The *Saccharomyces cerevisiae* HtrA orthologue, Ynm3, is a chaperone-protease that aids survival under heat stress

PhD Thesis

in partial fulfilment of the requirements for the degree “Doctor of Philosophy” in the Molecular Biology Program at the Georg August University Göttingen, Faculty of Biology

submitted by

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born in

Trivandrum, India

2008
I hereby declare that the PhD thesis entitled “The *Saccharomyces cerevisiae* HtrA orthologue, Ynm3, is a chaperone-protease that aids survival under heat stress” has been written independently and with no other sources and aids than quoted.

Nirmala Padmanabhan
To my grandfather, Late Mr. P. Rama Iyer
Acknowledgements

This work was performed in the Department of Molecular Microbiology and Genetics, Institute of Microbiology and Genetics, Georg August University, Goettingen.

I am grateful to my mentor and supervisor Prof. Gerhard H. Braus for giving me the opportunity to pursue my PhD work in his laboratory and for his constant encouragement and support.

I am thankful to Dr. Lars Fichtner for his involvement in my work and for his friendly support throughout my PhD.

My doctoral committee members, Prof. Christiane Gatz and Prof. Jörg B. Schulz have given me their time and support, for which I am thankful. Prof. Schulz was involved in my work from the very beginning and has invited me to regularly present my work in his departmental seminars.

I am beholden to the following people for their help and collaborations, without which this work would not be in its present form.

Ms. Maria Meyer for the vast amount of technical assistance and for organizing the activities of Lab 107, which made working easier. Dr. Achim Dickmanns for his interest in my work, which resulted in a fruitful collaboration. Ms. Annette Berndt for performing the protein purifications. Dr. Susanne Behrens-Kneip for teaching me how to perform the chaperone activity assay. Dr. Özgur Bayram for his willingness to help with difficult molecular biology techniques. Dr. Oliver Valerius for performing mass spectrometry during my search for protein interaction partners of Ynm3. Dr. Micheal Hoppert for performing electron microscopy during the initial phase of this work. Ms. Heidi Northemann and Ms. Nicole Scheiter for helping me with official tasks and ordering of chemicals. I thank all the lab rotation students who have worked with me and all the other members of the department who have helped me during the course of my work.

For an enjoyable working atmosphere, I wish to thank both past and present members of lab 107, Dr. Lars Fichtner, Ms. Maria Meyer, Ms. Seema Singh, Dr. Susanna Braus-Stromeyer, Mr. Tran Van Tuan, Mr. Christian Timpner, Ms. Peggy Findeisen, Mr. Christoph Braun, Mr. Marc Dumkow, Ms. Melanie Nolte and Ms. Katrin Hartwich.

I am thankful to Dr. Steffen Burkhardt, the co-ordinator of the International Molecular Biology Program for making life easier in Göttingen.

I would like to thank my husband Dr. Tabrez J. Siddiqui for his immense patience, love, encouragement and for being my pillar of support.

My deep fondness and gratitude for my mother Ms. Parvathy Padmanabhan and my father Mr. R. Padmanabhan for all that they have given me and my sister Ms. Sumathy Mohan for her love and support.

I remember with deep respect all my grandparents, all of whom are no more. I dedicate this work to my late grandfather, Mr. P. Rama Iyer who dreamed to see me fly in colours.

Lastly, I wish to thank all my relatives and friends for their faith in what I do.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAA+</td>
<td>ATPases associated with various cellular activities</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BFP</td>
<td>Blue fluorescent protein</td>
</tr>
<tr>
<td>CEN</td>
<td>Centromeric or centromere containing</td>
</tr>
<tr>
<td>CHIP</td>
<td>C-terminus of Hsc70-interacting protein</td>
</tr>
<tr>
<td>CS</td>
<td>Citrate synthase</td>
</tr>
<tr>
<td>DHR</td>
<td>Dihydrorhodamine 123</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic reticulum associated degradation</td>
</tr>
<tr>
<td>FKBPs</td>
<td>FK506 binding proteins</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HtrA</td>
<td>High temperature requirement A</td>
</tr>
<tr>
<td>Hsps</td>
<td>Heat shock proteins</td>
</tr>
<tr>
<td>IAPs</td>
<td>Inhibitor of apoptosis proteins</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>mnd2</td>
<td>Motor neuron degeneration 2</td>
</tr>
<tr>
<td>Nma111</td>
<td>Nuclear mediator of Apoptosis (111 kDa)</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Sequence</td>
</tr>
<tr>
<td>OMPs</td>
<td>Outer membrane proteins</td>
</tr>
<tr>
<td>PAN</td>
<td>Proteasome activating nucleotidase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>Description</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulphide isomerase</td>
</tr>
<tr>
<td>PDZ</td>
<td>protein-protein interaction domain; stands for PSD-95, <em>Drosophila Disc</em></td>
</tr>
<tr>
<td></td>
<td>large protein, zona occludans protein 1</td>
</tr>
<tr>
<td>PPIase</td>
<td>Peptidyl prolyl cis-trans isomerase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SC</td>
<td>Synthetic complete</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>TAP</td>
<td>Tandem affinity purification</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>TBS with 0.1% Tween</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast peptone-yeast extract-dextrose medium</td>
</tr>
</tbody>
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Abstract

Ynm3 is the only budding yeast protein possessing a combination of a serine protease and PDZ domains, a defining feature of the widely conserved HtrA (high temperature requirement A) protein family. The bacterial HtrA/DegP is involved in protective stress response to aid survival at higher temperatures by cleaving irreversibly unfolded proteins in the periplasm. At ambient growth temperatures, it acts as a chaperone and aids maturation of outer membrane proteins that have escaped folding mediated by the primary periplasmic chaperone SurA. Studies involving overexpression of mammalian mitochondrial HtrA2/Omi in cell culture propose a proapoptotic role for the protein. Mice lacking HtrA2/Omi or its protease activity, however, show no evidence of reduced rate of cell death. Instead, these mice suffer loss of a population of neurons in the striatum leading to a Parkinsonian phenotype. Two mutations in the gene encoding human HtrA2/Omi, leading to partial loss of protease activity have been identified as susceptibility factors for the development of Parkinson’s disease. There is mounting evidence that the mammalian HtrA2/Omi, a resident of the mitochondrial intermembrane space could have a protective role. The hypothesis that HtrA2/Omi might act as a chaperone-protease like its bacterial counterparts is indeed very attractive considering that mitochondria are evolutionary derivatives of ancestral α-proteobacteria. It is noteworthy that no such definitive function has been ascribed to any eukaryotic member. This work deals with unravelling the cellular role of Ynm3, the HtrA orthologue of the simple eukaryotic model, the unicellular *Saccharomyces cerevisiae* with respect to unfolding stresses.

The major finding of this work is that Ynm3, like the *E. coli* HtrA/DegP, is a dual chaperone-protease. The proteolytic activity conferred by the serine residue at position 236 of Ynm3 is crucial for executing its protective role upon heat stress. Ynm3 also exhibits strong ATP-independent general chaperone activity *in vitro*, a novel finding for a eukaryotic HtrA member. I propose that the chaperone activity of Ynm3 may be important to improve the
efficiency of proteolysis of aberrant proteins by preventing the formation of non-productive toxic aggregates and presenting them in a soluble state to its protease domain. Like in the case of the classic proteolytic complexes of bacteria and mitochondria, Ynm3’s associated chaperone activity may be indispensable for sequestering proteins that need to be cleaved, in their unfolded states, in order to accommodate them in its proteolytic chamber which may be too narrow to allow the entry of misfolded or folded conformers.

Suppression studies performed in this study led to the identification of Fpr3, a nucleolar peptidyl prolyl cis-trans isomerase (PPIase), as a partial knockout suppressor of the heat sensitivity of Δynm3. Further analysis demonstrated that Fpr3 acts as a strong chaperone, in vitro. A similar scenario exists in the bacterial periplasm where the bacterial HtrA/DegP partly compensates the folding activity of the major periplasmic chaperone SurA, which is also a PPIase. The observed interaction with the PPIase, Fpr3 further supports the role of the eukaryotic HtrA member Ynm3 in protein quality control analogous to the bacterial HtrA/DegP.

Ynm3 is primarily nuclear but a subpopulation is associated with mitochondria. This study provides initial evidence that Ynm3 is presumably involved in mitochondrial homeostasis during aging. Since the budding yeast has been effectively used to derive molecular mechanisms underlying apoptosis, unfolding stresses and human neurodegenerative disorders, this work could have significant bearing on the understanding of the role of HtrA2/Omi, the mammalian mitochondrial HtrA counterpart implicated in Parkinson’s disease, especially with respect to protein quality control.
1. Introduction

Polypeptides may have the potential to fold by themselves to their native conformation, the information for which is encoded in their amino acid sequences. But the intracellular milieu, owing to molecular crowding and a high risk of aggregation, is barely conducive for such spontaneous folding to occur. Many newly synthesized polypeptides are unable to achieve the appropriate amino acid contacts and find their native conformations until chain termination is achieved. Therefore, right from the time they emerge from the ribosome, polypeptides run a high risk of aggregation. Molecular chaperones prevent such an event by carefully assisting the folding of polypeptides to their native structures. By binding exposed hydrophobic patches, chaperones prevent the aggregation of newly synthesized proteins and assist in folding to their native conformations (Hartl & Hayer-Hartl, 2002). In a properly folded conformer, the hydrophobic residues of the protein are buried in the centre whereas the hydrophilic amino acids are exposed to the surface maintaining the protein in a soluble state.

1.1 Protein quality control

Successful initial folding of proteins does not guarantee their long-term stability. Proteins are constantly threatened by environmental insults like heat or chemical stresses that can lead to loss of their native conformation. Cells cope with such undesirable conditions by upregulating the synthesis of protein quality control factors: chaperones and proteases and also components of the ubiquitin-proteasome pathway in the case of eukaryotes (Imai et al., 2003; Morimoto, 1998). Damaged proteins and those proteins that have not achieved their native conformations due to mutations, inaccuracy in the transcription-translational machinery or post translational modifications or incorrect localization are subject to rescue by chaperones or degradation by quality control proteases. Failure of these quality control mechanisms often leads to aggregation due to unspecific interactions between exposed
hydrophobic patches of denatured proteins. Oligomers leading to aggregate formation are often toxic. Aggregation can lead to sequestration of important cellular proteins or protein complexes disrupting normal cellular function (Gidalevitz et al., 2006). Moreover, proteins within aggregates are generally refractory to proteolytic attack impeding the recycling of amino acids, which could otherwise be used for \textit{de novo} protein synthesis.

