PAB1 also stabilizes the mRNA by preventing decay. mRNA degradation generally starts with the releasing of PAB1 followed by the shortening of the poly(A) tail (deadenylation). (Zhang C. et al 2013)
Eukaryotic Translation Termination

Translation termination takes place when one of the stop codons enters the ribosomal A site. Eukaryotic termination factor 1 (eRF1), a protein factor structurally similar to a tRNA, is responsible for recognizing and interacting with the stop codons. (Fig. 1.2) Driven by the hydrolysis of GTP by eRF3 (bound to eRF1), the newly synthesized peptide chain is released. The ribosome is disassembled by eRF3 and may be recycled back to the same mRNA for another round of translation, if the mRNA does not enter degradation (Pisarev et al., 2007). However, the ribosomal recycling factors (RRFs) mediating the recycling process are only found in prokaryotes: to date no homologs of RRF have been discovered in eukaryotes. Instead, eukaryotic ribosomal recycling is promoted by the ABC family protein ABCE1 (RLI1 in yeast) (Hirokawa et al., 2005; Hirokawa et al., 2006). The detailed mechanism of eukaryotic translation termination is still not fully understood yet.

Fig. 1.2 Translation termination
mRNA Turnover

mRNA Degradation

The degradation of mRNA plays an important role in translational regulation as well. In eukaryotic cells, mRNA degradation is usually initiated by shortening of the 3’ poly(A) tail, a process known as deadenylation, followed by the removal of the 5’ cap structure (decapping) by DCP1/DCP2 and 5’-3’ exonucleolytic digestion of the mRNA by XNR1. An alternative 3’-5’ degradation by exosome may also occur, at a slower rate.

P-body mRNA degradation

Previous studies show that translation and mRNA degradation are usually two competitive pathways in cells. As described, the removal of the cap structure, which is a key factor in translational initiation (known as “cap” dependent translational initiation), is also a key step in mRNA degradation. Moreover, initiation factors binding to the mRNA block the decapping of mRNA by DCP1/DCP2 (Dunckley, T. 1999), and translation inhibitors stimulate mRNA degradation (For review, see Balagopal, V. et al. 2009). Studies in yeast suggest that the degradation of mRNA starts with the transition of translation mRNP into degradation mRNP, with the translation machinery being replaced by the degradation machinery. The degradation mRNPs are then concentrated in mRNP granules called processing bodies (P-bodies) (Fig. 1.3). Although their complete composition is not yet known, P-bodies are found to including the decapping enzymes DCP1/DCP2, the activators of decapping DHH1/RCK/p54, PAT1, SCD6/RAP55, EDC3, the LSM1–7 complex, and the exonuclease, XRN1 (Parker, R. 2007). In general, P-bodies are considered to be essential to the degradation of mRNA and also to
play roles in nonsense-mediated mRNA decay, adenylate-uridylate-rich element mediated mRNA decay, and microRNA induced mRNA silencing (Kulkarni M, et al 2010). However, mRNAs entering P bodies do not all end up being degraded. Evidence shows there is some mRNA concentrated in P-bodies that can reenter translation when translation is restored (Brengues M et al 2005). So far, it is not clear what mechanism is determining the fate of mRNA in P-bodies. As an important part of translational regulation, further studies on P-bodies are necessary to be done.

**Stress Granules**

Another cytoplasmic granule (stress granule) formed under stress is observed that often docks with the P-body (Nover, L. et al 1989). Similar to the P-body, the stress granule is also a dynamic cluster of non-translating mRNP. Instead of associating with degradation factors as in the P-body, mRNA in stress granules stimulated by heat shock (for instance) is associated with the 40S ribosomal subunit and a subset of initiation factors eIF4E, eIF4G, PAB1 (Anderson P. et al 2008). Although, the exact composition of stress granules is not fully understood yet, several findings suggesting stress granules have variable compositions under different types of stress. Stress granules formed under glucose deprivation do not contain the 40S subunit, as an example. This difference in composition is a result of different blocks in translational initiation caused by the different stresses (Wang et al., 2012).

According to their composition and behavior, stress granules are likely to temporarily hold the mRNPs. mRNA in stress granules may either turn back to translation after removal of the
stress, or enter P-bodies, with translational initiation factors being replaced with degradation factors, in which it proceeds to decay. A reverse transition is also observed during glucose deprivation in which stress granules are formed after the formation of P-bodies, suggesting the flow of mRNA in the direction of translation → P-body → stress granule (Buchan JR. 2008).

Fig. 1.3 The Life-Cycle of mRNA
Previously known as SSB1, SBP1 is a single-stranded RNA binding protein that contains two RNA recognition motifs (RRM) domains (Fig. 1.4). Initially, SBP1 was localized to the nucleolus and was found to associate with small nucleolar RNA snoRNA10 and snoRNA11 (Clark MW et al 1990). These data suggested that SBP1 was involved in ribosome biogenesis. However, deletion of SBP1 did not lead to any notable phenotype in yeast. Later studies have shown that in addition to the ability to bind with RNA, SBP1 could interact directly with eIF4G to suppress translation (Rajyaguru P et al 2012). SBP1 could also suppress a conditional allele in a decapping enzyme. Overexpression of SBP1 significantly reduced the overall half-life of mRNA by promoting both 5’-3’ and 3’-5’ mRNA degradation, thereby rescuing strains with a lethal degradation defect. These data suggested a role for SBP1 in both translational repression and mRNA degradation.

In support of these roles, SBP1 was also found to regulate the formation of P-bodies under the stress of glucose deprivation. For example, SBP1 was co-localized with P-bodies and stress granules, and deletion of Sbp1 could affect the aggregation of P-bodies (Segal SP et al 2006, Mitchell SF et al 2013). Furthermore, our lab has previously identified SBP1 as a component of the mRNP active in translation, supporting a role for SBP1 in the process of translation (Zhang C et al 2014).
For the first chapter of this thesis, the primary subject was to identify the role of SBP1 in translation. This problem was addressed in particular by determining the composition and function of the mRNP complexes that co-immunoprecipitate with SBP1. We had successful experience on performing affinity purification on protein complexes associating with FLAG peptide tagged translational protein factors in previous studies in our lab. As described above, AU analysis would allow us to distinguish protein complexes by size from a mixture of complexes. In this project, a Flag peptide tagged SBP1 expressing plasmid was constructed and several strains of yeast expressing FLAG-SBP1 were created. In contrast with our previous findings using FLAG-PAB1 to identify translation complexes (Wang et al 2012 and Zhang et al 2014), the complexes associating with SBP1 were found to have low abundance of other protein factors considered to be critical to translation initiation. These data suggested that such FLAG-SBP1 complexes are more likely close to the end of the mRNA life after the initiation factors have been removed from the mRNA. Stoichiometric analysis using AU-FDS conducted with these strains suggests that SBP1 is associating with a different set of translating mRNPs than is PAB1.
Materials and Methods

Cloning of FLAG-SBP1

Vector pWX01 used for expressing FLAG-SBP1 was a derivative of vector pG-1 (Fig. 1.5), which is a high copy expressing vector containing a yeast glyceraldehyde-3-phosphate dehydrogenase gene (GPD) promoter and the TRP1 gene for yeast transformant selection (Schena M et al 1991). A FLAG-peptide gene sequence and four extra cloning sites were inserted in the pG-1 vector between BamHI and SalI, to create a vector that fuses a FLAG-tag to the N’ terminal of the proteins expressed with this plasmid.

pWX01 insert sequence:

\[
\begin{align*}
5' & \cdots \text{GGATCC} \text{ATG GACTACAAGGATGACGACGACAAG GGTACC CCATGG} \\
& \text{BamHI} \quad \text{FLAG} \quad \text{KpnI} \quad \text{NcoI} \\
& \text{GAGCTC CTCGAG GTCGAC} \cdots 3'
\end{align*}
\]

\( \text{SacI} \quad \text{XhoI} \quad \text{SalI} \)

Fig. 1.5 pG-1 expression vector

The fragment of the SBP1 gene was amplified from a previous SBP1-GFP construct by PCR,
with following primers:

Sbp1-Upstream 5’-GGCA **GGTACC** ATGTCTGCTGAAATTGAAGAA-3’

\[ \text{KpnI} \]

Sbp1-downstream 5’-CCG **CTCGAG** TTCTTGCTTTTCTTCAGAACC-3’

\[ \text{XhoI} \]

In this case, digestion with restriction enzyme **KpnI** allowed insertion of the 5’end of **SBP1** and with **XhoI** at the 3’end.

The plasmid pWX03 was constructed with the insertion of the **SBP1** gene between designated sites of the vector pWX01. The expression of FLAG-SBP1 was verified by western blot analysis with Anti-FLAG antibody. A trp1-I/URA3 (pWX03tU) conversion was made by using ’marker swap’ plasmids (Cross FR, 1996) through homologous recombination.

**Yeast growth conditions and treatments**

Yeast strains used in this study are listed in Table 1. All the yeast strains are isogenic (except for RP2191), with the DNA sequence encoding GFP fused to a different gene for each individual strain as described. Yeast cells were grown at 30ºC in synthetic complete (SC) medium until mid-log phase (OD600 = 0.8-1.2), with 2% glucose and appropriate amino acids deficiency for strain selection, respectively (Kaiser C et al 1994). For glucose deprivation samples, yeast pelleted cells were washed twice with glucose-free medium and incubated for an extra 10 minutes without glucose. For samples involving reinitiation of translation after glucose depletion, 2% glucose was added back to the culture after the incubation of 10 minutes in glucose-free medium, with an extra incubation of 1 or 10 minutes depending on the experiment. In AU analysis, 200 mL yeast cultures of each samples were
used, yeast pellets were collected by 8000 g centrifugation for 3 minutes and generally were stored at 80°C for later use. The protein content of yeast cells was extracted by the glass bead method with cell lysis buffer containing 1mg/mL of PMSF, 1:500 dilution of Protease Inhibitor Cocktail (Sigma-Aldrich P8215), 0.05 M of Tris Base, 0.15 M of KCl, 2 mM of MgCl₂ and 10% glycerol.

The FLAG-tag and associated mRNPs were purified by affinity purification with 100 μL of ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich) at 4°C for 2 hours, followed by a wash with cell lysis buffer without the Protease Inhibitor Cocktail. The mRNPs were eluted with 500 μL of 0.2mg/mL FLAG® Peptide (Sigma-Aldrich) at 4°C for 40 minutes.