Chaperones act by binding to sticky hydrophobic patches on proteins, maintaining them in a soluble state and giving them another chance to refold to their native state. When chaperone-mediated rescue fails, quality control proteases recognize and bind irreversibly unfolded proteins facilitating their degradation. A balance among these mechanisms determines the efficiency of protein quality control (Wickner et al., 1999). Thus chaperones and proteases function hand-in-hand in relieving the cell from the load of unfolded or misfolded proteins (Hinault & Goloubinoff, 2006). Lack of these quality control factors or mutations in the genes encoding these factors often lead to accumulation of intracellular protein aggregates and cell cycle arrest, especially after heat stress.

\section*{1.2 Classic chaperones and proteases}

There is significant convergence in the quality control mechanisms operating in prokaryotes and eukaryotes. The chaperone and protease machineries operating in prokaryotes and mitochondria are strikingly similar. This is not surprising since mitochondria are evolutionary derivatives of ancestral \(\alpha\)-proteobacteria. In addition to the ATP-dependent proteolytic complexes in their cytoplasmic organelles, eukaryotes contain a huge mega-complex called the proteasome for degradation of proteins in the nucleus and cytoplasm. Related AAA+ proteins and 20S proteasomes are also found in the archea.
1.2.1 Molecular chaperones

Molecular chaperones are composed of several classes of proteins performing diverse functions ranging from assistance in protein folding and translocation across organellar membranes, prevention of aggregation, refolding of un/misfolded proteins and dissaggregation of proteins (De Los Rios et al., 2006; Hinault et al., 2006). Most of them are induced by stress and hence called heat shock proteins (hsp). They have been classified depending on their apparent molecular weight on gels. The classic chaperones operating in *E. coli* are DnaK/Hsp70 and its cochaperones, DnaJ and GrpE (Deuerling et al., 1999; Saibil, 2008; Teter et al., 1999) and GroEL/Hsp60 and its cochaperone GroES (Bochkareva & Girshovich, 1992; Chapman et al., 2006; Horwich et al., 2006; Horwich et al., 2007). These are ATP-dependent chaperones, which function not only in de novo protein folding but also during cellular stresses by preventing aggregation and aiding the refolding of denatured proteins (Ben-Zvi et al., 2004). Hsp60 related heat shock inducible proteins are also present in Archea (Kapatai et al., 2006). Homologues of Hsp70 are present in both the cytosol and mitochondria of eukaryotes. Chaperones belonging to the Clp family in *E. coli* act either independently or in conjunction with a protease component. Hsp104 is a Clp family homologue in the eukaryotic cytosol and mtHsp78 is the Clp homologue present in mitochondria. Some of these chaperones, eg, yeast Hsp104 and bacterial ClpB, not only prevent aggregation of proteins but also aid in dissaggregation (Bochkareva & Girshovich, 1992; Glover & Lindquist, 1998; Goloubinoff et al., 1999; Leidhold & Voos, 2007; Motohashi et al., 1999; Sanchez & Lindquist, 1990).

1.2.2 ATP-dependent proteases

There are three major families of ATP dependent multisubunit proteolytic complexes in the cytosol of *E. coli* and in organelles like mitochondria and chloroplasts of eukaryotes. These belong to the Clp family, the membrane anchored FtSH/AAA protease family and the
Lon protease family (Reviewed in Mogk et al., 2008; Snider et al., 2008). The Lon (La) protease is a 600 kDa ATPase complex that rapidly degrades abnormal proteins in *E. coli* (Chung, 1993; Goldberg, 1992; Maurizi, 1992). The mitochondrial Lon homologue is a matrix protein and is necessary for viability because it prevents accumulation of aberrant proteins within the organelle (Langer et al., 2001; Suzuki et al., 1997). Members of the Clp family, in *E. coli* are ClpAP (Kessel et al., 1995; Wickner et al., 1994) and ClpXP (Wawrzynow et al., 1995) in addition to ClpB. As mentioned before, the chaperone Hsp104 is the Clp homologue in the eukaryotic cytosol (Chernoff et al., 1995; Parsell et al., 1994) and mtHsp78 is the mitochondrial Clp homologue (Moczko et al., 1995). Members of the FtsH/AAA family are membrane anchored but contain cytoplasmic domains with ATPase sites and Zn$^{2+}$ metalloprotease active sites (Tomoyasu et al., 1993). Mutations in the genes encoding the yeast mitochondrial FtsH family Yta10/Yta12 protease complex block degradation of abnormal proteins and mitochondrial membrane protein assembly (Pajic et al., 1994; Tauer et al., 1994). All these proteolytic complexes are composed of ATPases and proteolytic subcomplexes. ATP hydrolysis is necessary for unfolding of substrates and for their delivery into the proteolytic chambers where they are processively degraded to small peptides (Reviewed in Baker & Sauer, 2006; Mogk et al., 2008). These proteases also fall into the category of heat shock proteins (hsp90s) as they are induced along with molecular chaperones under stress. Archea encode proteins similar to FtsH ATPase and the Lon protease. ClpP proteases are not present in the archea excepting *M. thermoautotrophicum*, which appears to encode a Clp-like ATPase (Maupin-Furlow et al., 2000).

### 1.2.3 The 26S proteasome

Eukaryotic cells have a single large multisubunit ATP-dependent proteolytic complex, the 26S proteasome, in their nucleus and cytoplasm unlike in prokaryotes and mitochondria where several specialized ATP-hydrolyzing proteases are in operation. The proteasome
contains sites that cleave after hydrophobic, acidic and basic residues enabling degradation of various types of substrates fed into it by multiple ubiquitin ligases with distinct specificities (Goldberg, 2003; Voges et al., 1999). The proteasome has a domain organization similar to the bacterial ClpAP but contain additional regulatory components. The 26S proteasome is composed of a core of 20S particles which contains the proteolytic sites and one or two 19S regulatory particles responsible for substrate recognition and delivery into the core particle (Goldberg, 2003; Voges et al., 1999). The architecture of both the bacterial ATP dependent proteases and the proteasome allows the degradation of unfolded proteins in a central channel secluded from the cellular environment. In all these energy dependent proteolytic machineries, ATP hydrolysis is necessary to unfold the substrate and channel it into the proteolytic sub-compartment whose dimensions do not allow the entry of native proteins. This is mediated by the ATPase component of bacterial or organellar proteases, for example, the ClpA or ClpX component of ClpAP and ClpXP respectively mediate unfolding of substrates (Hoskins et al., 2000; Kim et al., 2000). In the base of the 19S regulatory subunit of the 26S proteosome, a ring of 6 ATPases are present that unfold ubiquitinated substrates, facilitate gate opening and translocate them into the proteolytic compartment in the 20S particle (Benaroudj et al., 2003; Smith et al., 2006). Archea also have a proteasome in addition to other AAA+ family related proteases. Protein degradation by the proteasome activating nucleotidase (PAN) regulatory complex of archea and its 20S proteasome also involve substrate recognition, ATP-dependent unfolding, translocation and opening of the gated channel in the proteasome (Smith et al., 2006; Zwickl et al., 1999).

1.3 Substrate recognition by chaperones and proteases

Similar fundamentals direct substrate recognition, binding and unfolding by chaperones and the ATPase components of energy dependent proteases. These quality control proteins have evolved to recognize surface exposed hydrophobic residues on proteins that enable them
to distinguish between native and non-native forms. Classic chaperones, namely the DnaK/Hsp70 system and the GroEL/Hsp60 system repeatedly bind hydrophobic patches on unfolded proteins and release partially folded or native conformers in an ATP dependent manner (Bukau et al., 2006; Sigler et al., 1998). Similarly, the ATPase components of proteases must recognize abnormal proteins from the cellular protein pool. The ATPase components of heterooligomeric proteases like ClpAP (Hoskins et al., 2000) or ClpXP (Schirmer et al., 1996; Wawrzynow et al., 1995) and the ATPase domains of homooligomeric proteases like the FtsH/AAA (Tomoyasu et al., 1993) or Lon protease also possess ATP-dependent chaperone activities capable of remodeling or reactivating unfolded or misfolded proteins (Gottesman et al., 1997a; Mogk et al., 2008; Snider et al., 2008).

An attractive ‘triage’ model has been proposed explaining how classic chaperones or the regulatory ATPase components of energy dependent proteases partition substrates for either refolding or degradation by the protease components (Gottesman et al., 1997b) (Figure 1). The meaning of “triage” in the medical dictionary is “sorting of patients and allocation of treatment depending on the degree of their wounds”. According to the model, if attempts at folding newly synthesized proteins or refolding damaged proteins by chaperones fail, the irreversibly unfolded or misfolded proteins are committed for degradation by the recruitment of proteases or the ubiquitination machinery, which would ultimately eliminate them. Thus chaperones or chaperone components of proteases first give aberrant proteins a chance to refold by repeated ATP dependent binding and release (Inobe & Matouschek, 2008). Once refolded, native conformers are released owing to their low affinity for chaperones. Damaged proteins that are difficult to refold may remain associated with a chaperone for a longer duration, which might stimulate their degradation by a physically associated protease subunit. Thus the kinetics of interaction of a protein with chaperones or chaperone components of proteases determines its fate (Murata et al., 2001; Sherman & Goldberg, 2001; Wickner et al., 1999).
Figure 1: The protein “tria
gre” model for quality control. Chaperones or the ATPase components of proteases bind to exposed hydrophobic patches on denatured proteins. Repeated cycles of ATP dependent binding and release allow unfolding intermediates to fold into their native conformations. If not, they remain in the pool of non-native proteins and may rebind another chaperone or a protease. Protease binding followed by ATP-dependent unfolding and subsequent degradation eliminates the protein. In eukaryotes, ubiquitination marks substrates for recognition by the proteasome. If non-native proteins escape quality control mediated by chaperones or proteases, they eventually aggregate. Proteins in aggregates are refractory towards proteolysis. Some chaperones aid in proteolysis by simply preventing aggregation. Some of them are also able to dissolve aggregates (Adapted from Wickner et al., 1999).

1.4 Role of chaperones in protein degradation

In many cases, molecular chaperones are also necessary for elimination of damaged proteins by proteases. In *E. coli*, the rapid degradation of certain abnormal proteins requires the presence of Hsp70(DnaK)/Hsp40(DnaJ) and degradation of others requires the Hsp60(GroEL)/Hsp10(GroES) chaperone systems (Huang *et al.*, 2001; Kandror *et al.*, 1994). The yeast mitochondrial Hsp70 and Mdj1(DnaJ) are required for degradation of certain
abnormal proteins by the Lon protease homologue, Pim1 which would otherwise form large aggregates (Leidhold & Voos, 2007; Wagner et al., 1994). There are several different ways in which molecular chaperones can facilitate degradation of proteins. In some cases, molecular chaperones simply avert the massive aggregation of proteins thus increasing their chance of being degraded. In other cases, chaperone activities of regulatory subunits that are physically associated with proteases help in modulating the structure of the substrate, maintaining it in an unfolded form thus enabling its entry through the narrow channel leading to the proteolytic chamber, which cannot accommodate folded conformers (Bukau et al., 2006). The hexameric ring of AAA+ ATPases at the base of the 19S regulatory lid of the proteasome binds and unfolds proteins and translocates them into the 20S protease chamber (Lee et al., 2001). Similarly, homomeric rings of the bacterial ClpA or ClpX unfold and translocate substrates into the associated ClpP protease which cannot function alone as a protease without the regulatory ATPase components (Hoskins et al., 2000; Kim et al., 2000; Leidhold & Voos, 2007; Thompson & Maurizi, 1994). These regulatory ATPase components may also provide substrate specificity to the protease (Thompson & Maurizi, 1994; Wawrzynow et al., 1995).

Chaperone involvement in the promotion of ubiquitination of substrates for proteasome degradation has been documented. In yeast, the DnaJ homologue, Ydj1 facilitates ubiquitination whereas another DnaJ homologue, Sis promotes degradation of ubiquitinated proteins by the proteasome (Lee, D.H., M.Y. Sherman, and A.L. Goldberg. 1995. Cold Spring Harbor Symp. Quant. Biol. VX:111a.). Hsp70 and Hsp90 chaperones have been implicated in promoting substrate recognition by the E3 ligase, CHIP (Murata et al., 2001).

1.5 Protein folding catalysts

In addition to ATP-dependent chaperones mentioned in the earlier sections, another set of enzymes, the peptidyl prolyl cis-trans isomerases (PPIases) and protein disulphide isomerases (PDI) accelerate the proper folding of proteins to their native conformations.
These catalysts perform steps that involve covalent changes in the folding process. PDIs present in the periplasm of bacteria and the endoplasmic reticulum of eukaryotes help proteins attain correct disulphide linkages between cysteine residues that are required to attain the final folded state (Wilkinson & Gilbert, 2004). PPIases catalyse the rate-limiting cis-trans isomerization in a peptidyl-prolyl bond (Lu et al., 2007). One example of a PPIase is the prokaryotic trigger factor, which acts in a redundant manner with Hsp70/DnaK in binding and aiding the folding of newly synthesized polypeptides (Deuerling et al., 1999; Teter et al., 1999). This is especially important in the case of larger polypeptides and secretory proteins in which folding is delayed or when assembly into larger complexes is required (Wickner et al., 1999). PPIases fall into three categories: the cyclophilins, FK506 binding proteins (FKBPs) and parvullins. Role of the PPIase activity ranges from assisting in global protein folding, like the trigger factor to acting as a molecular switch in signal transduction pathways. Some of the PPIases like the budding yeast Cpr6, Cpr7 and the bacterial periplasmic SurA have general chaperone activity in addition to the PPIase activity (Behrens et al., 2001; Buchner et al., 1998; Mayr et al., 2000).

1.6 Autophagy

In addition to the quality control mechanisms described so far, another pathway exists in eukaryotes for the clearance of macromolecular aggregates or damaged intracellular organelles. This involves the recognition and engulfment of targets into membrane bound cytoplasmic structures called autophagosomes, which eventually fuse with lysosomes, or vacuoles that are enriched in digestive enzymes that cleave the substrates and generate building blocks for de novo synthesis. It is a tightly regulated process important for cellular homeostasis, growth and development. More than about 20 Atg proteins mediate this process. It is now increasingly becoming evident that autophagy is the major pathway for elimination
of ubiquitin tagged protein aggregates, especially in neurodegenerative diseases (Komatsu et al., 2007).

1.7 Quality control of secretory proteins

Proteins targeted to the outer membrane or periplasm of bacteria have an N-terminal cleavable signal sequence that directs them to the secretory pathway for translocation through the inner membrane via the Sec machinery (Dalbey & Chen, 2004). The periplasm of Gram-negative bacteria such as *E. coli* is in direct communication with the environment by the constant exchange of small molecules through the pores in the outer membrane. Therefore this compartment is easily affected by environmental fluctuations in temperature or pH that lead to protein unfolding and aggregation. Quality control factors are therefore copiously represented in the bacterial periplasm. The major difference between the periplasmic chaperones and the classic cytoplasmic chaperones described in the previous sections is that they must act in an ATP independent manner unlike the latter due to the absence of ATP in the periplasm. This makes them mechanistically distinct from the classic chaperones described so far.

Several chaperones like Skp, DegP and SurA which have general chaperone activity and folding catalysts like PPIases and PDIs have been identified in the periplasm (Duguay & Silhavy, 2004). The chaperones responsible for the folding of soluble periplasmic proteins are not well understood unlike those that aid folding of envelope proteins, which have to first traverse the periplasmic space before reaching their final destination. The integral β-barrel proteins are a major class of outer membrane proteins (OMPs) in Gram-negative bacteria such as *E. coli*. These proteins first pass through the periplasm to reach their assembly site on the outer membrane. Under normal growth conditions SurA functions as the major periplasmic chaperone responsible for the maturation of OMPs. The function of the chaperones DegP and Skp, under normal conditions is to handle only those proteins that have escaped SurA
mediated folding (Sklar et al., 2007). DegP is a member of the widely conserved HtrA family of proteins. It is a unique ATP-independent chaperone-protease, which undergoes a temperature dependent switch from a chaperone to a protease (Spiess et al., 1999). The function of DegP and Skp becomes prominent under stressful conditions when the load of misfolded proteins in the periplasm increases or in the absence of SurA (Sklar et al., 2007).

In eukaryotes, the endoplasmic reticulum (ER) is responsible for the structural maturation of secretory proteins. The ER is enriched in general chaperones Hsp70 and Hsp90 and foldases, PDIs and PPIases that aid in the rapid folding of secretory proteins. The ER also has its own surveillance mechanisms. The lectins, calnexin and calreticulin recognize specific N-linked oligosaccharides on misfolded proteins. The ER employs two distinct quality control mechanisms- the unfolded protein response (UPR) and ER- associated degradation (ERAD) during stress (Ellgaard & Helenius, 2003). The UPR leads to an increase in the transcription of ER chaperones, which then aid in relieving the unfolding stress. Terminally unfolded proteins are transported back into the cytosol by the ERAD pathway for degradation by the 26S proteasome (Bukau et al., 2006).

1.8 Failure of protein quality control: implications for the development of neurodegenerative diseases

When the cell’s quality control system fails to tackle the buildup of aberrant proteins due to prolonged stress or pathogen attack, the default pathway of aggregation is followed which may be a dead end for proteins. This can lead to growth arrest manifested in unicellular organisms due to loss of a specific function or due to the general accumulation of aggregates. In higher animals, the failure of protein quality control can have dire consequences manifested as progressive neurodegenerative diseases because neurons are most affected since they are post-mitotic cells, which have lost the ability to dilute the concentration of aberrant
proteins via multiplication (Winklhofer et al., 2008). Accumulation of unfolded proteins could also trigger an apoptotic pathway in higher animals.

The cells capacity to handle aberrant proteins also diminishes with age. Various observations indicate that aged organisms or senescent cultures are not able to efficiently induce Hsps (Heydari et al., 1994; Rattan & Derventzi, 1991). Aged cells may also have a reduced degradative capacity (Ciechanover, 2006; Shang et al., 1997). This could explain the late onset of neurodegenerative diseases like Parkinson’s (PD) or Alzheimer’s (AD) where failure of protein quality control resulting in the formation of oligomers or amyloid inclusions of the associated proteins, α-synuclein in PD or Aβ and tau in AD, are the key pathogenic features. Proteins such as tau and α-synuclein are natively unfolded. Under stressful conditions or due to certain toxic gain-of-function mutations, they tend to form misfolded structures rich in β-sheets. These misfolded proteins escape the quality control system either because they have surface structures that are not recognized by chaperones or proteases or because the speed at which aggregates form due to initial seeding events overwhelms the capacity of the cell’s quality control system to eliminate them. Aggregates have exposed hydrophobic surfaces that can interact with other misfolded proteins and membranes. The toxic oligomers can cause membrane damage and result in ion leakage (Lashuel et al., 2002).

Another example of an amyloid disease is Huntington’s caused by the expansion of polyglutamine tracts beyond a repeat number of 40. In all these diseases, soluble early intermediates and not the insoluble aggregates have been identified as the toxic species. However, the aggregates may act as reservoirs for release of soluble oligomers. Aggregates contain ubiquitinated proteins implying that the misfolded species have been recognized by the ubiquitin conjugating system (Bence et al., 2001; Bennett et al., 2005). But these inclusions result in inhibition of the proteasome probably due to “choking” of the proteasome chamber (Venkatraman et al., 2004). Evidence is now accumulating that ubiquitin
conjugation of aggregates may target them for elimination by the autophagic pathway (Iwata et al., 2005; Ravikumar et al., 2004).