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP1950/WX03tU</td>
<td>*Mata ura3 leu2 his3 met15 eIF5B-GFP-[HIS3] [URA3-Flag-SBP1]</td>
</tr>
<tr>
<td>RPL6B/WX03tU</td>
<td>Isogenic to RP1950 except *RPL6B-GFP-[HIS3]</td>
</tr>
<tr>
<td>RPL7A/WX03tU</td>
<td>Isogenic to RP1950 except *RPL7A-GFP-[HIS3]</td>
</tr>
<tr>
<td>RPL25A/WX03tU</td>
<td>Isogenic to RP1950 except *RPL25A-GFP-[HIS3]</td>
</tr>
<tr>
<td>RP1946/WX03tU</td>
<td>Isogenic to RP1950 except *CDC33-GFP-[HIS3]</td>
</tr>
<tr>
<td>RP1947/WX03tU</td>
<td>Isogenic to RP1950 except *TIF4631-GFP-[HIS3]</td>
</tr>
<tr>
<td>RP2191/WX03tU</td>
<td>*Mata leu2-3,112 trp1 ura3 his4 cup1::LEU2/PGK1 pG/MFA2 pG/PAB1-GFP(NEO) [URA3-Flag-SBP1]</td>
</tr>
<tr>
<td>RP2137/WX03tU</td>
<td>Isogenic to RP1950 except *SBP1-GFP-[HIS3]</td>
</tr>
<tr>
<td>GSSD1/WX03tU</td>
<td>Isogenic to RP1950 except *SSD1-GFP-[HIS3]</td>
</tr>
</tbody>
</table>

Table 1.1, Yeast strains used in this study.
AU analysis

Samples were loaded to the two-channel charcoal/Epon60K sedimentation velocity cells, with approximately 350 µL per channel. The centrifugation was started with a speed of 15000 RPM, which was previously shown to be suitable for differentiating complexes ranged from 20S to 250S in size (Wang X. et al 2012). All the experiments were conducted at a temperature of 20°C. For the AU-FDS analysis, at least 200 scans of samples were taken from each experiment, in about 5 hours, a period of time enough for the major concentration boundary to migrate to the bottom of the cell. For AU-Abs analysis, because only one sample can be scanned in each scan, the scan numbers of each individual samples are determined by the numbers of samples loaded to the device. A total of 300 scans were typically used for the analysis. The raw data was analyzed by Sedfit (Schuck P. 2000), with the model c(s) distribution and proper parameters (Fig. 1.6). The calculated data set of S-values was adjusted by multiplying the viscosity factor of 1.52 for the presence of 10% glycerol in the buffer.
Stoichiometry Analysis

The stoichiometry of the mRNP components in the 77S translation complex and other complexes was calculated based on the fact that mRNP component quantities are proportional to the size of the relevant peaks in the AU results. The size of a peak was calculated by adding up all the c(s) values within the peak. The amount of a certain protein in the mRNP, \( A_{(FDS)} \), can be calculated from the AU-FDS result of the GFP tagged strains, and the amount of total mRNP, \( A_{(Abs)} \), can be calculated from the AU-Abs results of the samples, respectively. Therefore, the abundance of the protein in the complex can be regarded as the peak size ratio of \( A_{(FDS)} \) to \( A_{(Abs)} \) (Fig. 1.7). For a component known to be solely present in a complex, such as ribosomal proteins in the 77S monosomal translation complex, the \( A_{(FDS)}/A_{(Abs)} \) ratio can be used as a standard of 100% to normalize with other components with unknown stoichiometry in a particular complex.
Fig. 1.7 The abundance calculated from the area of peaks
**Results**

SBP1 is associating with the active translation mRNP

Because our previous studies had shown that SBP1 was a component of the 77S translating ribosomal complex using FLAG-PAB1 to identify mRNA complexes, we were interested in determining the type of complexes SBP1 was a part of. To investigate SBP1-containing complexes, a FLAG-SBP1 fusion was created. By purifying the subsequent FLAG-SBP1 complexes, we would be able to identify the macromolecular complexes containing SBP1, determine their characteristics, and potentially identify their components. I initiated these studies by expressing FLAG-SBP1 in yeast, obtaining crude extracts from the yeast, and affinity purifying FLAG-SBP1 complexes with Anti-FLAG agarose. The purified sample was analyzed by AU-A260 to detect SBP1 complexes containing RNA.

Overall, a good absorbance signal was detected by AU-260 analysis (AU raw data, not shown), indicating that the Anti-FLAG purification was successful and there was a decent amount of RNA associating with SBP1. The calculated results (Fig. 1.8A) show a major peak below 80S, suggesting the presence of a large abundance of ribosome in the complexes, as well as two peaks at 60S and 40S that had more minor signals. Noticeably another major peak was observed with an S-value below five. Because centrifugation speeds of 15000 rpm were used in these experiments, complexes sized 20S-150S would be most readily differentiated. Peaks below this range are generated by the Sedfit program with insufficient sedimentation differentiation and were generally not studied further. A trace amount of complexes with 110S were also barely visible.
The major peak migrating just below 80S had a calculated S value of 74.6±2.74 based on five experiments (Table 1.2). This size corresponds to the 77S monosomal translating complex that has been previously identified. The identity of this putative 77S peak was confirmed by two types of experiments. First, I combined FLAG-SBP1 with GFP-tagged ribosomal components, RPS4B (small subunit, Fig. 1.8C) and RPL6B (large subunit, Fig. 1.8D) and conducted AU-FDS analysis on the FLAG-purified material. In both cases, the major peak observed migrated at 77S (summarized in Table 1.2), suggesting that both 40S and 60S ribosomes were part of the 77S peak identified with FLAG-SBP1.

<table>
<thead>
<tr>
<th></th>
<th>S values</th>
<th>Mean 77S complex size ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A260</td>
<td>77.8 80.1 74.7 73.2 76.3</td>
<td>76.4±2.68</td>
</tr>
<tr>
<td>RPS4B-GFP</td>
<td>75.5 73.2 70.9 73.2 75.5</td>
<td>73.7±1.92</td>
</tr>
<tr>
<td>RPL6B-GFP</td>
<td>77.8 73.2 75.5 70.9 70.9</td>
<td>73.7±3.00</td>
</tr>
</tbody>
</table>

Table 1.2. S values of the 77S peak in different tests

Legend: S values were determined following AU-A260 or AU-FDS analysis. The mean is given ± the Standard Error of the Mean (S.E.M.).
However, according to previous data (Wang X. *et al* 2012), both the free ribosome (80S) and the monosomal translating 77S mRNP complex could be sedimenting in the 77S region. To differentiate these possibilities I conducted a second analysis. The type of complex in this region can be determined by ascertaining the effect of glucose depletion on the abundance of the 77S peak. As shown previously, cellular translation stalls when glucose is removed from the medium, the translating mRNP dissembles, and the free ribosomes are released in less than 10 minutes. This results in an increase in the free 80S ribosome and a decrease in the translating 77S mRNP (Wang X. *et al* 2012).

Using AU-260 analysis, the yeast sample treated with glucose deprivation (Glu+-) underwent a dramatic decrease in the major peak around 80S (Fig. 1.8B), suggesting that SBP1 is a component of the 77S monosomal translating mRNP complex that was previously identified (Wang *et al* 2012). In contrast, there is no significant difference between the untreated and treated results in the abundance of the minor 40S and 60S peaks, suggesting SBP1 is also associating with a small portion of small and large ribosomal subunits which are translationally inactive. This result could be linked with SBP1’s putative role in ribosome biogenesis, which is previously reported associating with snoRNA10 and snoRNA11 (Clark MW *et al* 1990).

Similarly, the abundance of the putative 77S complex observed following AU-FDS analysis of RPS4B-GFP and RPL6B-GFP was found to be reduced following glucose deprivation (see Fig. 1.8C and 1.8D, respectively). These results establish that the 77S peak identified in
FLAG-SBP1 purified extracts is a translating monosomal complex and functionally related to the 77S complex previously identified (Wang et al 2012). Importantly, FLAG-SBP1 co-purified with little material greater than 110S, unlike FLAG-PAB1 purifications. The latter observation suggests that SBP1 does not primarily associate with polysomal material that migrates at 110S and larger S values (Wang et al 2012).
Fig. 1.8 (A, B) AU-A260 of Flag-Sbp1 expression strain. AU-A230 and AU-FDS analysis was conducted on FLAG-SBP1 purified material and analyzed by AUC as described in the Materials and Methods. glu + refers to cells grown under steady-state elongation conditions in the presence of glucose; glu + - refers to cells grown under glucose growth conditions followed by depletion of glucose from the medium for 10 min.
Fig. 1.8 (C, D) AU-FDS of FLAG-SBP1 expression strain
The 77S mRNP associating with FLAG-SBP1 is distinctive from the previous 77S mRNP associating with FLAG-PAB1

The 77S monosomal translating complex identified with FLAG-PAB1 contained not only the 80S ribosomal components but a number of other factors: translation initiation factors PAB1, eIF4E, eIF4G1, eIF4G2, translation termination factors eRF1 and eRF3, and the proteins SSD1, SLF1, and PUB1 (Wang et al 2012; Zhang et al 2014). The presence of each of these factors in the 77S complex identified with FLAG-SBP1 was subsequently determined. Surprisingly, several essential translation initiation factors were only found in very low abundance in this 77S complex, suggesting SBP1 may be primarily present in a 77S mRNP that is different than that found with FLAG-PAB1. As shown in Fig.1.9 A, although the 77S complex was present, the signal levels (peak heights) were significantly lower than the abundance of ribosomal components when PAB1-GFP, eIF4E-GFP, eIF4G1-GFP and eIF4G2-GFP were examined (Fig 1.9). Importantly, because the abundance of this 77S peak observed for eIF4E, eIF4G1, and eIF4G2 was not significantly different as compared to the no Flag control, the 77S peaks observed in these cases appear to result from non-specific immunoprecipitation. Moreover, in the FLAG-SBP1 pull-downs, the abundance of the peaks in the 77S region for each of the closed-loop factors was not affected by the glucose deprivation treatment. These results suggest that whatever 77S monosomal translating complexes that were being identified with FLAG-SBP1 that contained PAB1 and possibly eIF4E and eIF4G were not translationally active. In contrast, as shown previously with FLAG-PAB1, the abundance of all of these factors existing in the 77S monosomal translating complex was correspondently reduced by glucose deprivation treatment for ten minutes.
Therefore, the 77S monosomal translating complex identified with FLAG-SBP1 contains the 80S ribosomal proteins but low abundances of these other factors.

Fig.1.9 (A, B) Effect of glucose deprivation on PAB1-GFP/FLAG-SBP1 (A) and eIF4E-GFP/FLAG-SBP1 (B). Analysis was conducted as described in Figure 1.8.
Fig. 1.9 (C, D) Effects of glucose deprivation on translation factors eIF4G1 (C) eIF4G2 (D)
Analysis of eRF1 presence in the FLAG-SBP1 complexes

In addition to the translation initiation factors PAB1, eIF4E, and eIF4G, the termination factor eRF1-GFP was studied for its presence in FLAG-SBP1 complexes. As shown in Figure 1.10A, eRF1-GFP exhibited a different pattern than the initiation factors with FLAG-SBP1. eRF1 is known as a termination factor that recognizes the STOP codon and initiates the release of translation machinery from the mRNA. eRF1 was previously identified as a component of the 77S complex (Zhang C et al. 2014) and in vitro studies indicate that it is present during translation initiation (AMRANI N et al. 2008). When purified by FLAG-SBP1, a set of eRF1-GFP peaks were observed. A 70S peak was observed, possibly the 77S complex, and there were also two large peaks running at 15S and 25S, which are possibly merged from multiple smaller smear peaks based on the shape of the peaks. However, in contrast to the decrease of total 77S monosomal translating complex with FLAG-SBP1 following the glucose deprivation treatment (Figure 1.8), the overall signal of eRF1-GFP including the putative 77S peak increased. The increase 77S peak indicates that eRF1 is not part of the 77S monosomal translating complex, but it may be present with SBP1 in some other type of monosomal complex.

Translation repressor DHH1 does not co-purify with SBP1

Because SBP1 is a translational repressor, it was possible that other translational repressors might be associating with it. DHH1 is one such repressor that has been shown to function relatedly to SBP1. DHH1 is described as a P-body component involved in translation repression and RNA degradation (Chang LC et al 2012; Carroll JS et al 2011). Like SBP1,
deletion of \textit{DHH1} also affects the rate of mRNA degradation, while double deletion of \textit{DHH1} and \textit{SBP1} completely disrupts the ability of mRNA degradation and results in lethality to yeast cells (Segal, SP \textit{et al} 2006). \textit{DHH1} and \textit{SBP1} are considered to be involved in two pathways of mRNA degradation which are independent to each other. However, as analyzed by AU-FDS, \textit{DHH1-GFP} gave no detectable signal in the \textit{FLAG-SBP1} purifications (Fig. 1.10B). Our experimental results showing that \textit{DHH1} does not associate with \textit{SBP1} supports the current understanding of that these two factors may be functioning in distinct pathways.
Fig. 1.10 AU-FDS analysis of translation terminator eRF1 and repressor DHH1
mRNA binding proteins SLF1, SSD1 and PUB1 are not present in the SBP1 complexes

In addition to SBP1, three other mRNA binding proteins, SLF1, SSD1 and PUB1 were found previously to be present in FLAG-PAB1 purified 77S translating complexes (Zhang et al 2014; Richardson et al 2013). SLF1 is a regulator of mitochondria and is involved in the oxidative stress response. SLF1 is further related to the response of oxidative stress by activating a set of antioxidant and other proteins required for oxidant tolerance (Chatenay-Lapointe M et al 2011; Kershaw CJ et al 2015). SSD1 controls cell wall remodeling by repressing translation of proteins involved in wall expansion (Uesono Y et al 1997; Jansen JM et al 2009; Wanless AG et al 2014). PUB1 is a highly abundant poly-U binding protein co-localizing in stress granules which binds to the 77S mRNP during translation as well as stress conditions. Binding of PUB1 to the AREs of 3’UTR protects mRNA from decaying. (Duttagupta R et al 2005). I subsequently tested whether any of these three factors were present in the FLAG-SBP1 77S complex (Fig. 1.11 A, B and C). None of these mRNA binding proteins were identified as components of the protein complexes purified by FLAG-SBP1. Unlike translation initiation factors eIF4E, eIF4G and PAB1, these three factors were completely excluded from the 77S mRNP associating with SBP1. It is currently not clear the role of these three factors in the 77S mRNP, but their absence in FLAG-SBP1 purified complexes supports the finding that the FLAG-SBP1 77S mRNP is in a different state of translation than is the FLAG-PAB1 77S complex.
Fig. 1.11 AU-FDS analysis of GFP-tagged strains SLF-GFP (A), SSD1-GFP (B), PUB1-GFP (C)
Stoichiometry analysis of the SBP1 associated 77S complex

To more specifically analyze the difference of the 77S complex that co-purify with the FLAG-PAB1 and FLAG-SBP1 proteins, the stoichiometry of the different GFP-tagged proteins in the respective 77S complexes was calculated. As described in the Materials and Methods section, the absolute abundance of a target protein can calculated based on the area of the 77S peak observed in the AU-FDS analysis of the GFP-tagged protein as normalized by to the total protein content of the 77S complex in the same sample as analyzed by AU-260. The stoichiometry of each of the GFP tagged proteins was then calculated setting RPS4B-GFP as 100% (Table 3). Importantly, most ribosomal components have more than one genomic copy within the genome. We chose the RPS4B molecule as our standard for one protein per 77S ribosome, as it has previously been shown that the RPS4A paralog of RPS4B is not efficiently expressed in yeast (573 PRS4A molecules versus 102000 RPS4B molecules per cell) (Ghaemmaghami S, et al. 2003). Another internal reference, RPL7A, was also used as it was also dominantly expressed as compared to RPL7B (101000 RPL7A versus 7800 RPL7B molecules per cell). Finally, as shown in Table 3, the large subunit component RPL6B has only half of the abundance in the 77S complex of that of RPS4B. This is because a paralog RPL6A without GFP-tag is expressed at a similar level in the cell (37100 RPL6A versus 38400 RPL6B molecules per cell). Despite possible experimental errors and slight variations among these three ribosomal GFP-tagged proteins, the calculated stoichiometry of ribosomal components to each other is close to our expectation.

In Table 1.3, the stoichiometry data from previous FLAG-PAB1 analysis (Wang X, 2013) is
also listed. In FLAG-PAB1 pull-downs, 56% of the 77S complex contains eIF4E and 34% of them contains eIF4G (20% of eIF4G1 and 14% of eIF4G2), which is consistent with a translating mRNP. In contrast, the 77S complex in FLAG-SBP1 pull-downs contains only 3% eIF4E and 1.2% eIF4G. These values for eIF4E and eIF4G are probably overestimates given that no significant detection of these proteins were observed above the background control. These results indicate that the majority of this FLAG-SBP1 77S complex is not at the same stage of translation as is the FLAG-PAB1 77S complex. In addition, there is no detectable signal of SLF1, SSD1 and PUB1 in the FLAG-SBP1 complex, which are all observed in the FLAG-PAB1 complexes that contain 9% of SLF1 and 3% of SSD1. Finally, a small fraction of eRF1 is present in both the FLAG-PAB1 and FLAG-SBP1 pull-downs (2% and 3%, respectively), suggesting that the majority of complexes are not undergoing termination.
<table>
<thead>
<tr>
<th>Component</th>
<th>FLAG-SBP1 complex</th>
<th>FLAG-PAB1 complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPS4B</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>RPL6B</td>
<td>49</td>
<td>49</td>
</tr>
<tr>
<td>RPL7A</td>
<td>113</td>
<td>130</td>
</tr>
<tr>
<td>PAB1</td>
<td>9</td>
<td>N/A</td>
</tr>
<tr>
<td>SBP1</td>
<td>N/A</td>
<td>7</td>
</tr>
<tr>
<td>eIF4E</td>
<td>3</td>
<td>56</td>
</tr>
<tr>
<td>eIF4G1</td>
<td>0.7</td>
<td>20</td>
</tr>
<tr>
<td>eIF4G2</td>
<td>0.5</td>
<td>14</td>
</tr>
<tr>
<td>SLF1</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>SSD1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>PUB1</td>
<td>0</td>
<td>Less than 0.5</td>
</tr>
<tr>
<td>DHH1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>eRF1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 1.3. Stoichiometry comparison of 77S complex components associating with SBP1 and PAB1, in percentage.


**Discussion**

In the first part of this thesis, I used AUC to analyze a novel 77S complex associating with FLAG-SBP1. The overall results support the idea that SBP1 is associating with a translationally active 77S mRNP, which appears to be distinctive from the previous 77S complex purified by FLAG-PAB1. This finding provides insight as to the role of SBP1 in translation and more importantly improves the current understanding of the 77S mRNP in translation.

**SBP1 is an abundant component of the 77S mRNP**

According to the previous publications in our group, SBP1 is one of the minor components of the 77S mRNP co-purified with FLAG-PAB1, present within about 7% of the FLAG-PAB1 complex. With the use of FLAG-SBP1 as the purification handle instead, I detected a decent amount of 77S complexes as determined from both AU-A260 and AU-FDS results. Although SBP1 is not believed to be a critical factor in translation, as deletion of SBP1 does not cause any noticeable phenotype, my results suggest SBP1 is abundantly associating with the 77S mRNP. Moreover, in FLAG-SBP1 complexes, the stoichiometry of PAB1 is 9%, a close amount to the presence of SBP1 in the FLAG-PAB1 77S complex. Assuming there is no binding preferences of the 77S mRNP to FLAG-SBP1 or FLAG-PAB1, the overall abundance of 77S mRNP associating with FLAG-SBP1 within the cell should be also close to what is associating with FLAG-PAB1.
SBP1 is participating in the 77S mRNP in the late elongation and termination phase

Previously, the abundance of the eIF4E, eIF4G1, and eIF4G2 factors in the FLAG-PAB1 77S monosomal translating complex was determined under both steady state elongation conditions and during elongation soon after initiation of translation (Wang 2013). Dr. Wang’s results indicated that there were 50% to 66% more of these initiation factors present in the 77S complex early in initiation as compared to steady state elongation conditions. These results were interpreted to indicate that these initiation factors come off the 77S translating complex moves from initiation through elongation to termination. These results were used as a basis to understand the low abundances of these initiation factors in FLAG-SBP1 77S complexes.

Translation initiation factors eIF4E, eIF4G1 and eIF4G2 have very low abundance in the 77S mRNP associated with FLAG-SBP1, with the stoichiometry of 3%, 0.7% and 0.5% as compared to RPS4B, respectively. This result suggests that most of the FLAG-SBP1 77S mRNP are not at the early stages of elongation following translation initiation, but likely are fairly far advanced in the elongation phase after the initiation factors have been removed (Wang X, 2013). There is very little overlap between SBP1 and the initiation factors and PAB1, which also indicates that the translation initiation factors are exiting the 77S mRNP during elongation as the poly (A) tail is being shortened and either prior to or at the same time that SBP1 binds to the mRNP. These observations support the model that SBP1 preferentially binds the 77S complex after the initiation factors have been removed. Whether SBP1 plays an active role in this removal remains unknown.
Following the glucose deprivation treatment when translation ceases, the abundance of eRF1-GFP was increased in FLAG-SBP1 complex but decreased in the FLAG-PAB1 complex (Zhang C et al 2014), possibly suggesting that more termination factors are binding to the FLAG-SBP1 77S mRNP to terminate translation. This possibility also supports that the FLAG-SBP1 77S complex is in a late stage of elongation.

Moreover, little of the SBP1 77S mRNP contains PAB1, which specifically and strongly binds to the poly (A) tail. This result suggests that the mRNP has shortened its poly (A) tail to less than 24 residues. Without the poly (A) tail this would suggest that the mRNA is not able to form the close-loop structure or to be translated again. An alternative possibility is that an unknown mechanism prevents PAB1 binding to the poly (A) tail in this FLAG-SBP1 77S complex. Such factors could be deadenylating factors that SBP1 may have some functional relation to. A third possibility is that SBP1 binding directly prevents PAB1 binding, although there is no evidence for this. In either case, the mRNA without PAB1 and associating with SBP1 may be close to the end of its translational life and may soon enter P bodies or stress granules and targeted for degradation. The role of SBP1 in this 77S mRNP is possibly connected, therefore, with the promotion of translation termination and mRNA degradation.

**PAB1 co-localizes with SBP1 in a variety of complexes including the 77S mRNP**

According to the initial scans of during the AU-FDS analysis of the raw data (data not shown) PAB1-GFP was observed to have high affinity to FLAG-SBP1. However, there was only a small amount of complexes sedimenting to the bottom of the centrifugal cell, with the
majority of the fluorescent signal only travelling a short distance during the experimental period (4-5 hours of centrifugation at 15000 rpm). As a result, only a few complexes with low signal level are observed in our interested size range (20S to 250S). There is a strong signal below 10S (full peak not shown in the current scale), which is the undifferentiated small complexes (including possibly monomeric PAB1-GFP) in the sample (Fig. 10 A). According to previous findings, SBP1 and PAB1 are both mRNA binding proteins, and it is possible that these complexes are the mRNA molecules that bind with both FLAG-SBP1 and PAB1-GFP. Another set of very small peaks running higher than 100S are also present in the results. These seem to be related to the polysomes. There is no peak between 30S to 70S observed, indicating that these putative pre-initiation complexes do not contain both SBP1 and PAB1 during the translation. This conclusion is consistent with the previous suggestion of SBP1 association with the 77S complex during translation termination and mRNA degradation.

A small fraction of the FLAG-SBP1 77S mRNP containing initiation factors is not sensitive to glucose deprivation

Following AU-FDS, PAB1-GFP, eIF4E-GFP and eIF4G-GFP had similar band patterns and the 77S signals remained the same after glucose deprivation as in non-glucose deprived cells (steady state conditions). These observations are contrary to our current understanding that the 77S complex is the mRNP complex that is actively performing translation. In this case glucose deprivation should suppress the overall rate of cellular translation which should be reflected in the dramatic reduction of the 77S peak. This is verified by AU tests of all the FLAG-PAB1 strains (Wang X et al 2012), as well as the GFP-tagged ribosomal components
of FLAG-SBP1 strains. As a result, the 77S complex co-purified with the FLAG-SBP1 is possibly containing two types of complexes. The majority is late-elongating mRNP that have no initiation factors associated and a few (the glucose deprivation non-responsive mRNP) contain different translation complexes. Since there might be other factors enter and exiting between these two types of mRNP, the shape and molar mass may remain the same, resulting in both of the mRNP running closely at 77S.