Failure of mitochondrial quality control is also a major reason for the development of neurodegenerative diseases such as PD. Mitochondria are susceptible to various extra- and intracellular stresses and form a major interface in determining life or death as they play a central role in many apoptotic pathways. Deficiency of mitochondrial Complex I (Sherer et al., 2003), located in the inner mitochondrial membrane that catalyzes the transfer of electrons from NADH to coenzyme Q, and oxidative stress can trigger Lewy body formation (Ischiropoulos & Beckman, 2003). Dysfunction of mitochondrial energy metabolism can lead to elevation in the production of reactive oxygen species (ROS), decreased ATP production and impaired calcium buffering. All these factors can either lead to necrotic or apoptotic cell death. Elevated ROS levels lead to membrane lipid peroxidation and proteolytic stress and can in turn lead to mutations in mtDNA. Again neurons in the brain are most affected because of their high oxygen consumption rate and the presence of high content of peroxidizable lipids. Mutations in several genes that affect mitochondrial homeostasis, namely those encoding Parkin, PINK1, DJ1 and HtrA2/Omi have been associated with PD. Parkin encodes an E3 ubiquitin ligase. Deletion of exon 3 of Parkin in mice results in reduced expression of several proteins involved in mitochondrial function and oxidative stress. Parkin may also be involved in the elimination of oxidatively damaged proteins. Mutations in DJ1 are extremely rare. It has been proposed to act as a redox sensor or an antioxidant protein. It has been detected in the mitochondria in addition to its cytoplasmic localization (Abou-Sleiman et al., 2006).

There is increasing interest in the function of HtrA2/Omi, a close homologue of the bacterial periplasmic HtrA/DegP (introduced in section 1.7), which is a well-characterized chaperone-protease. HtrA2/Omi is a resident of the mitochondrial inter-membrane space, analogous to the localization of bacterial HtrA/DegP in the periplasmic space. Considering
that mitochondria are evolutionary derivatives of ancestral $\alpha$-proteobacteria, it seems possible that HtrA2/Omi could also function as a quality control factor in the mitochondrial intermembrane space.

1.9 The HtrA family of serine proteases

The HtrA (high temperature requirement) family constitutes of proteins that have a characteristic combination of a protease domain with at least one PDZ domain (Pallen & Wren, 1997). HtrA proteins are widely conserved, present in most prokaryotic and eukaryotic species. Several prokaryotic HtrAs have been attributed in stress tolerance and pathogenicity (Ponting, 1997). The genome of the worm Caenorhabditis elegans lacks any HtrA homologue in contrast to Drosophila melanogaster (Challa et al., 2007). Vertebrates possess three secretory paralogs in addition to mitochondrial HtrA2/Omi (see phylogram in Figure 2). Some archea also appear to encode a HtrA-like protein (http://supfam.mrc-lmb.cam.ac.uk/).

1.9.1 Bacterial HtrAs

The E. coli HtrA/DegP was initially identified by two phenotypes of $htrA$ null mutants and named accordingly. Null mutants were unable to grow at elevated temperatures (Lipinska et al., 1989) or failed to digest misfolded proteins in the periplasm (DegP) (Strauch et al., 1989). The genome of E. coli encodes three paralogues of HtrA: HtrA/DegP, DegQ and DegS. HtrA/DegP is an ATP-independent dual chaperone-protease. As discussed earlier (section 1.7), it is unique in that it acts as a chaperone at ambient temperatures but undergoes a temperature dependent switch to a protease at elevated temperatures cleaving irreversibly unfolded substrates in the periplasm. HtrA/DegP is therefore indispensable for survival under heat stress (Spiess et al., 1999). HtrA/DegP forms a self-compartmentalized structure similar to other energy dependent proteases. Because the periplasmic space lacks ATP, DegP does not rely on energy dependent unfoldases and rather cleaves heat-denatured proteins (Kim et al., 1999). In contrast, the archeal HtrA owing to the lack of signal sequences may form a
self-compartmentalized structure in the cytosol depending on association with AAA+ type proteins for hydrolysis of proteins (Maupin-Furlow et al., 2000).

Figure 2: Phylogram showing selected HtrA members. The HtrA family members are widely conserved among both prokaryotes and eukaryotes. *E. coli* has three paralogues namely, HtrA/DegP, DegQ and DegS, humans have four: HtrA1-4 and the budding yeast *Saccharomyces cerevisiae* has one orthologue called Ynm3. The phylogram was produced by Clustal W (Adapted from Clausen et al., 2002).

The bacterial DegS is a stress sensor. When misfolded outer membrane proteins bind to its C-terminal PDZ domain, it cleaves its only substrate, the anti-σ factor RseA that releases the alternative sigma factor σE, which then induces the expression of several stress responsive proteins in the periplasm including DegP (Walsh et al., 2003). The function of DegQ is less understood. It is believed to have roles redundant with DegP and DegS. Overexpression of DegQ can substitute for the lack of DegP in *E. coli* (Waller & Sauer, 1996).

1.9.2 Mammalian HtrA2/Omi

The mammalian HtrA member, HtrA2/Omi, resides in the mitochondrial intermembrane space which corresponds to the periplasmic localization of HtrA/DegP in bacteria (Verhagen et al., 2002). Cell culture studies suggest a proapoptotic role for this protein. A mature form of HtrA2/Omi is generated in the mitochondrial intermembrane space revealing an amino
terminal motif similar to the *Drosophila* death promoting proteins Reaper, Grim and Hid. During apoptosis, mature HtrA2/Omi is released into the cytosol where it binds inhibitor of apoptosis proteins (IAPs) via its N-terminal IAP binding domain (Hegde *et al.*, 2002; Suzuki *et al.*, 2001; Verhagen *et al.*, 2002). IAPs are proteins that inhibit caspases. Binding of HtrA2/Omi to IAPs presumably triggers its serine protease activity leading to their cleavage thereby promoting apoptosis (Hegde *et al.*, 2002; Martins *et al.*, 2002; Verhagen *et al.*, 2000). This view of the function of HtrA2/Omi, which arose from cell culture studies, has been contradicted by the phenotype displayed by mice lacking HtrA2/Omi or its protease activity. The *mnd2* (*motor neuron degeneration 2*) mutant mice, in which the corresponding gene encodes an intact IAP binding domain but carries a protease inactivating point mutation (S276C), suffer neurodegeneration leading to juvenile death (Jones *et al.*, 2003). Interestingly, the HtrA2 knockout mice also show a similar phenotype (Martins *et al.*, 2004). Both these mice exhibit characteristic Parkinsonian symptoms like tremor, decreased mobility and lack of coordination. Cells from these mice are more susceptible to apoptotic stimuli. A certain percentage of cells from the HtrA2/Omi knockout mice exhibit abnormal mitochondrial morphology combined with a decreased mitochondrial density (Martins *et al.*, 2004). This suggests a more protective than a proapoptotic role for mammalian HtrA2/Omi under physiological conditions, which is more reminiscent of its bacterial homologues.

Mutations in the gene encoding HtrA2/Omi have been identified in patients suffering from PD (Strauss *et al.*, 2005). This gene has been allocated to the locus PARK13. A study showed that PINK1 dependent phophorylation of HtrA2/Omi at a residue adjacent to one of these mutations might modulate its proteolytic activity (Plun-Favreau *et al.*, 2007). Indeed, lower HtrA2/Omi phosphorylation was detected in PD patients having mutations in PINK1 (Plun-Favreau *et al.*, 2007). Thus, mutations in the gene encoding either PINK1 or HtrA2/Omi that affect this phosphorylation event could abolish HtrA2/Omi induction. This could possibly lead to mitochondrial stress and neurodegeneration (Vande Walle *et al.*, 2008).
Recent studies report that HtrA2/Omi is activated following cytosolic proteasome inhibition and may act as a second checkpoint to counter the accumulation of its substrates in the mitochondria (Radke et al., 2008).

### 1.9.3 The *Saccharomyces cerevisiae* HtrA orthologue Ynm3

The eukaryotic model organism, *Saccharomyces cerevisiae* encodes an HtrA-like protein called Ynm3 or Nma111. It has an HtrA-like serine protease domain followed by two PDZ domains (Apweiler et al., 2000), one present immediately proximal to the protease domain and the other at the C-terminal end of the protein. The role of this HtrA-like protein in yeast is still obscure, because seemingly contradictory functions have been ascribed to it in earlier reports. It was originally proposed to be a pro-apoptotic serine protease and hence called Nma111, which stands for nuclear mediator of apoptosis 111 kDa protein. It was reported that the absence of the corresponding gene *NMA111* rendered yeast resistant to apoptosis induced by H$_2$O$_2$. Its overexpression was shown to induce apoptosis (Fahrenkrog et al., 2004). Moreover Bir1, a protein homologous to inhibitor of apoptosis proteins (IAPs) was identified as a substrate of Nma111, *in vitro*. It was shown that overexpression of Nma111 counters the anti-apoptotic effect which results from the overexpression of Bir1 (Walter et al., 2006). The same protein, termed Ynm3 was described as a modulator of fatty acid metabolism. Furthermore, deletion of the *YNM3* locus in the yeast strain YB322 was shown to result in the inability to use non-fermentable carbon sources pointing to a possible mitochondrial role for Ynm3 (Tong et al., 2006).
1.10 Aims of this study

Failure of mitochondrial quality control has been found to be one of the primary reasons for the development of neurodegenerative diseases. Among the various genes implicated in PD is the mitochondrial HtrA2/Omi whose bacterial counterparts are well-studied quality control factors. The bacterial HtrA/DegP aids cell survival under heat stress by irreversibly cleaving misfolded proteins in the periplasm and acts as a chaperone at ambient growth temperatures (Spiess et al., 1999). The function of the mammalian HtrA2/Omi is controversial. The proposed pro-apoptotic function in vitro (Verhagen et al., 2002) has been contradicted by studies on mice lacking HtrA2/Omi (Martins et al., 2004) or its serine protease activity (Jones et al., 2003), which exhibit neurodegeneration due to progressive mitochondrial impairment leading to a Parkinsonian phenotype. Recent reports indicate that Htra2/Omi may be important for protein quality control in the mitochondria following cytosolic proteasome inhibition (Plun-Favreau et al., 2007; Radke et al., 2008). The prospect that the mammalian HtrA2/Omi might act as a quality control chaperone-protease like the bacterial HtrA/DegP is immensely attractive though as yet untested. It is noteworthy that such a role is not explicit for any eukaryotic HtrA member.