Implications for future study

With the interpretation of FLAG-SBP1 and FLAG-PAB1 results, the 77S mRNP exhibits polymorphism in its composition and to be highly diversified in different conditions. The 77S mRNP, despite the core constituents such as ribosomes, requires all sorts of “add-on” proteins that temporarily associate with it in different stages. These “add-on” proteins may be critical to controlling the assembly and disassembly of the mRNP and to turning it on and off during translation. With single purification handles such as FLAG-SBP1 and FLAG-PAB1, we are able to capture only a fraction of the total 77S mRNP at a certain stage. These purified complexes would provide better subjects to the study of translational regulation and the interactions of translational factors within a specific stage.

However it also brings a challenge to the study of the 77S mRNP comprehensively. As there is no factor known to exclusively bind to all of the 77S mRNP, other than the ribosomes themselves, a major problem is to obtain the stoichiometry information of target proteins in the total 77S mRNP. With FLAG-SBP1 or FLAG-PAB1, only certain types of mRNP can be
purified and analyzed in the AU assay. Consequently, there is a great chance that some of the mRNP have neither of these factors and remain undiscovered. The stoichiometry data we currently have obtained are incomplete which can only represent a certain type of mRNP. The abundance of the total 77S mRNP and proteins in the unpurified 77S mRNP remain unknown. Without an ideal purification handle, the total 77S mRNP can only be closely approached by a strategy similar to the “shotgun” method of DNA sequencing, which is interpreting the data from multiple factors binding to the 77S mRNP at different stages to reconstruct the whole figure of this complex. The partial stoichiometry information of each type of 77S mRNP can also be used to study the transition of factors during different translation stages and environmental conditions.

In summary, using AU analysis on FLAG-SBP1 strains we have revealed the role of SBP1 in translation, determined the timing of SBP1 binding to the 77S mRNP. We also captured and analyzed a new type of 77S mRNP at a late elongation and early termination phase, adding to our current knowledge of the translational complexes and providing a possible methodology for future analysis.
Chapter II  Characterization of the role of molecular chaperones in the amyloid aggregation of Huntington’s disease

Introduction

Huntington’s disease (HD) is a fatal neurodegenerative disorder that is caused by the loss of neuronal structure and functions. There is no cure for HD, with only a few clinical treatments available to reduce the severity of HD symptoms. Unlike other commonly known neurodegenerative diseases, including Alzheimer’s disease, Parkinson disease and Amyotrophic lateral sclerosis, HD is inheritable but, related to these other diseases, is linked with the abnormal accumulation of certain mutant proteins in neural cells, affecting the normal functions of the central nervous system. HD can be symptomatic to patients at all ages, but most commonly displays its phenotypes in their middle age. The onset of HD mostly affects the basal ganglia (Fig. 2.1) which leads to conditions such as progressive dementia, psychiatric symptoms, and choreiform movements, that culminates in premature death. Once they begin to manifest symptoms, HD patients have an average of 17-20 years to live until death (Myers RH, 2004).

Fig. 2.1 HD causes widespread deterioration of brain tissue, particularly of the basal ganglia.
**HD genetics**

In HD patients, a mutation in the protein huntingtin (HTT), causing the expansion of the polyglutamine (poly-Q) region of HTT, is found to be strongly related to the disease (The Huntington’s Disease Collaborative Research Group, 1993). HTT is a 350 kDa cytoplasmic protein expressed in human and all mammalian cells and is particularly abundant in brain and nerve cells. Other than HTT causing HD, the actual function of HTT remains unclear. There are several difficulties in investigating the function of HTT because HTT has no other homologous proteins, it is widely expressed and is localized in many subcellular compartments, and the deletion of HTT in mice is lethal to the embryos. Intracellularly, HTT is present in nuclei, cell bodies, dendrites and nerve terminals. It has been found to associate with vesicular and microtubule and to potentially have function in cellular trafficking and in cytoskeleton anchoring (Hoffner G. et al, 2002; DiFiglia M, et al, 1995). Reducing the levels of HTT leads to several phenotypes including reduced vesicle and mitochondria transport in Drosophila melanogaster, abnormal brain development and apoptosis in the brain and testes, and abnormal distribution and morphology of cellular organelles (Hilditch-Maguire, P. et al 2000; White, J. K. et al 1997; Auerbach, W. et al 2001).

HTT normally contains a region of polyglutamine (poly-Q) repeat close to the N-terminus (exon 1) that varies from 11 to 34 glutamine residues. The mutant HTT (mHTT), however, has an expanded poly-Q region that can be as long as 103Qs. In the HTT gene, the trinucleotide CAG repeats which encodes the poly-Q region show instability during replication: larger repeats tend to be more unstable. The instability of CAG repeats mostly
leads to a further expansion of the repeats (73% of the time) while contraction can also occur (23% of the time) (Chattapadhyay B et al 2005; Djoussé L et al 2004; MacDonald ME et al 1999). The severity of the disease is found to be proportional to the length of the expansion of the poly-Q. Thus, the expansion of CAG repeats to a certain point leads to the onset of HD as well as the increased trend of making further expansions of itself and further development of the disease.

Being one of the autosomal-dominant diseases, HD is not induced by terminal deletion or physical disruption of the HTT gene. Structural studies reveal that HTT confers a toxic gain of function in the mutation. *In vitro* experiments suggest that once expansion of the poly-Q tract reaches a critical number of glutamine residues, reportedly to be 37 residues, the mutant protein starts misfolding and aggregating. The rate of aggregation increases with the length of the poly-Q as well as with the protein concentration. Consequently, central nervous system cells with high expression levels of HTT are most vulnerable to the poly-Q aggregates. Individuals with 36-40 glutamine residues have variable penetrance of the HD phenotype, whereas HD develops full penetrance with more than 40 glutamine residues. Although no HD cases had been diagnosed with poly-Qs below 36, the offspring of individuals with 28-35Qs are at risk to inherit HD due to the expansion of poly-Q sequences.
Amyloid aggregation

The aggregation of HTT results in insoluble prion-like amyloid aggregates, which accumulate in the affected cells and can be visible by microscopy. In general, amyloids are elongated, fibers, β-sheet-rich protein aggregates which are characterized by their ability to self-propagate and to be resistant to detergents. These attributes are found in all types of neurodegenerative disease (Fig. 2.2A). However, it is currently not clear concerning the mechanism of amyloid toxicity, or the role of amyloids in neurodegenerative diseases. The mHTT amyloid has been reported to promote the aggregation of normal HTTs which do not aggregate (Busch A et al 2003; Saleh AA et al 2014). The HTT amyloid is also able to co-aggregate with other proteins rich in glutamine and/or asparagine, sequestrating the normal functions of these proteins, which is believed contributory to the toxicity of mHTT in neurodegeneration (Steffan JS et al 2000). Interestingly, the presence of wtHTT in the mHTT amyloid slows the effect of altering the conformation of the aggregates by increasing the solubility and reducing the toxicity caused by mHTT (Saleh AA et al 2014). Moreover, overexpression of a Q-rich prion-like protein is also reported to neutralize the poly-Q toxicity (Ripaud L et al 2014; Kayatekin C et al 2014). In summary, it is still controversial about the role of amyloid aggregates in the process of neurodegeneration.
Fig. 2.2 (A) Amyloid deposit of neurodegenerative disease in neuron cells (Soto C, 2003); (B) The structure of amyloid aggregates (Eisenberg D and Jucker M, 2012)
Although correlated with neurodegenerative diseases, amyloids are defined by a unique structure pattern referred as cross-β fiber diffraction pattern in which the amyloid fiber are formed by protofibrils consisting of parallel or similarly arranged β-strands perpendicular to the length of the fiber (Fig. 2.2B). During aggregation, a segment of the protein backbone may expose the N-H groups and the C=O groups, allowing the formation of the hydrogen bonds with other chains. The exposure of backbone amide groups can be caused by several different conditions including denaturing of normally folded proteins (Chiti F et al, 1999); overwhelming chaperones by overexpression of a protein (Wang L et al, 2008); cleaving a peptide (such as Aβ) from a folded protein; or expansion of a specific region of a protein (poly Q diseases). In addition, amyloid aggregation also requires sufficient concentration of the exposed proteins. For the amyloid aggregation, it is necessary to form a core aggregate consists of three or four protein molecules. The core aggregate also reduces the free energy required for normal proteins to expose the protein amid backbone, significantly accelerating the rate of aggregation (Nelson R et al, 2005).

Despite the appearance in neurodegenerative disease, there are several types of amyloid found with biological functions. For example in yeast, translation termination factor Sup35p (eRF3 in humans) has two states: normal state [PSI] and prion state [PSI*]. Yeast strains carrying the [PSI*] have the ability to convert [PSI] strains into its prion form. The [PSI*] state deactivates the normal function of Sup35p as a translation terminator, allowing ribosomes to read through early termination mutations. Although more than half of the artificially induced [PSI*] strains are lethal or weak (Nakayashiki T, et al 2005; McGlinchey...
RP, et al 2011), they gain growth advantages under specific conditions including that of ethanol or lithium presence (True HL et al 2000). As a result, except for the Sup35p loss-of-function, the amyloid aggregate appears harmless to yeast cells.

Molecular chaperones alter the toxicity of the mHTT

Because amyloid aggregation is believed to be related to misfolding, molecular chaperones are considered to be involved in this process. Molecular chaperones are proteins with the primary function of assisting newly synthesized proteins to fold into the correct three-dimensional conformation. Chaperones are hence involved in a general mechanism of preventing protein misfolding and aggregation. Originally, molecular chaperones were discovered as heat shock proteins (HSPs), which are induced by heat shock. Heat stress increases the chance of protein misfolding and aggregation, whereas molecular chaperones are proven to provide protective mechanisms against heat stress (Ellis RJ et al 1991). Chaperones, in particular Hsp70 and co-chaperone Hsp40 families, recognize and bind to the exposed hydrophobic amino-acid residues and unstructured backbones of a misfolding peptide, which has a great tendency of aggregate with each other in a concentration-dependent manner and to refold the peptide to the correct conformation with an ATP-driven mechanism (Hartl FU et al 2011; Kim YE et al 2013). Moreover, Hsp70 and Hsp100 chaperones are also able to synergistically disaggregate the insoluble aggregates formed by misfolding proteins. Hsp70 disentangles the peptides from aggregates while Hsp100 further unfolds the peptides by translocation through the central channel of Hsp100 complex in an ATP-driven mechanism (Liberek K et al 2008; Nillegoda NB, Bukau B 2015;
Nillegoda NB et al 2015b).

Research shows molecular chaperones are also involved in the aggregation of the amyloid in HD and other neurodegenerative diseases, although with diversified effects. The overexpression of Hsp70 chaperone family members suppresses the cytotoxicity of mHTT without altering the amyloid formation in mammal cell models. The aggregation of amyloid is suppressed only if a member of Hsp40 co-chaperone is simultaneously expressed (Zhou H, et al 2001; Rujano MA, et al 2007; Jana NR, et al 2009; Ormsby AR 2013). The overexpression of Hsp40 family members YDJ1 and SIS1 in yeast also alters the localization of amyloid formation between cytosol and nucleus. Amyloid deposits in the latter case is more harmful to the cell. Interestingly, deletion of Hsp40 or Hsp70 in yeast also rescues yeast from lethality caused by mHTT (Meriin AB et al 2002). In addition, a yeast specific chaperone Hsp104, which is has no homologs in metazoans, is not only important to preventing the formation of the aggregates in yeast but also suppresses the toxicity of mHTT in other model species including C. elegants, D. melanogaster and transgenic mice (Jackrel ME et al 2013; Lee DH et al 2010; Vacher C et al 2005). Dramatically, both overexpression and deletion of Hsp104 in the yeast model prevents the aggregation of mHTT (Krobitsch S et al 2000). In addition, deletion of Hsp104 in yeast increases the size and decrease the number of [PSI⁺] prion aggregates and may eventually convert [PSI⁺] yeast into [PSI⁻]; overexpression of Hsp104, on the other hand, results in “diffusion” of smaller aggregates that can be possibly degraded by proteolytic mechanisms (Wegrzyn RD et al 2001; Cox B et al 2003).
These findings suggest Hsp104 is able to disaggregate large amyloid deposits into smaller aggregates, and more interestingly, indicates that the toxicity of amyloids may be size related: break down of large aggregates produces propagation “seeds” to increase the rate of aggregation but also increases the chance of elimination of small aggregates by proteolytic mechanisms. In summary, molecular chaperones play a critical role in prion propagation. Moderate level of chaperones increase the size, amount, and toxicity of the amyloid aggregates; deletion or overexpression of these chaperones, in contrast, suppress the toxicity by altering the size, amount and distribution of the amyloid aggregates.

The poly-Q tract and the flanking sequences also determines the toxicity of mHTT

Additionally, the cytotoxicity in HD may be caused by a fragment of the mHTT, for the full length HTT, regardless of wild-type or mutant form, appears benign to the cells. Since the full length HTT is a huge protein, with its correct three-dimensional confirmation there is little chance for the aggregation and self-propagation of the poly-Q tract to occur. In fact, the “stacked” β-sheet confirmation of amyloid is highly sequence sensitive and requires a large number of glutamine residues for its structure, as only the poly-Q tract is aggregated in the amyloid. The N-terminus of the mHTT containing an expanded poly-Q sequence is prone to be cleaved and released by caspases or other proteases. The cleaved N-terminal fragment, which is mostly the exon I of mHTT alone, is observed to be self-aggregating and capable of inducing the neurodegeneration phenotype. As expected, inhibition of caspase activities reduces both toxicity and the amyloid aggregation of mHTT in tested subjects (Graham RK, et al 2000; Wellington CL, et al 2002). Nevertheless, expansion of the CAG repeats also
promotes aberrant splicing of the HTT gene and results in the synthesis of an exon I fragment which is similar to the caspase cleaved fragment (Sathasivam K, et al. 2013). Overexpressing exon I of mHTT appears to result in the same effect of causing neurodegeneration.

The flanking sequences to the poly-Q tract profoundly alter the toxicity of mHTT. Although being regarded as the primary cause of HD, poly-Q expansion is not the only determining factor of the disease. In fact, there are at least nine different neurodegenerative diseases that are cause by poly-Q expansion in different proteins. The different types of poly-Q aggregates affect different regions of the central nervous system, resulting in different phenotypes of neurodegeneration. Research suggests that the difference among the poly-Q containing proteins may also contribute to the tissue preference of amyloid formation. In HD, the flanking sequences adjacent to the poly-Q in exon I product fragment N17 and C38, the 17 amino-acid residues in the N-terminus and the 38 amino-acid residues in C-terminus respectively, are also critical to the amyloid formation and toxicity of the poly-Q region. With the presence of the N17 sequence, the rate of poly-Q aggregation is significantly increased while deletion of N17 suppresses aggregation (Tam S, et al 2009). Interestingly, it is not necessary for the N17 to be adjacent to the poly-Q tract to display this effect: N17 expressed at the C-terminus of mHTT is similarly effective in promoting aggregation. Analysis on the N17 structure suggests that the amphipathic residues of N17 are essential for poly-Q aggregation. Replacing them with alanine also dramatically blocks the poly-Q aggregation and cytotoxicity (Tam S, et al 2009). Moreover, replacing N17 with FLAG-peptide (DYKDDDDK) is also found to promote the poly-Q cytotoxicity in yeast models, with a
similar locus independent manner (Duennwald ML, et al 2006). The C38 fragment, nevertheless, stabilizes the poly-Q from aggregation and inhibits the cytotoxicity of poly-Q expansion (Duennwald ML, et al 2006). The C38 fragment is mostly made up with proline and contains 28 proline residues. The presence of this proline-rich fragment inhibits the cytotoxicity of the expanded poly-Q fragment, and deletion of C38 stimulates the aggregation of poly-Q as expected. Furthermore, attaching an oligoproline to the carboxyl-terminus of a poly-Q protein can decrease the rate of amyloid aggregation (Duennwald ML, et al 2006, Wetzel et al 2006). Noticeably, many other poly-Q proteins also contain proline-rich regions adjacent to the poly-Q tracts, which is likely an evolutionary derived stabilizer of the poly-Q tract for preventing aggregation in cells (Bhattacharyya A. et al 2006).

Modeling of HD

During past decades, several model systems of HD with a variety of species have been developed. The studies of HD in model organisms are invaluable in elucidating the molecular basis, genetics, behavior, and clinical treatment of HD. One popular model organism is that of the budding yeast Saccharomyces cerevisiae. Expressing of mHTT in yeast resulted in amyloid aggregation and cytotoxicity, while the wild-type HTT remained soluble and non-toxic (Meriin AB et al 2002). This research suggested yeast was a possible model of HD research. Yeast has already been extensively used in other protein misfolding and cellular damage studies. A vast majority of cellular processes, such as protein synthesis, translational control and chaperones, are highly conserved between human and yeast cells. Yeast is the first eukaryote to have the whole genome sequenced, and the yeast genome is one of the best
studied genomes, which provides us sufficient background information about all the factors we may find to be involved in the amyloid formation. In addition, yeast also contains several native prion-like proteins including SUP35, URE2, RNQ1 and SWI1, which can potentially aggregate into amyloids as described above (Inge-Vechtomov SG et al 2007; Du Z et al 2008). Indeed, one of the native prions, RNQ1, is required for the mHTT to aggregate in the yeast model (Meriin AB et al 2002). Using yeast models may provide additional information about the interactions between the mHTT amyloid and internal prions. Although with the limitations of yeast as a unicellular organism, there is no applicable way to study the effects of HD on neuron-specific factors involved in HD, the yeast model is still sufficient for the study of HD at the molecular level. In this thesis, yeast HD model has been chosen to conduct the research on the aggregation patterns of mHTT amyloid using AU analysis.

Summary
Since neurodegeneration in HD is highly related to the propagation of mHTT amyloids, it is necessary to investigate the factors involved in this process, in particular, the intermediate misfolding complexes between free mHTT and insoluble amyloids. The intermediate complexes are at the cross roads of aggregation and elimination. Investigations of the properties of these complexes should be beneficial to the development of interventions in HD. However, no previous study had been done on the characterization of these complexes in part due to limitations in methods used to study these complexes. Taking the advantage of the novel technique AU-FDS, it is possible to distinguish the soluble intermediate complexes during amyloid aggregation, determining their sizes, components and regulation. It is also
possible to study the role of chaperones in amyloid aggregation and localize their presence in the intermediate complexes. Moreover, because chaperones are already known to have an effect on the toxicity of mHTT, studies with AU may help us to connect the toxicity of mHTT with the intermediate complexes and establish a quantification standard on the amelioration of HD, which can be further used as a screening platform of pharmaceutical compounds.

In this part of the thesis, I studied the soluble portion of mHTT during propagation in a time course manner. As compared with wild-type HTT, mHTT forms a set of complexes between 20S to 80S which appear to be involved in mHTT misfolding, amyloid aggregation, and cell toxicity. Concomitantly, the effect of altering the gene dosage of several different chaperones was also tested. My results indicate that altering the levels of chaperones could also affect the soluble intermediate complexes in terms of size and abundance.
Material and Methods

Plasmid configuration

All of the mHTT expressing plasmids used in this thesis were generously provided by Dr. Michael Sherman of Boston University. To recapitulate the toxicity of mHTT in the yeast model system, only the Exon I of the mHtt gene is necessary to be transformed into host cells (Mangiarini L et al 1996). Importantly, without altering the toxicity of mHTT, a FLAG peptide was fused to the N-terminus of the protein, which provided a reliable purification handle for the protein. The proline-rich C38 fragment which suppresses the toxicity was deleted, and an eGFP fluorescent tag was fused to the C-terminus to provide convenience in detecting the protein. Finally, the fragment expressing the fused protein FLAG-PolyQ-GFP was inserted into yeast expression vector pYES2 which carried a GAL1 promoter and URA3 selection marker. In particular, the following plasmids had been received and were used in this thesis: pYES2-Flag-25Q-Gfp (25Q), pYES2-Flag-47Q-Gfp (47Q) and pYES2-Flag-103Q-Gfp (103Q).

Fig. 2.3 Insert fragment of the plasmid pYES2/25Q (Addgene.org)
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</tr>
<tr>
<td>TRY124uT</td>
<td>Isogenic to TRY123 except <em>hsp104:TRP1</em></td>
</tr>
<tr>
<td>TRY125</td>
<td>Isogenic to TRY123 except <em>ssa1:HI3 ssa2:LEU2</em></td>
</tr>
<tr>
<td>TRY123/25Q</td>
<td>Isogenic to TRY123 except <em>FLAG-25Q-GFP:URA3</em></td>
</tr>
<tr>
<td>TRY123/47Q</td>
<td>Isogenic to TRY123 except <em>FLAG-47Q-GFP:URA3</em></td>
</tr>
<tr>
<td>TRY123/103Q</td>
<td>Isogenic to TRY123 except <em>FLAG-103Q-GFP:URA3</em></td>
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<td>TRY124/103Q</td>
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<td>Isogenic to TRY123/25Q except <em>SSA1:LEU2</em></td>
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<td>TRY123/Ssa1/103Q</td>
<td>Isogenic to TRY123/103Q except <em>SSA1:LEU2</em></td>
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<td>TRY123/Ydj1/103Q</td>
<td>Isogenic to TRY123/103Q except <em>YDJ1:LEU2</em></td>
</tr>
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Table 2.1 Yeast strains and genetic background.
Yeast growth conditions and sample preparation

Yeast strains used in this study are listed in Table 2.1. All of the strains tested are derived from the strain TRY123, with the same genetic background. Yeast cells were grown at 30ºC in glucose free synthetic complete (SC) medium until mid-log phase (OD600 = 0.8-1.2), with 1% sucrose, 1% raffinose and with the appropriate amino acids deficiency for plasmid selection, respectively (Kaiser C et al 1994, Krobitsch S et al 2000). To induce the production of poly-Q proteins, galactose was added to a finial concentration of 2%. The incubation was continued for designated period of time and then cell pellets were collected by 8000 g centrifugation for 3 minutes and stored at 80ºC. For each AU-FDS sample, 100 mL of yeast culture were collected, regardless of the final cell concentration at the time of collection. The protein content of yeast cells was extracted by the glass bead method with cell lysis buffer containing 1mg/mL of PMSF, 1:500 dilution of Protease Inhibitor Cocktail (Sigma-Aldrich P8215), 0.05 M of Tris Base, 0.15 M of KCl, 2 mM of MgCl₂ and 10% glycerol. The FLAG-tag and associated mRNPs were purified by affinity purification with 100 µL of ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich) at 4ºC for 2 hours, followed by a wash with cell lysis buffer without the Protease Inhibitor Cocktail. The mRNPs were eluted with 500 µL of 0.2mg/mL FLAG® Peptide (Sigma-Aldrich) at 4ºC for 40 minutes.