The budding yeast, Saccharomyces cerevisiae is a useful model to study various aspects of neurodegenerative diseases like protein aggregation, protein misfolding, oxidative stress and apoptosis. Features of diseases like Parkinson’s and Huntington’s have been successfully modeled in the budding yeast. The basic cellular machinery for neuronal function is conserved in yeast making it an attractive unicellular, genetically and biochemically tractable model organism to study pathways underlying neurodegeneration.

In this study, I address the question whether a eukaryotic homologue of the HtrA family, the budding yeast Ynm3, has retained the characteristic chaperone-protease function of its prokaryotic counterparts. This possibility is dealt with by both genetic as well as biochemical methods. The role of Ynm3’s serine protease activity as well as its chaperone
activity is assessed. From the results, the mutual importance of both chaperone and protease activities in maintaining quality control are analyzed.

Both structural and functional studies have revealed that the PDZ domains of the bacterial and mammalian homologues have substrate binding as well as regulatory functions (Clausen et al., 2002; Krojer et al., 2002; Li et al., 2002). To understand the importance of the duplicated PDZ domains of Ynm3, genetic experiments are carried out with variants lacking either of them, which could provide important hints that would become the basis for future structural studies.

Attempts are made to find *in vivo* substrates as well as physical and genetic interactors of Ynm3. Any potential partner of Ynm3 is assayed in genetic as well as a biochemical experiments. I also assess whether the interaction of Ynm3 with a potential interactor is evolutionarily conserved. Finally, on the basis of my findings, a cogent working hypothesis for the function of Ynm3 and its potential interactor(s) is presented.
2. Materials and Methods

2.1 Materials

2.1.1 Yeast strains

All yeast strains used in this study are listed in Table 1. All yeast strains are congenic to the wild type YB322 strain (Johnson et al., 1994) unless otherwise stated. The yeast strain RH3340 was obtained by replacing the \textit{YNM3} genomic locus with a module conferring Kanamycin resistance. The \textit{YNM3} coding sequence was deleted by homologous recombination using the kanamycin resistance cassette, amplified by PCR, containing sequences homologous to the 5’ upstream and 3’ downstream regions of the gene. Chromosomal DNA was isolated from the yeast colonies that grew on YPD+200 \(\mu\)g/ml Geneticin G418 sulphate (Gibco, UK). Successful disruption of \textit{YNM3} by the cassette was confirmed by PCR using two sets of primers: one set homologous to a sequence in the 5’ upstream region of \textit{YNM3} and the 5’ end of the drug resistance cassette and the other set homologous to a sequence in the 3’ downstream region of \textit{YNM3} and the 3’ end of the drug resistance cassette.

The yeast strain RH3343 was obtained by PCR based tagging of the \textit{YNM3} genomic locus at its 5’ region with the \textit{natNT2-GAL1-yeGFP} module which was PCR amplified from the plasmid, pymN25 as described (Janke et al., 2004). The module was amplified using a pair of primers annealing to 45 – 55 bases upstream of the ATG (including ATG start codon) of \textit{YNM3}, followed by 5’-CGTACGCTGCAGGTCGAC-3’ and the reverse complement of 45 – 55 bases downstream of the ATG (start-codon) of \textit{YNM3} (excluding ATG), followed by 5’-CATCGATGAATTCTCTGTCG-3’ (Table 4). The resulting PCR product was transformed into the wild type YB322 strain. Genomic DNA was isolated from colonies that grew on YPD+100 \(\mu\)g/ml Nourseothricin (ClonNAT, Werner BioAgents, Jena-Cospeda, Germany).
Materials and Methods

Verification by PCR was done using a pair of primers annealing to a upstream region of \textit{YNM3} and the 5’ end of the module as before since all these drug resistance cassettes have exactly the same promoter and terminator sequences.

The yeast strains RH3344 and RH3345 were obtained by replacing the genomic \textit{FPR3} locus in the wild type YB322 or in RH3340 respectively with the nourseothricin-resistance cassette amplified from the plasmid \textit{pFA6a-natNT2} (Janke et al., 2004) using primers containing sequences homologous to the upstream and downstream region of \textit{FPR3} (Table 4). The strains transformed with the PCR product containing the disruption cassette were plated on YPD+100 µg/ml Nourseothricin. Genomic DNA was isolated from colonies that grew on YPD+100 µg/ml Nourseothricin. Verification by PCR was done using a pair of primers annealing to an upstream region of \textit{YNM3} and the 5’ end of the cassette (Table 4). \textit{YCA1} was also disrupted in the strain YB322 in a similar manner (For primers see Table 4). The deletion strains of the BY4741 background were obtained from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html).

Table 1. List of yeast strains used in this study

<table>
<thead>
<tr>
<th>Yeast Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH3339</td>
<td>YB322 wild type, \textit{Mat a,ura3,his3,ade2,lys2,leu2, trp1}</td>
<td>(Johnson et al., 1994)</td>
</tr>
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<td>RH3340</td>
<td>YB322, \textit{Mat a,ura3,his3,ade2,lys2,leu2, trp1, ynm3\Delta:: kanMX4}</td>
<td>This study</td>
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<tr>
<td>RH3342</td>
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<tr>
<td>RH3343</td>
<td>YB322, \textit{Mat a,ura3,his3,ade2,lys2,leu2, trp1, natNT2-GAL1\textit{UaseGFP::YNM3}}</td>
<td>This study</td>
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</table>
2.1.2 Growth conditions

The yeast strains were grown in standard yeast extract-peptone-dextrose (YPD: 1% yeast extract, 2% peptone, 2% dextrose) or yeast extract-peptone-galactose (YPGal: 1% yeast extract, 2% peptone, 2% Galactose) supplemented with adenine or in synthetic complete (SC) media (YNB: 1.5 g/l yeast nitrogen base lacking amino acids, 5 g/l ammonium sulfate, 2% glucose or galactose and supplemented with amino acids).

2.1.3 Plasmids

All plasmids used in this study and a concise description about their construction are listed in Table 2. The plasmids obtained from other sources are listed in Table 3.

2.1.4 Oligonucleotides

The oligonucleotides used in this study are listed in Table 4.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Insert(s)</th>
<th>Background Vector</th>
<th>Preparation of insert</th>
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<tbody>
<tr>
<td>pME3325</td>
<td>YNM3</td>
<td>pRS416MET25</td>
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<td>pRS426GAL1</td>
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<td>GFP</td>
<td>pRS416MET25</td>
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<tr>
<td>pME3449</td>
<td>YNM3GFP</td>
<td>pRS416MET25</td>
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<td>pME3360</td>
<td>YNM3 (1-1946bp)</td>
<td>pUC19</td>
<td>YNM3 (1-1946) insert cloned into BamH1/Hind11 site of pUC19</td>
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<td>pME3361</td>
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<tr>
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<td>pRS416MET25</td>
<td>Replacement of YNM3 sequences (1-1946bp) of pME3325 with YNM3S235A(1-1946bp) obtained by BamH1/Hind111</td>
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<tr>
<td>Constructs</td>
<td>Description</td>
<td>Source or Method</td>
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<tr>
<td>pME3329</td>
<td>YNM3ΔPDZ2</td>
<td>pRS416MET25 BamH1/Xho1 restricted YNM3ΔPDZ2 PCR product obtained by overlap PCR as described.</td>
<td></td>
</tr>
<tr>
<td>pME3455</td>
<td>YNM3ΔPDZ1-GFP</td>
<td>pRS416MET25 pME3459 containing Spe1/Sma1 restricted YNM3ΔPDZ1 PCR product amplified from pME3328 as template using NP26/NP25 primers.</td>
<td></td>
</tr>
<tr>
<td>pME3457</td>
<td>YNM3ΔPDZ2-GFP</td>
<td>pRS416MET25 pME3459 containing Spe1/Sma1 restricted YNM3ΔPDZ2 PCR product amplified from pME3329 as template using NP26/NP25 primers.</td>
<td></td>
</tr>
<tr>
<td>pME3481</td>
<td>YNM3S236C-GFP</td>
<td>pRS426MET25 pME2564 containing Spe1/Sma1 restricted YNM3S236C PCR product amplified using NP26/NP25 primers.</td>
<td></td>
</tr>
<tr>
<td>pME3364</td>
<td>YNM3ΔN100aa-GFP</td>
<td>pRS426MET25 pME2564 containing Spe1/Sma1 restricted YNM3ΔN100aa PCR product amplified from pME3325 as template using NP25/NP64 primers.</td>
<td></td>
</tr>
<tr>
<td>pME3566</td>
<td>YNM3ΔN100aa-GFP</td>
<td>pRS416MET25 pME3459 containing Spe1/Sma1 restricted YNM3ΔN100aa PCR product amplified from pME3325 as template using NP25/NP64 primers.</td>
<td></td>
</tr>
<tr>
<td>pME3341</td>
<td>YNM3</td>
<td>pGDBU-C1 BamH1/Xho1 restricted YNM3 inserted</td>
<td></td>
</tr>
<tr>
<td>Plasmid</td>
<td>Insert</td>
<td>Vector</td>
<td>Restriction Sites</td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
<td>--------</td>
<td>------------------</td>
</tr>
<tr>
<td>pME3342</td>
<td>YNM3S236C</td>
<td>pGDBU-C1</td>
<td>BamH1/Xho1</td>
</tr>
<tr>
<td>pME3567</td>
<td>YNM3(1-100bp)</td>
<td>pRS426MET25</td>
<td>BamH1/Hind111</td>
</tr>
<tr>
<td>pME3345</td>
<td>htrA/degP</td>
<td>pRS426GAL1</td>
<td>BamH1</td>
</tr>
<tr>
<td>pME3348</td>
<td>HTRA2/OMI</td>
<td>pRS426GAL1</td>
<td>XbaI/SalI</td>
</tr>
<tr>
<td>pME3363</td>
<td>FPR3</td>
<td>pRS416MET25</td>
<td>BamH1/Xho1</td>
</tr>
<tr>
<td>pME3568</td>
<td>RPL6A</td>
<td>pRS416MET25</td>
<td>BamH1/Xho1</td>
</tr>
<tr>
<td>pME3460</td>
<td>YNM3</td>
<td>pGEX-6P1</td>
<td>BamH1/Xho1</td>
</tr>
<tr>
<td>pME3461</td>
<td>YNM3S236C</td>
<td>pGEX-6P1</td>
<td>BamH1/Xho1</td>
</tr>
<tr>
<td>pME3462</td>
<td>YNM3S236A</td>
<td>pGEX-6P1</td>
<td>BamH1/Xho1</td>
</tr>
<tr>
<td>pME3463</td>
<td>FPR3</td>
<td>pGEX-6P1</td>
<td>BamH1/Xho1</td>
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Table 3: Plasmids from other sources used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>pRS416</td>
<td>*URA3, CEN, Amp&lt;sup&gt;R&lt;/sup&gt; (bla), ori</td>
<td>(Sikorski &amp; Hieter, 1989)</td>
</tr>
<tr>
<td>pRS426</td>
<td>*URA3, 2µ, Amp&lt;sup&gt;R&lt;/sup&gt; (bla), ori</td>
<td>(Sikorski &amp; Hieter, 1989)</td>
</tr>
<tr>
<td>pFA6a-natNT2</td>
<td>Contains nourseothricin resistance cassette.</td>
<td>(Janke et al., 2004)</td>
</tr>
<tr>
<td>pYM-N25</td>
<td>Contains GAL1-yeGFP-natNT2 promoter module.</td>
<td>(Janke et al., 2004)</td>
</tr>
<tr>
<td>pRS416MET25</td>
<td>pRS416 containing MET25 promoter and CYC1 terminator.</td>
<td>(Mumberg et al., 1994)</td>
</tr>
<tr>
<td>pRS426MET25</td>
<td>pRS426 containing MET25 promoter and CYC1 terminator.</td>
<td>(Mumberg et al., 1994)</td>
</tr>
<tr>
<td>pRS426GAL1</td>
<td>pRS426 containing GAL1 promoter and CYC1 terminator.</td>
<td>(Mumberg et al., 1994)</td>
</tr>
<tr>
<td>pME2564</td>
<td>pRS426MET25 containing GFP encoding sequence in its Sma1/Cla1 site.</td>
<td>(Bomeke et al., 2006)</td>
</tr>
<tr>
<td>pME3365</td>
<td>(pJS13) pT7-5 derivatives; Amp resistance; htrA/degP insert cloned into BamH1/Hind111 sites.</td>
<td>Kindly provided by Dr. Joana Skorko-Glonek, University of Gdansk, Poland.</td>
</tr>
<tr>
<td>pME3366</td>
<td>Plasmid encoding mitochondria targetted BFP</td>
<td>Kindly provided by Dr. Stefan Jakobs, MPI (Biophysical Chemistry), Göttingen</td>
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### Table 4: List of oligonucleotides