AU analysis

Samples were loaded to the two-channel charcoal/Epon60K sedimentation velocity cells, with approximately 350 µL per channel. The centrifugation was started with a speed of 25000 RPM, at a temperature of 20ºC. For the AU-FDS analysis, at least 200 scans of samples were
taken from each experiment, in about 5 hours, a period of time enough for the major concentration boundary to migrate to the bottom of the cell. The raw data was analyzed by Sedfit (Schuck P. 2000), with the model c(s) distribution and proper parameters. The calculated data set of S-values was adjusted by multiplying the viscosity factor of 1.52 for the presence of 10% glycerol in the buffer.
**Results**

The AU-FDS analysis of mHTT

Based on previous results from Dr. Xin Wang who identified the unique patterns of soluble aggregates with HTT-25Q and HTT-103Q following AU-FDS analysis (X. Wang, pers. comm.), I first compared the aggregation pattern of an N-terminal HTT fragment carrying different lengths of poly-Q: 25Q, 47Q and 103Q (Fig. 2.4). These HTT fragments carry a FLAG tag at the N-terminus of HTT, the 17 N-terminal residues of HTT, the different lengths of poly-Q, and GFP fused at its C-terminus (Figure 2.3). As described previously, the HTT-25Q can be considered to be the non-toxic wild-type HTT, the HTT-47Q is an intermediate mutant and the HTT-103Q is the severely toxic mutated form of HTT.

Upon 90 minutes of induction, the three strains expressed the designated FLAG-HTT-PolyQ-GFP protein that can be purified by anti-FLAG agarose as detected by AU-FDS and gave the same type of results previously observed by Xin Wang in our laboratory. All three HTT proteins displayed a major peak below 5S (presumably monomeric forms of the HTT-GFP proteins). All three poly-Q constructs also significantly displayed a major peak at about 22S, and several peaks dispersed of lesser abundance in the higher range of S values. The estimated molecular weight for the 22S peak, if it were spherical, would be approximately 580 kDa. This size indicates that the HTT-GFP proteins are aggregating, given that the estimated size for each individual poly-Q would be about 32KDa, 34KDa and 42 KDa, respectively. There was no significant difference in the peak patterns and signal strength of each individual poly-Q tested, indicating that soon after induction of these
The polyQ aggregation differs over time

As Dr. Xin Wang had shown that the aggregation pattern for HTT-103Q changes with time in the first 24 hours after induction, and remains the same afterwards in 48 hours and 66 hours. I subsequently conducted a time-course assay to investigate the effect of poly-Q length on aggregation over an expanded time period of induction. The samples of two additional post-induction time points, 6 hours and 24 hours, were collected. With HTT-25Q, the AU-FDS results at 6 hours and 24 hours displayed no significant differences from that of the 90 minute result, except for higher peaks indicating an increased amount of HTT-25Q protein was produced (Fig. 2.5A). In contrast, AU-FDS analysis of the HTT-103Q construct resulted in a dramatic shift in peaks patterns at both the 6-hour and 24-hour times of induction (Fig. 2.4 AU-FDS analysis of HTT-25Q, -47Q and -103Q at 90 min after induction.)
2.5B). There are four peaks close to 80S, 110S, 150S and 170S that appeared in the 6-hour sample that were not present in the HTT-25Q 6 hour sample. These four peaks disappeared in the later 24-hour sample of HTT-103Q. The 22S peak in both time points was also significantly lower than that of the HTT-25Q. A different set of peaks between 30S and 60S also appeared in the 6-hour sample for HTT-103Q that was different at 6 hours for the HTT-25Q sample. These 30-60S peaks remained in the HTT-103Q samples after 24 hours with some shifts in abundance and perhaps sizes.

In summary, comparing the 24-hour HTT-103Q sample with that of the HTT-25Q, the HTT-103Q sample had a dramatic decrease in the 22S peak, all of the peaks above 60S disappeared while three peaks migrating approximately at 30S, 40S and 50S do not appear similar to the peaks found in the HTT-25Q analysis. These new peaks that appeared in the 6-hour and 24-hour samples for HTT-103Q are therefore likewise related to the complexes involved in the propagation of HTT-103Q.
Fig. 2.5 Time-course analysis of the aggregation of HTT-25Q and -103Q
The effect of altering HSP70 gene dosage on the aggregation of HTT-polyQ

As described above, deletion and overexpression of Hsp70 family proteins alone alters the toxicity of mHTT but does not affect the propagation of amyloid fibrils. I subsequently examined the effects of deleting the HSP70 genes on the HTT-103Q aggregation pattern. In yeast there are two HSP70 genes, SSA1 and SSA2. Deletion of both of these genes resulted in several effects on both HTT-25Q and HTT-103Q aggregation phenomenon (Fig. 2.6 A and B). First, the overall fluorescent signal that was obtained for either HTT-25Q or HTT-103Q was reduced. However, as observed in the wild-type strain, HTT-25Q had the same set of peaks at all time points with the hsp70 deletion, but the signal strength of 6-hour sample was similar to the 90-minute curve. This lack of increase in abundance with time for the HTT-25Q peaks differs from that of the wild-type strain (Fig. 2.5 A).

Moreover, a more significant change was observed for HTT-103Q with the hsp70 deletion. At the 6-hour time point, the peaks discovered in the wild-type strain (Fig. 2.5 B) were completely absent and the peak pattern was similar to that observed for the HTT-25Q samples. Although the lower range of peaks eventually came back at 24-hour for the HTT-103Q sample, they lacked the 50S peak compared with the wild-type strain at 24 hours. This observation suggests HSP70 is one of the factors controlling the aggregation pattern of HTT-103Q and is important to the control of degree of aggregation for both HTT-25Q and HTT-103Q.
Effect of overexpression of SSA1 on HTT aggregation

In addition, strains overexpressing HSP70 (SSA1) were also analyzed. In this case, the HTT-25Q samples displayed reduced abundance in the 6-hour sample, and overall the peaks appeared to be similar to that observed in the wild-type (Fig. 2.6 C), suggesting alterations in the synthesis or degradation of HTT-25Q. Interestingly, overexpression of HSP70 also significantly affected the peak patterns observed with the HTT-103Q sample at the 6-hour time point, in which only the 22S peak and a 30S peak were present. All the rest of the peaks observed in the wild-type strain were absent (Fig. 2.6 D). At the 24-hour time point the effect of overexpression of HSP70 was similar to that found for HTT-103Q in the wild-type strain in that the 22S, 30S, 40S and 50S peaks, with an additional peak observed at approximately 75S, were observed. As overexpression of HSP70 is also reported to be suppressing the toxicity of mHTT (Zhou H, et al 2001; Rujano MA, et al 2007; Ormsby AR 2013), these results on the deletion and overexpression of Hsp70 family member SSA1 share the similarity at the 6-hour time point that a series of peaks in the higher S value range (80S and above) were suppressed in both cases, supporting these as aggregation-related complexes linked to toxicity. For the 20 to 80S region, HSP70 deletion reduced these complexes, whereas overexpression had less effect, especially at 24 hours post induction.
Fig. 2.6 (A, B) AU-FDS analysis of HTT-25Q and HTT-103Q in the hsp70 deletion strain.
Fig. 2.6 (C, D) AU-FDS analysis of HTT-25Q and HTT-103Q in a strain overexpressing SSA1 (HSP70)
**Effects of deleting HSP104 on polyQ aggregation**

As described above, HSP104 is a yeast specific chaperone found to promote disaggregation of amyloids. Deletion of the *HSP104* gene also repressed the aggregation of mHTT in yeast (Meriin AB 2002). In the following experiment, the deletion of *HSP104* and the effect of two variants of the *HSP104* gene strains were analyzed by AU-FDS for effects on HTT-103Q aggregation. In the *hsp104* deletion strain, no major effects on HTT-25Q were observed except for the reduction of signal strength at the 24-hour time point (Fig. 2.7 A and B). In contrast, the *hsp104* deletion displayed strong effects on the HTT-103Q peaks at the 6-hour and 24-hour time points: all of the peaks in the high range (above 80S) presumably involved in the aggregation of HTT-103Q amyloids were significantly repressed. For the HTT-103Q peaks of smaller size, only a small 60S peak was observed at the 24-hour time point. These results indicate a strong correlation between the soluble aggregates observed with HTT-103Q and their effects on amyloid formation and cytotoxicity.

In addition, two variants of HSP104, carrying mutations of A503V and V426L were tested in the study. *HSP104A503V* and *HSP104V426L* are two optimized variants of *HSP104* which display enhanced function in chaperone activities. These *HSP104* variants were reported to antagonize the toxicity of several different types of neurodegenerative diseases (Jackrel ME *et al* 2014). Following AU-FDS analysis, these two variants exhibited similar effects on the aggregation of HTT-103Q, while no alterations on HTT-25Q aggregation were observed (Fig. 2.7 C, D, E and F). In both variants for HTT-103Q, a pair of major peaks running at 60S and 80S appeared in the 6-hour time point and significant material larger than 80S was observed.
This pattern is most similar to that of the strain carrying the wild-type \textit{HSP104}. These larger peaks also disappeared at the 24-hour time point similar to wild-type. Additional peaks running at 30S to 80S were also observed at the 24-hour time point. It should also be noted that in the 30-60S region for the 24-hour time point for these two variants that the results display similarities to the wild-type results at 24 hour. Overall these two \textit{HSP104} variants are displaying more modest effects on HTT-103Q aggregation patterns.

Fig. 2.7 (A, B) AU-FDS analysis of HTT-25Q and HTT-103Q in an \textit{hsp104} deletion strain.
Fig. 2.7 (C, D) AU-FDS analysis of HTT-25Q and HTT-103Q in HSP104A503V mutation strain.
Fig. 2.7 (E, F) AU-FDS analysis of HTT-25Q and HTT-103Q in *HSP104V426L* mutation strain.
Overexpression of Hsp40 family member *YDJ1*

The co-chaperone YDJ1 of the Hsp40 family is a regulatory factor that interacts with the Hsp70 chaperones to regulate their activity. Overexpression of Hsp40 along with Hsp70 reduces the size and amount of amyloids formed in different neurodegenerative diseases (Rujano MA, *et al* 2007; Ormsby AR 2013). The effect of *YDJ1* overexpression on HTT aggregation was subsequently analyzed (Fig. 2.8). No effect of *YDJ1* overexpression was observed on HTT-25Q aggregation. In terms of HTT-103Q aggregation, overexpression of *YDJ1* resulted in a set of peaks similar to the results of two *HSP104* variants and wild-type: similar major peaks were present at 30S, 40S, 60S and 80S at the 6-hour time point. The 24-hour result for the *YDJ1* overexpression was also similar to that observed for the *HSP104* variants and the wild-type strain. These results imply that overexpression of *YDJ1* is having little effect on HTT soluble aggregation.
Fig. 2.8 AU-FDS analysis of the effects of overexpression of *YDJ1* on the aggregation of HTT-103Q.
Overexpression of heat shock transcription factor HSF1

The heat shock factor (HSF1) is a transcription factor inducing the expression of heat shock proteins. It is also found to be a regulator of aging that increases the lifespan in yeast (Hsu AL et al 2003; Ohtsuka H et al 2011; Riva L et al 2012). Overexpression of HSF1 increases the overall expression of HSPs and suppressed the age depending aggregation of HTT-47Q (Cohen A et al 2012). To begin to look at possible effects of aging on HHT-103Q aggregation, the effects of overexpression of HSF1 were analyzed.

As shown in Figure 2.9, HSF1 suppressed the aggregation complexes observed with HTT-103Q following AU-FDS analysis. For HSF1 overexpression only the 22S, 30S, 40S and 60S peaks but no peaks in between 80S and 180S were present in the 6-hour sample. Additionally, the overall peak pattern of the 6-hour samples was similar to that observed for the HTT-25Q sample, except for the slightly increased 60S peak. This indicates that the aggregation was less pronounced at this earlier stage than for other strains tested. Similar effects were also observed in the hsp70 deletion and hsp104 deletion strains. Unfortunately, because the 24-hour sample of HTT-103Q was lost during the experiment, the overall effect of HSF1 overexpression cannot be completely evaluated here.
Deletion of *sir2* results affects the HTT-103Q aggregation pattern

SIR2 is another anti-aging factor that promotes the activities of chaperones under stress. The deletion of *sir2* was found to promote the aggregation of poly-Q in yeast cells (Sorolla MA *et al* 2011; Cohen A *et al* 2012). I subsequently tested the effect of deleting the *SIR2* on HTT-103Q aggregation. Deletion of *SIR2*, as expected, dramatically changed the complexes
found with HTT-103Q while the HTT-25Q aggregates remained unaffected (Fig. 2.10). For HTT-103Q at the 6-hour time point, only three major peaks running between 20S and 60S were observed in the AU-FDS results. None of the larger 80-180S peaks were observed that was present in the wild-type SIR2 yeast strain (Figure 2.5B). At 24 hours, the three peaks were observed in the same pattern with increased abundance, although a slight shift in sizes of the peaks was also observed, which is most likely due the human and instrumental errors in determining the meniscus of the sample. Because the 24-hour sample had a clearer background signal without the disturbance of the undifferentiated complexes below 5S, the sizes of the three complexes should be 22S, 30S and 40S. These three peaks may be related to the three peaks observed in the HTT-103Q sample at 24 hours in the wild-type strain. Because the sir2 deletion stain is supposed to have a faster rate of poly-Q aggregation, my results are consistent with the sir2 deletion resulting in the 24 hour pattern characteristic of HTT-103Q being obtained at a much earlier time point (6 hours in this case). These data further suggest that this unique peak pattern may be related to a specific HTT-103Q aggregation pattern.
Fig. 2.10 PolyQ aggregation in a sir2 deletion strain.
Discussion

In this chapter using AU-FDS analysis, we have observed a set of protein complexes involved in HTT protein aggregation. The role of chaperones in HTT aggregation was subsequently addressed, allowing us to further characterize which HTT aggregates were playing critical roles in the aggregation process.

The effect of expressing mHTT in yeast

According to previous research, expressing mHTT results in a single large cytoplasmic aggregate in yeast cells, in contrast normal HTT (HTT-25Q) did not aggregate (Krobitsch S and Lindquist S 2000). With the expression of the prion protein RNQ1, expressing mHTT was toxic to yeast cells, as they stopped growing after the mHTT expression was induced (Meriin AB et al 2002). In my experiments, the strain TRY123/103Q primarily used as the positive control of mHTT toxicity also developed similar phenotypes of toxicity after induction of HTT-103Q expression. When plated with low concentration on selective galactose plates, no observable colonies were formed by TRY123/103Q after a significant period time of incubation (>72 hours). Surprisingly in liquid medium, when the induction was performed in mid-log phase, the growth of the TRY123/103Q strain appeared to be impaired but not stopped, as the OD600 of the culture was still increasing. Meanwhile a large amount of yeast cells were observed to be dramatically increased in their sizes, after 24 hours of induction. The differentiated morphology of yeast cell under microscopy is likely to be caused by the instability of the 103Q plasmid. As reported, only a small portion of yeast cells were able to retain the 103Q plasmid after a long period of 103Q synthesis, even if incubated
with a selective medium (Merin AB et al 2002). With the stress from both selective medium
and mHTT aggregation, in either case, the growth of the yeast in the inductive medium
should be significantly reduced. As a result, the increase of OD600 in the cell culture may be
occurring not only because of increasing cell numbers but also by the increase in the sizes of
the yeast cells.

The general distribution of HTT-25Q

For every strain expressing HTT-25Q, despite overall differences in the c(s) levels (which
varied between strains and time points) a unique peak pattern was observed. This pattern
included a huge undifferentiated peak below 5S, a 22S peak with moderate abundance and a
series of dispersed low abundance peaks gradually descending from 30S to 180S. The
undifferentiated 5S peak was calculated from the background fluorescent signal which did
not sediment during centrifugation. This peak is likely to representing the monomeric
HTT-25Q proteins in the solution. The 22S peak and the subsequent dispersed peaks, in
contrast, represent a set of complexes much larger in size, indicating some of the HTT-25Q
was aggregated into large oligomers. It is necessary to note that this type of aggregation was
of a different type of aggregation of poly-Q that has previously been shown to not result in
amyloid formation and therefore, would not be toxic to the cells.

It should be noted that since the complexes 22S in size and larger contain presumably
multiple molecules of HTT-25Q, the peak abundances of these complexes as detected by
AU-FDS may actually be exaggerated by multiple copies of the HTT-25Q-GFP proteins.
What this means is that, if we assume that the increase in S value is due to increased numbers of HTT-25Q associating in a particular complex, as the S value increases and the mass increases, the fluorescent signal increases. This results in the fluorescent signal providing higher than expected values and the actual abundances of the complexes as they increase in S value would be lower than visualized in the Figures presented.

Two types of aggregation in HTT-103Q expressing strains

As compared to the results observed with HTT-25Q, the aggregation of HTT-103Q was much different. While at 90 min post-induction, all of the HTT-103Q complexes had the same set of peaks as observed with the HTT-25Q results, later time points identified a number of large complexes. According to the abundance changes of HTT-103Q monomers over time in the wild-type strain, the aggregation of the misfolding HTT-103Q mainly occurred during the 90 min to 6 hr period. This is reflected in the unique peak patterns of the 6 hr results, in which the 22S peak became reduced, a set of discrete peaks were observed in the range of 80S to 180S, and a new pattern was observed in the 30-50S region. In contrast, by 24 hr, the large peaks above 80S became absent and the abundances of the 30-50S peaks were different than observed at 6 hr. Therefore, these large transient complexes (greater than 60S) are likely to be the intermediates of amyloid formation; whereas the 30S-50S peaks present in both time points are more likely to be the terminal soluble aggregation form for HTT-103Q and appear to coexist with the presence of the amyloids. Therefore, in the wild-type strain, the presence of this set of large complexes suggests a different type of soluble protein aggregation that leads to the formation of insoluble amyloids occurring after 90 min of induction, before
which, the aggregation pattern was similar to that of HTT-25Q.

Since I found these peaks between 20S and 80S varied in abundance by altering the dosages of different chaperones, these results indicate that these peaks are related to the misfolding of HTT-103Q. As a result, HTT-103Q is likely to be synthesized in an unstable native state which behaves and looks similar to that of HTT-25Q and which forms similar types of oligomeric structures in the 22S to 60S region. Following 90 min, the misfolding of HTT-103Q takes place, as observed at the 6 hr time point. This misfolding resulted in the aggregation of multiple larger complexes both in the 30-50S region and definitely in the 80S and larger region and eventually the formation of insoluble amyloid material.

The amyloid aggregation of HTT-103 results in significant loss of the monomeric form of the protein

Based on the results obtained, the signal migrating below 5S, which is presumably the monomeric form of poly-Q, increased over time and remained in high abundance in all HTT-25Q samples. In contrast, in the wild-type strain, the monomeric HTT-103Q had reduced abundance in 6 hr and 24 hr as compared to the 90 min samples. This effect was enhanced in the overexpression or increased activity of chaperones, and was suppressed in the strains carrying deletion of HSP70 and HSP104 and in the HSF1 overexpression strain (Fig 2.11, only HTT-103Q results are re-scaled). The reduction of the monomeric HTT-103Q was also coupled with the presence of the large complexes related to the misfolding aggregation of HTT-103Q. With only soluble complexes being analyzed, the majority of the HTT-103Q
protein may be present in the amyloid, which is too large for FLAG agarose purifications. Therefore, the complexes observed in the results that I present are likely to be the intermediate aggregates of HTT-103Q in the process of amyloid formation.
Fig 2.11 Rescale of the 2.5S peak of HTT-103Q in different strains.
Absence of the HSP70 and HSP104 chaperones reduce the overall rate of amyloid aggregation.

Although regarded as a self-propagating process, during the time points tested in this thesis, the amyloid aggregation of poly-Q relied on the presence of chaperones and other cellular factors. Deletion of chaperone HSP70 strongly prohibited this overall process, with very low abundance of all aggregation complexes (both the 30-50S material at both the 6 hr and 24 hr time points and the material larger than 80S at 6 hr). There was also more monomeric HTT-103Q observed at these time points as compared with the wild-type (Fig. 2.11). These results are consistent with the model that the HSP70 gene is required for HTT-103Q monomer to oligomer aggregation. Because it has previously been shown that deletion of HSP70 resulted in smaller amyloids of HTT-103Q that suppressed the toxicity of mHTT in yeast (Meriin AB et al 2002), my results indicate that the repression of toxicity by hsp70 is likely due to the overall slower rate of poly-Q aggregation in this chaperone deletion strain.

The deletion of HSP104 also had major effects on HTT-103Q aggregation. In this case, and unlike that of deleting HSP70, the overall pattern looks more similar to that of HTT-25Q than it does to the HTT-103Q pattern in the wild-type strain. Also, the hsp104-containing strain did not display the reduction of the monomeric HTT-103Q material with time that was observed in wild-type. Therefore, deletion of HSP104 appears to strongly block the aggregation process and suppresses the formation of all soluble aggregates greater than 22S. Because deletion of HSP104 blocks HTT-103Q toxicity and results in the formation of a single non-spreading amyloid, my results are consistent with these hsp104 effects being the
result of the blockage of the soluble oligomerization process that we have observed.

The formation of soluble aggregates was affected by SSA1 overexpression

Despite suppressing the toxicity of HTT-103Q, overexpression of HSP70, or enhancement of HSP104 did not significantly suppress the formation of the HTT-103Q amyloid (Zhou H, et al 2001; Rujano MA, et al 2007; Jana NR, et al 2009; Ormsby AR 2013; Jackrel ME et al 2013; Lee DH et al 2010; Vacher C et al 2005). For my results, overexpression of SSA1 at the 6 hr time point resulted in blockage of all large aggregates (greater than 60S) and significant reduction in the 30-50S aggregates (Figure 2.6D). AT 24 hr, however, the pattern displayed with SSA1 overexpression was similar to that of the peak patterns as compared to the wild-type strain with only one additional 75/80S peak being present. These results suggest that the aggregation process is being affected, which might be the cause of SSA1 effects on HTT-103Q toxicity. However, given that the 24 hr soluble aggregation pattern is similar for SSA1 overexpression as it is for wild-type, there may be no effect of SSA1 overexpression on insoluble amyloid formation, which is in agreement with published results. Therefore, the soluble complexes may be more related to HTT-103Q toxic effects than the large insoluble amyloid.

In contrast to these results, overexpression of YDJ1 displayed less noticeable effects on the 6h and 24 hr HTT-103Q aggregation patterns (Figure 2.8B). Both the larger than 60S complexes were observed at 6 hr and the 30-50S complexes appeared similar to that of wild-type at both 6 and 24 hr. Since overexpression of YDJ1 does not affect HTT-103Q
toxicity or amyloid formation, these results again are consistent with the soluble aggregates that HTT-103Q forms as being important to both toxic effects and amyloid formation.

*HSF1* overexpression can have effects on HTT-103Q aggregation either from its control of the expression of multiple chaperones or from effects on aging.