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence (5’-3’)</th>
<th>Use</th>
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<tr>
<td>NP01 27mer</td>
<td>GCTGAGTCGACCTCCAACACTGTTC</td>
<td>Check disruption of ( YNM3 ) locus with ( Kan^R ) cassette in the 5’ region.</td>
</tr>
<tr>
<td>NP08 18mer</td>
<td>GCCACAGTCACATCATGC</td>
<td>same as above</td>
</tr>
<tr>
<td>NP02 28mer</td>
<td>GTTCACCTAGGGAAGCTCAACGGCTTC</td>
<td>Check disruption of ( YNM3 ) locus with ( Kan^R ) cassette in the 3’ region.</td>
</tr>
<tr>
<td>NP09 17mer</td>
<td>CCTCGACATCATCTGCC</td>
<td>same as above</td>
</tr>
<tr>
<td>NP14 30mer</td>
<td>GCTAAGTCGACGCCAAGCTCAACCGCCA</td>
<td>Amplification of ( Kan^R ) disrupted ( YNM3 ) locus from the genomic DNA of ( BY4741 \Delta ynm3 ).</td>
</tr>
<tr>
<td>NP15 31mer</td>
<td>GTTCAGGATCCCGCACGGTTAACC</td>
<td>same as above</td>
</tr>
<tr>
<td>NP16 69mer</td>
<td>ACTCTGAATACACGCTAGGATTACAGTCGAC</td>
<td>Amplification of the ( natNT2-GAL1_{UAS-EGFP} ) module.</td>
</tr>
<tr>
<td>NP17 69mer</td>
<td>CGGAATTTTAGATTGCTCCTTTTCTTTATATTGCTAAGATATGTCATCGATGAAATTCTCTGTCG</td>
<td>same as above</td>
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<tr>
<td>NP22 21mer</td>
<td>GATTAAGTGGGTAACGCCCAG</td>
<td>Sequencing primer for pRS202 library</td>
</tr>
<tr>
<td>NP23 21mer</td>
<td>GTGTGGAAATTGTGAGCGGATA</td>
<td>same as above</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NP39 71mer</td>
<td>TCGAAGTTTATCTAAACTACCACCAAAGAAGACCGACTAGATTACAAATCATGCGTACGCTGCAGGTCGAC</td>
<td>Amplification of NatR cassette for disruption of the YCA1 locus.</td>
</tr>
<tr>
<td>NP40 73mer</td>
<td>ACATACTACACCAGTCTGAATACATCTACCAACGTACACATCCATATTTCTAATCGATGAATTGAGGCTCG</td>
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<tr>
<td>NP107 71mer</td>
<td>TTGTGTGAAAGTTTATACATAATTGAAAGCAAGCTACCAACCAGCGCCAAATATCGTACGCTGCAGGTCGAC</td>
<td>Amplification of NatR cassette for disruption of the FPR3 locus.</td>
</tr>
<tr>
<td>NP108 76mer</td>
<td>ATATTATGTGAAAAAAGGATATATATATATACATCTATCCGTACGAGCGCGTGTACTAATCGATGAATTGAGGCTCG</td>
<td>same as above</td>
</tr>
<tr>
<td>NP109 26mer</td>
<td>CAATGCTCCAAACATTCTACTATCAC</td>
<td>Check disruption of FPR3 locus with NatR cassette in the 5’ region along with NP08.</td>
</tr>
<tr>
<td>NP20 39mer</td>
<td>TTATATGGATCCATGCATATCGTTGAGCAATATAAAG</td>
<td>Forward primer used to PCR amplify YNM3 or its mutants for cloning. Contains BamH1 site.</td>
</tr>
<tr>
<td>NP21 37mer</td>
<td>ATTATTCTCGAGCTATTTTTCACATTCGTCGTTGCGTGTTGCCG</td>
<td>Reverse primer used to PCR amplify YNM3 or its mutants for cloning. Contains Xho1 site.</td>
</tr>
<tr>
<td>NP102 36mer</td>
<td>GCATCTGCTTCTGCGTGCAAGCTGGTT</td>
<td>Primer for SDM (YNM3S235A)</td>
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### Materials and Methods

<table>
<thead>
<tr>
<th>Primer ID</th>
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<th>Sequence</th>
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<tr>
<td>NP103</td>
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<td>CTCCAGTC</td>
<td>GACTGGAGAACCTTGACCCAGAGCAGATGC</td>
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<td>NP104</td>
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<td>NP61</td>
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<td>CTCCAGTC</td>
<td>GCATCTGCTTCTGGCGGTCATGTGGTTAGCAGATGC</td>
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</tr>
<tr>
<td>NP26</td>
<td>38mer</td>
<td>CAATATAAAG</td>
<td>AGCTTACTAGTATGACCATATCGTTGAG</td>
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<tr>
<td>NP25</td>
<td>33mer</td>
<td>GCCGG</td>
<td>AGCACCCGGGTTTTTCACCTTGCTTGTT</td>
</tr>
<tr>
<td>NP11</td>
<td>25mer</td>
<td>GCCAGTGACTCCACCGCTGTC</td>
<td>GAATTCTAGACTCCACCGGTTCGTT</td>
</tr>
<tr>
<td>NP30</td>
<td>23mer</td>
<td>GACC</td>
<td>GCCAGTGACTCCACCGGTTCGTT</td>
</tr>
<tr>
<td>Seq ID</td>
<td>Description</td>
<td>Primer Sequence</td>
<td>Notes</td>
</tr>
<tr>
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| NP33 54mer | Overlap PCR to delete PDZ1 in YNM3. | GCAGGTGACAGTACATTGACACCGCC CTTTGAACCTTGATCGACCAGGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT GT
| NP34 22mer | same as above | AGGGGCCTTGTGCAGATGTACTG |
| NP57 23mer | Overlap PCR to delete PDZ2 in YNM3. | TTCGGGACACCTCTAATTCTGG |
| NP58 72mer | same as above | TCGGTTCAATTTCGTTCGGAAGCCAG |
| NP63 34mer | Reverse primer used to PCR amplify DNA encoding first 100 amino acids of Ynm3 along with NP26 as forward primer. Contains Sma1 site. | AGCACCCCGGGCAAGCAGAATCGCAATCGAATGG |
| NP64 37mer | Forward primer used to amplify YNM3ΔN100 along with NP25 as the reverse primer. Contains Spe1 site. | AGCTTACTAGTATGGTATCTGAAGCTACCGGATTTGT |
| NP92 36mer | Forward primer used to PCR amplify FPR3 for cloning. Contains BamH1 site. | ACATGGATCCATGTCTGATTGTTACCACTAGCTAC |
| NP91 38mer | Reverse primer used to PCR amplify FPR3 for cloning. Contains Xho1 site. | ACATTCGAGCTAGTTTTTCATAGAAACCAATTAACG |
| NP93 34mer | Forward primer used to PCR amplify RPL6A for cloning. | ACATGGATCCATGAGTGCCCAAAAAGTATGTTCCATGTCTGATTGTTACCACTAGCTAC |
2.2 Methods

2.2.1 DNA sequence analysis

Vectors and other DNA sequences were analysed using the LASERGENE software package from DNASTAR. Budding yeast chromosomal DNA sequences were analysed using the tools available in the *Saccharomyces* genome database (SGD) (www.yeastgenome.org).

2.2.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) (Higuchi *et al.*, 1988) was performed for rapid amplification of DNA for various purposes. For cloning, either KOD HiFi DNA Polymerase (NOVAGEN, Darmstadt, Germany) or Phusion\textsuperscript{R} (NEW ENGLAND BIOLABS, Frankfurt Am Main, Germany) were used. The PCR reaction constitutions and conditions were based on manufacturers’s protocols. For amplification of DNA for site-specific homologous recombination, either KOD HiFi DNA Polymerase or self-made Taq polymerase available in the laboratory was used. For analytical purposes, the self-made Taq polymerase with an initial denaturation at 95°C for 2 minutes followed by 30 cycles of denaturation at 95°C for 1 minute, annealing for 45 seconds and extension at 72°C for 1 minute/kb. This was followed by a final extension at 72°C for 5 minutes. PCRs were performed either in Mastercycler Gradient (EPPENDORF, Hamburg, Germany) or in the PCR cycler (MWG-BIOTECH Primus or Primus 96\textsuperscript{plus}, Ebersberg, Germany). The annealing temperatures were generally between 60-65°C.
2.2.3 Restriction digestion of DNA

For cloning and for analytical confirmation of the resultant clones, DNA was digested using restriction enzymes (FERMENTAS, St.Leon-Rot, Germany). For analytical purposes, generally 200-500 ng of plasmid DNA was digested for 1-2 hours in a total volume of 20 µl. For cloning, about 500 ng of plasmid DNA was digested for 2-3 hours in a total volume of 20 µl.

2.2.4 Preparation of DNA for ligation

For cloning, the restricted vector DNA was first dephosphorylated in order to remove 5’ phosphates to avoid unnecessary re-ligation. This was done by incubation at 37°C for 45 minutes with 1 µl shrimp alkaline phosphatase (SAP) (FERMENTAS, St.Leon-Rot, Germany) which was added directly to 20 µl of the restriction digestion reaction. The restricted and dephosphorylated vector DNA was then purified following 1% agarose gel electrophoresis using the QIAspin Nucleotide Removal Kit (QIAGEN, Hilden, Germany) according to instructions provided in the manual. For preparation of insert, the PCR product was first purified after 1% agarose gel electrophoresis using the QIAspin Nucleotide Removal kit followed by restriction digestion. The restricted insert DNA was once again purified using the QIAspin Nucleotide Removal Kit. For ligation, about 50 ng of restricted vector DNA was incubated with at least 5 times the amount of restricted insert DNA along with 1 µl of T4 DNA ligase (FERMENTAS, St.Leon-Rot, Germany) in 1X T4 DNA ligase buffer in a total volume of 10-20 µl.

2.2.5 Preparation of chemically competent E.coli cells

About 10 colonies of E. coli were inoculated into 250 ml of SOB medium (2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄) in a 1 l flask. The culture was incubated at 20°C for about 20 hours until the OD₆₀₀ reached approximately 0.6. The culture was cooled on ice for 10 minutes and centrifuged at 2,500 g for 10 minutes at
4°C. The pellet was then resuspended in 80 ml TB (10 mM PIPES, 15 mM CaCl₂, 250 mM KCl; the pH was then adjusted to 6.7 with KOH followed by the addition of 55 mM MnCl₂). This cell suspension was incubated on ice for 10 minutes followed by centrifugation at 2,500 g for 10 minutes at 4°C. The pellet obtained was then resuspended in 20 ml TB followed by the slow addition of DMSO with swirling to obtain a final concentration of 7%. This cell suspension was further incubated for 10 minutes on ice and dispensed in 0.5 ml aliquots which were first subjected to shock freezing in liquid N₂ before storage at -80°C.

2.2.6 Transformation of chemically competent E.coli cells

An aliquot of competent cells was first allowed to thaw on ice. About 3-4 µl of the ligation reaction was added to about 200 µl of the competent cells and incubated for 30 minutes on ice. The cells were then subjected to heat shock at 42°C for 45 seconds and then placed on ice for 5 minutes. 800 µl of SOC medium (SOB + 20 mM Glucose) was added to the mixture followed by recovery at 37°C with shaking at 180 rpm for 1 hour. The cells were then centrifuged at 2,500 rpm for 3 minutes on a tabletop centrifuge (Biofuge pico, HERAEUS, Hanau, Germany) and plated on LB agar supplemented with ampicillin to a final concentration of 100 µg/ml.

2.2.7 Plasmid DNA purification

Plasmid DNA was purified using the QIAPrep Spin Miniprep Kit (QIAGEN, Hilden, Germany). A single colony of E. coli was inoculated into 5 ml LB medium supplemented with Ampicillin to a final concentration of 100 µg/ml and incubated for 16 hours at 37°C with shaking (180 rpm). The cells were harvested by centrifugation at 13,000 rpm on a tabletop centrifuge (Biofuge pico, HERAEUS, Hanau, Germany). The remaining steps were exactly based on the instruction manual provided.
2.2.8 DNA sequencing

Crucial cloning steps were validated by sequencing on an ABI Prism 310 capillary sequencer (PE Biosystems). DNA sequences were verified using the 4Peaks software (www.mekentosj.com).

2.2.9 Site directed mutagenesis

Site directed mutagenesis was used to mutate codons encoding Ser235 or Ser236 of Ynm3 to Cysteine or Alanine. The QuikChange II Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used for this purpose. The primer design and reaction conditions were according to the instructions provided in the manual. Two primers containing the desired mutations were designed spanning the target region in opposing directions (Table 4). A region of \textit{YNM3}, 1-1946 base pairs, containing the codon to be altered was cloned into a smaller PUC 19 vector so that the dsDNA template size does not exceed the limit prescribed in the manual. A series of different concentrations of the dsDNA (5, 10, 20 and 50 ng) were used in the reaction to which the various components of the kit were added as prescribed in the manual, including the primers at 125 ng/reaction. Lastly, 1 µl of PfuUltra HF DNA polymerase was added and the reaction mixture was cycled in a PCR thermal cycler (MWG-Biotech). The annealing temperature used was 55°C and the number of cycles used was 16 decided based on the instructions manual. After this, the reaction was cooled below 37°C and 1 µl of Dpn I restriction enzyme was added to each reaction and incubated at 37°C for 1 hour. The Dpn I enzyme is specific to methylated and hemi-methylated DNA and therefore digests the parental DNA template but does not digest the mutant-synthesized DNA. As most \textit{E.coli} strains produce methylated DNA, they are not resistant to Dpn I digestion. Undigested DNA was then used to transform the XL1-Blue supercompetant cells provided with the kit. The cells were aliquoted into Falcon 2059 polypolyene tubes prior to transformation. Transformed cells were incubated in SOC medium for 1 hour in an orbital shaker and then
plated on LB agar containing ampicillin (final conc. of 100 mg/ml).

Plasmids were isolated from the resulting colonies and sequenced to confirm the presence of the desired mutation. The mutated PUC19 insert was then excised and ligated with BamH1/Hind111 digested pME3325 (Table 2) in order to replace the 1-1946 base pairs of wild type YNM3 with the region containing the desired mutations.

2.2.10 Overlap extension PCR

An overlap extension PCR method was used to delete the region encoding either of the two PDZ domains of YNM3. For generation of YNM3ΔPDZ1 or YNM3ΔPDZ2, two individual PCRs were performed on YNM3 template to amplify only those regions 5’ and 3’ of the region encoding the PDZ domain which is to be excluded. The 5’ end of one of the primers spanning a sequence immediately before or after the region encoding the PDZ domain to be deleted contains an added sequence that overlaps with a small part of the other PCR product. These overlapping fragments of YNM3 obtained by individual PCR reactions were then used as templates in a single PCR reaction in which only the 5’ and 3’ end-specific primers of YNM3 (Table 4) were used for amplification, thus generating a product that lacks the region encoding a PDZ domain.

2.2.11 Amplification of DNA libraries following electroporation

DNA libraries were transformed by high-voltage electroporation into electro-competent E. coli cells (ElectroMAX™ Stb14™, INVITROGEN, Karlsruhe, Germany). The cells were thawed on ice and mixed with 1 µl of the library DNA and mixed gently. The mixture was transferred into a gene pulser cuvette (BIORAD, Herts, UK) already placed on ice. The cells in the cuvette were then subjected to a 1.2 KV single pulse lasting for 5 ms in a Gene Pulser (BIORAD, Herts, UK). The cells were incubated for 1.5 hours in 1 ml SOC medium in a 15 ml reaction tube at 37°C on a shaker (180 rpm). The contents were inoculated into 500 ml
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Terrific Broth (12 g Tryptone, 24 g Yeast Extract, 4 ml Glycerol in a total volume of 900 ml H_2O to which 100 ml of sterile 10X TB salt solution containing 2.312 g KH_2PO_4 and 12.514 g K_2HPO_4 was added after autoclaving followed by cooling). The culture was incubated overnight at 37°C on a shaker. The library DNA was isolated using the QIAGEN Maxi Prep Kit according to the instructions provided in the manual.

2.2.12 Transformation of yeast cells

2.2.12.1 Preparation of competent yeast cells

10 ml of YPD was inoculated with the yeast strain to be transformed. The culture was incubated on a shaker at 30°C overnight. The culture was then centrifuged at minimal speed (SIGMA 4K15C) and resuspended in 10 ml 100 mM LiOAc/TE (5 ml 1M Tris-Cl pH: 8.0, 1 ml 0.5 M Na.EDTA pH:8.0, 100 mM LiOAc in a total volume of 50 ml H_2O). The suspension was allowed to shake slowly for 1 hour at 30°C, then centrifuged at minimal speed and resuspended in 1ml 100 mM LiOAc/TE.