In contrast, overexpression of *HSF1* had shown promising effect on the prevention of amyloid aggregation in 6 hr (Cohen et al 2012). HSF1 is regarded as the “master” regulator of all chaperones, overexpression of *HSF1* increases the expression of all heat shock proteins (Morimoto RI et al 1992). Multiple chaperones are known to control the misfolding process, and therefore a global regulator like HSF1 may be having more general effects than singly overexpressing any one chaperone. HSP70 corrects the misfolding proteins with the assistance of co-chaperone HSP40, while HSP104 has the function of disassociating amyloids (Zhou H, et al 2001; Rujano MA, et al 2007; Ormsby AR 2013; Jackrel ME et al 2013; Lee DH et al 2010; Vacher C et al 2005). These chaperones are likely to have synergistic effects on amyloid formation in that HSP70 corrects the misfolding protein disassociation from the large aggregates of amyloid by HSP104. Overexpressing the chaperones individually may be effective on suppressing the toxicity but the effect will be restricted by the limited presence of other chaperones it cooperates with.

My results with the overexpression of *HSF1* resulted in very high abundance of the 60S peak at the 6-hour time point, whereas the 22S, 30S, and 40S, peaks appeared more similar to that observed for HTT-25Q. This means that overexpression of *HSF1* is delaying the onset of
HTT-103Q aggregation, a result consistent with its known effect on amyloid formation (Riva L et al 2012; Cohen A et al 2012).

Deletion of SIR2 significant effects on the aggregation patterns of poly-Q

Two anti-aging factors HSF1 and SIR2 were previously shown to alter the aggregation rates of HTT-poly-Q (Cohen A et al 2012). In my results the deletion of SIR2 resulted in rapid aggregation of HTT-poly-Q at an early stage, which was observed to generate a unique peak pattern of aggregation with abnormally high abundance of the 40S and 30S complexes, and very low abundance of 60S and 80S complexes. On the other hand, the overexpression of HSF1 resulted in decreased aggregation at 6 hr as described above. Since the 40S and 30S peaks were observed in the wild-type strain expressing HTT-103Q but in moderate abundance, the excessive amount of these two complexes in the sir2 deletion strains correlate with the increased toxicity linked to sir2, which is therefore linked to the amyloid aggregation of HTT-103Q.

The enhanced function variants of HSP104 had little effect on aggregation

Two variants of HSP104 (HSP104A503V and HSP104V426L) were analyzed because previous results had indicated that they enhanced disaggregation of misfolded complexes, reducing the toxicity of α-syn of Alzheimer’s disease, and FUS and TDP-43 of ALS disease (Jackrel ME et al 2014). However, my results indicate that neither variant seemed to display large effects on HTT-103Q soluble aggregates. It is possible that these variants are unable to function with HTT whereas they work well dissociating other types of misfolded proteins.
The formation of 50/60S complex may be an early event of the aggregation of the amyloid

The 6-hour time point for the effect of HSF1 overexpression on the HTT-103Q aggregation pattern and the 24-hour time point of the hsp104 deletion shared a similar pattern of peaks that appeared identical to the HTT-25Q analyzed sample except for an increased 60S peak. Additionally, these two particular results both suggested that the loss of HTT-103Q monomers was delayed, as reflected in the background signal below 5S. These observations suggest that the change in the 60S peak is an early stage of the misfolding poly-Q aggregation pattern. Although the 60S peak was only observed in part of the strains at a particular time point, several other strains contained a 50S peak instead. Because the sizes of these peaks were not precisely determined, it is possible that the 50S and 60S peak were representing the same complex (see below discussion of a similar situation with the 75S and 80S peaks). This conclusion is supported by the observation that the hsp70 deletion strain, which had a very strong effect on the aggregation process, also did not display the 60S peak. The sir2 deletion strain, which is supposed to have rapid aggregation, also displayed a significant 50S/60S peak at 6 hr. These observations suggest that the 50S/60S peak may be an early intermediate in the aggregation process, but more analysis of time points between 90 min and 6 hr would be necessary to further test this hypothesis.

It should be mentioned that the 75S and 80S peaks observed are also likely to represent the same peak. For AU-FDS analysis conducted at 25000 rpm, 80S is a little larger than the range of S values which is best resolved at this rotor speed. It is likely that systematic errors perhaps exaggerated by human errors in these experiment result in small inconsistency of the
size of the peaks in this range.

The linkage between HTT-103Q toxicity, amyloid formation and the AU-FDS observations

Although regarded as the most critical feature of neurodegenerative diseases, the formation of insoluble amyloid is not directly linked to cytotoxicity. In fact, natural amyloids widely exist in a variety of organisms with different biological functions (Hammer ND et al 2008). In the case of HD, several factors had been discovered to suppress the toxicity or symptoms of mHTT without altering the aggregation of amyloids, yet suppressing amyloid formation will definitely ameliorate the disease (Zhou H, et al 2001; Rujano MA, et al 2007; Ormsby AR 2013). My results also supported that suppressing amyloid formation is not necessarily required for the prevention of HTT-103Q toxicity, as soluble aggregates may be affecting toxicity. For example, deletion of HSP70 or HSP104 suppressed amyloid formation and toxicity, and I showed that they had major effects on the unique soluble aggregation observed with HTT-103Q. Therefore, the toxicity of HTT-103Q may be determined by the type of amyloid it forms, and whether the presence of excessive abundance of chaperones alters the concentration of soluble aggregates. Interestingly, in the HSP70 overexpression strain, which has reduced toxicity with little alteration in amyloid, I observed several differences in peaks from the wild-type strain in both 6 hr and 24 hr samples with AU-FDS. Although the compositions and functions of these peaks are not yet determined, their presence might be relevant to the reduction of amyloid toxicity in the samples.
Aging significantly promotes the amyloid aggregation and toxicity

Surprisingly, both of the two aging factors tested in this study greatly altered the aggregation of HTT-103Q. A majority of neurodegenerative diseases patients get the disease symptoms at mid-age or later, suggesting aging may be contributing to the development of the disease. Additionally, without the replacement of new cells, most of the neuron cells have an extra-long life span. Aging can be one of the determining factors in the neural loss of these neurodegenerative diseases. Among the tested strains, overexpression of HSF1 had one of three best suppression effects on the soluble aggregation pattern observed among the strains tested, the other two being the deletion of HSP70 and the deletion of HSP104. Relatedly, the deletion of SIR2 increased the aggregation and toxicity of the HTT-103Q amyloid (Cohen et al 2012) and exhibits a unique peak pattern of soluble aggregates under AU-FDS. These two results are consistent with the soluble aggregates being directly linked to toxicity. As a result, more anti-aging factors should be tested in the future. Practically, to produce remedies against HD, overexpression of anti-aging factors is a more likely approach, since deletion of chaperones might clearly bring additional problems with the misfolding clearance of all cellular proteins. Also, altering the abundance of anti-aging factors may also bring other benefits to cells.

Future prospects

Based on the current findings on the aggregation of HTT-103Q with different factors, further investigations are necessary to be conducted. Some of the possibilities are suggested below.

1) Increase the amount of timings on sample collection. The timing of samples I currently
used was discrete but not capable of capturing the full description of this process. Adding more time points between 90 min and 6 hr may help us determine the early events of and peak patterns of soluble aggregate formation. Because HD in a yeast model is a highly accelerated process, compared to that in humans, due to the high expression levels of mHTT and short lifespan of yeast, detecting a small delay in the start of aggregation may correspond to years for HD patients.

2) Determine the compositions of the (20S to 80S) peaks observed in these experiments. It is important to know the composition and stoichiometry present in each of the peaks that HTT-103Q is forming. As these peaks are likely to be linked with the amyloid formation or the toxicity of HTT-103Q, such investigations may help to discover the actual roles of the complexes in this process, and develop intervention methods that could affect their abundances.
General Discussion

In chapter I, I used AU-FDS analysis to characterize the 77S monosomal translating mRNP associated with SBP1. I determined the composition and stoichiometry of other factors associated with this 77S complex and showed that the complex was distinct from that of the 77S complex that had been previously identified with FLAG-PAB1. Based on the fact that the 77S complex that was co-purified with FLAG-SBP1 lacked translation initiation factors led to the suggestion that this complex and SBP1 is representative of a translating monosome at a late elongation phase or early termination phase.

In chapter II, AU-FDS analysis was used to identify the soluble aggregates present in a yeast model of HD. Using HTT-103Q that forms amyloids and which causes cell toxicity, our results identified a series of intermediate complexes that were formed. These complexes, ranged from 30-180S in size, are presumably the precursors of insoluble amyloid aggregates previously observed by other researchers, as they occur soon after HTT-103Q induction of expression. In that soluble aggregates in the 30-50S size range remained after 24 hr of HTT-103Q induction, these 30-50S complexes may also be the degradation products of amyloids or possibly stably formed precursor complexes. The effects of altering chaperone and aging factors gene dosage on HTT-103Q aggregation was also analyzed. My results suggest that the presence of amyloid aggregation was not always linked with HTT-103Q toxicity in yeast, and the differences in the aggregation patterns may alter the toxicity of amyloid.
In both parts of the thesis, AU-FDS was used as the primary technique to conduct the studies. As described above, the advantages of AU-FDS make it a unique tool to the studies of macromolecular complexes in solution. In this respect, this technique worked well both in further characterizing the 77S monosomal translating complex and for the first time in identifying soluble complexes that are relevant to the HTT amyloid aggregation. However, there are also a couple of disadvantages of AU analysis. First of all, although AU is capable of analyzing samples in a broad range of sizes, the actual range of each individual experiment was restricted by centrifugal speed. Under a certain speed, complexes larger than the designated range will sediment to the bottom too soon, while complexes too small will result in little sedimentation occurring. In both cases, the sizes of these complexes cannot be precisely determined.

Additionally, AU analysis determines the S value assuming the shape of the molecule is spherical. For complexes with unknown structure, it is impossible to determine the actual size by AU, as the conversion of the S values to molecular weight requires defining the shape of the factor. This might result in two complexes with the same size sedimenting at dramatically different rates, because of their differences in the shape, or complexes migrating at the same S value being of different composition and mass, as observed with the 77S complex. Fortunately, carefully designed experiments may be able to minimize the error generated by these disadvantages.

Two other limitations with AU analysis were observed in these studies. While AU-FDS
analysis can detect unique complexes not previously visualized (Wang et al 2012), the composition and stoichiometry of the components in these complexes are more difficult to determine. Using a variety of GFP tagged proteins, composition and stoichiometry of components of the 77S complex were determined for both the FLAG-PAB1 and FLAG-SBP1 complexes. But this technique was limited by the fact that the protein composition was probed based on what we, the researchers, thought was in the complex. The presence of novel factors in the 77S complex could not be feasibly tested. In this case, mass spectrometric procedures would be best to determine what factors are in a particular complex. On the other hand, we were not able to determine the composition of HTT-103Q complexes. For this type of analysis new types of FLAG-tagged HTT constructs lacking the GFP component would have to be generated. However, even these studies may be problematic if the lack of the GFP were to affect HTT-103Q aggregation in yeast.

Finally, AU-FDS analysis cannot stand on its own to characterize completely the soluble complexes identified herein and in our other studies (Wang et al 2012). Obviously, mass spectrometric procedures would be helpful for characterizing the composition of these complexes. Unfortunately, AU analysis does not purify the complexes. This means other purification means (sucrose gradient analysis, possibly) would be needed to purify the soluble complexes identified with AU analysis. Whether sucrose gradient analysis can rigorously identify the complexes observed with AU analysis is not certain. In this regard, AU analysis can identify and differentiate complexes not previously visualized, but their further purification and characterization by standard procedures may not feasible.
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