2.2.12.2 Transformation of competent yeast cells

To transform linear DNA, the PCR product was precipitated using 1/20th of its volume of 3 M NaOAc and 2.5 times its volume of 100% ethanol for about 1 hour on ice. The DNA was pelleted by centrifugation at 13,000 rpm (Biofuge fresco, HERAEUS, Hanau, Germany) for 10 minutes. The pellet was dried in a vacuum dryer (Speed Vac RC3B plus, SAVANT) and dissolved in 20 μl of TE buffer. About 20 μl of carrier DNA (salmon sperm DNA) was added to it and mixed with 100 μl of competent yeast cells in a microfuge tube. 0.5 ml of 50% PEG 4000 was added and the tubes were inverted several times and incubated at 30°C on a shaker for 1 hour. The cells were then subjected to heat shock at 42°C for 10-15 minutes and centrifuged at minimal speed. The supernatant was discarded, the cells resuspended in 500 μl YPD and incubated at 30°C for 2 hours on a shaker. The cells were then pelleted at minimal speed for 2-5 minutes and resuspended in 200 μl of sterile H_2O and spread on YPD agar.
plates containing the appropriate drug for selection and incubated at 30°C for 4 days. Transformation of plasmids was done in a similar manner except that there is no requirement for DNA precipitation or for the presence of carrier DNA. About 1 µg of each plasmid was used for transformation and the yeast cells plated on selective SC medium lacking the amino acids to select for auxotrophic maker(s) on the plasmid(s).

2.2.13 Isolation of DNA from yeast cells

An overnight culture of the yeast strain in 10 ml YPD was centrifuged and the pellet resuspended in 0.5 ml H₂O. The suspension was again centrifuged for 5 seconds in a tabletop centrifuge (Biofuge pico, HERAEUS, Hanau, Germany). The supernatant was discarded and the pellet was disrupted by vortexing briefly. It was then resuspended in 200 µl breaking buffer (2% V/V TritonX 100, 1% V/V SDS, 100 mM NaCl, 10 mM Tris-Cl, pH8.0, 1 mM EDTA, pH8.0). About 0.3 g (200 µl) of 0.45 mm glass beads were added and the suspension was vortexed at high power for 5 minutes. 200 µl of TE buffer (10 mM Tris-Cl pH7.5/8.0, 1 mM EDTA, pH8.0) was added and the suspension was again vortex briefly. This was centrifuged for 5 minutes at 13,000 rpm at room temperature in a tabletop centrifuge (Biofuge pico, HERAEUS, Hanau, Germany). The aqueous layer was transferred to a clean microfuge tube to which 1 ml 100% ETOH was added and mixed gently by inversion. This was further centrifuged at high speed for 3 minutes at room temperature. The supernatant was removed and the pellet was resuspended in 100 µl TE after drying at room temperature. The DNA thus obtained was used for analytical purposes like PCR. If pure DNA is required, the RNA contaminants have to be removed. For this purpose, the DNA obtained after the previously described ETOH precipitation step was resuspended in 0.4 ml TE to which 30 µl of 1 mg/ml RNase A was added, mixed and incubated for 5 minutes at 37°C. 10 µl of 4M NH₄COOCH₃ and 1 ml 100% ETOH was then added to it and mixed gently by inversion. This was centrifuged at high speed for 3 minutes at room temperature. The supernatant was discarded.
and the pellet was allowed to dry at room temperature or at slow speed in a vacuum dryer (Speed Vac RC3B plus, SAVANT). The pellet was then resuspended in 100 µl of TE buffer. The amount of DNA present in the solution was determined spectrophotometrically (Ultrospec 4000, PHARMACIA BIOTECH, Munich, Germany) at a wavelength of 260 nm. A value of $A_{260} = 10$ corresponds to 50 mg/ml of double stranded DNA.

### 2.2.14 Genetic suppressor screen

For the gain of function screen, the pRS202 genomic DNA library was used (Connelly and Hieter, unpublished, June 1990). Inserts from this library are approximately 6-8 Kbps in length. The Δynm3 strain was transformed with 1 µl of the library, plated on SC-Ura as the plasmids in the library have a URA3 auxotrophic selectable marker and incubated for a week at 38°C. Twenty individual transformations were performed. Colonies that appeared large were picked and growth tests were performed (spot dilution assay) at 38°C to avoid any false positives. Plasmids contained in the colonies that grew better at 38°C were isolated and retransformed into the Δynm3 strain for a further verification of their ability to induce better growth at 38°C. The inserts contained in the plasmids were sequenced using specific primers (Table 4).

### 2.2.15 Growth tests

For spot dilution assays, 10-fold dilutions of overnight cultures of each strain starting with an OD$_{600}$ of 0.5 were made in sterile water. 10 µl of each dilution were spotted on plates containing appropriate selective media (SC-Ura or SC-Met-Ura or SC-Ura+galactose) or rich medium containing either glucose or galactose and incubated at the temperatures indicated for at least 2-3 days or otherwise indicated. For assaying growth in liquid culture, overnight cultures of each strain were diluted in 100 ml of the appropriate selective inducing medium (SC-Met-Ura) in duplicate in unbaffled flasks (300 ml) to obtain a final OD$_{600}$ between 0.1 and 0.2. The flasks were incubated at 39°C with shaking (220 rpm). OD at 600 nm was
measured every two hours for the next 12 hours in duplicate per flask resulting in four readings per strain. A graph was plotted using the OD\textsubscript{600} values.

**2.2.16 Survival after heat shock treatment**

Logarithmic cultures (OD\textsubscript{600} = 0.5) of the wild type BY4741 strain and the congenic Δ\textit{ynm3} strain in liquid YPD were subjected to heat shock at 50°C in a prewarmed water bath for the indicated durations. Serial 10 fold dilutions of each culture were then made starting with an OD\textsubscript{600} of 0.5 and spotted on YPD agar. The plates were incubated at 30°C for 1 day.

**2.2.17 Preparation of yeast cell crude extracts for Western blotting**

Yeast strains were allowed to grow in appropriate selective medium overnight. Cells were pelleted at minimal speed (SIGMA 4K15C) at room temperature and resuspended in 10 ml of the appropriate inducing medium depending on the promoter driving the expression of the protein that is to be detected. The culture was induced for at least 2 hours and the cells were pelleted at 4°C and stored at -80°C until use. For preparation of extracts, the cell pellets were allowed to thaw on ice. The cells were washed with 1 ml ice cold TE (10 mM Tris-Cl pH7.5/8.0, 1 mM EDTA, pH8.0) centrifuged at 13,000 rpm using a tabletop centrifuge (Biofuge pico, HERAEUS, Hanau, Germany) and resuspended in 200 µl of R-buffer (150 µl 1M Tris pH 7.5, 6 µl 0.5 M EDTA, 150 µl 1M DTT, 120 µl PIM complete (ROCHE, Mannheim, Germany). The suspension was transferred to a 2 ml microfuge tube filled with 250 µl of 0.45 mm glass beads and vortexed at maximum power for 5 minutes at 4°C and then centrifuged at 13,000rpm for 10 minutes at 4°C (Biofuge fresco, HERAEUS, Hanau, Germany). The amount of protein in the supernatant was determined using the Bradford method (ROTQUANT, ROTH, Karlsruhe, Germany). 50 µl of the supernatant was transferred to a 1.5 ml microfuge tube containing 10 µl of 6x SDS loading buffer and stored at -20°C until use.
2.2.18 Protein immunoblotting

Yeast cell extracts were prepared for Western blotting as described in section 2.2.7. Extracts containing equal amounts of protein from each strain were separated by 12% SDS-PAGE prior to Western blotting. The proteins were transferred on to a nitrocellulose membrane by Western blot (wet transfer) for 1hr at 100V. The membrane was stained with Ponceau S stain (0.2% Ponceau S in 3% TCA) to confirm the transfer of protein bands and then washed repeatedly with 1X TBST (0.1% Tween 20 in TBS: 25 mM Tris Base, 155 mM NaCl, pH adjusted to 7.4 with HCl) until the Ponceau S stain disappeared completely. Blocking was done for 1 hour at room temperature with blocking buffer (5% non-fat milk powder in 1X TBST). The membrane was probed with a 1:500 dilution in blocking buffer of mouse anti-GFP monoclonal antibody (CLONTECH, Heidelberg, Germany) or 1:2000 dilution of Rabbit polyclonal antiserum against Cdc28 either for 1.5 hours at room temperature or overnight at 4°C. The membrane was then washed for 45 minutes with repeated changes of 1X TBST. 1:2500 dilution of peroxidase coupled rabbit anti-mouse or goat anti-rabbit IgG were used as secondary antibodies (DIANOVA, Hamburg, Germany). The membrane was again washed for 45 minutes with repeated changes of 1X TBST and developed by the Enhanced Chemiluminisence (ECL) method. The ECL solution 1 (100 µl of 2.5mM Luminol and 44 µl of 40 µM Paracoumaric acid in a total volume of 10 ml of 10 mM Tris, pH 8.5) was mixed with ECL solution 2 (6.15 µl of 30% H2O2 in 10ml of 10mM Tris, pH 8.5) just prior to use. The membrane was incubated in the reagent for 1 minute and exposed to the ECL film (AMERSHAM BIOSCIENCES, Munich, Germany) for 1-10 minutes in the dark until the signal intensity on the film was appropriately strong for viewing. The film was developed using a film-developing machine (PROTEC PROCESSOR TECHNOLOGY).
2.2.19 Dihydrorhodamine staining

Free intracellular radicals were determined using dihydrorhodamine123 (ROCHE, Mannheim, Germany) according to the procedure described (Madeo et al., 1999). Logarithmic cultures of each strain were subjected to 1 mM H$_2$O$_2$ for 4 hours and stained for the presence of reactive oxygen species. Fluorescence images were taken with a ZEISS Axiovert S100 microscope supported with a HAMAMATSU OCRA-ER digital camera employing the Openlab tmV 5.0.1 software package (IMPROVISION, Coventry, UK).

2.2.20 Fluorescence microscopy of yeast cells

Yeast cultures were grown in appropriate selective medium containing glucose supplemented with amino acids. A drop of culture was spread on the slide and allowed to dry. 1-2 µl of mounting medium (VCTASHIELD HARD SET$^\text{TM}$, Vector Laboratories Inc, Burlingame, CA, USA) was added and a coverslip was placed and sealed avoiding air bubbles. Cells were viewed with either the GFP filter or DAPI filter using a ZEISS Axiovert S100 microscope. Images showing colocalization of Ynm3-GFP with mito-BFP were taken using a LEICA DM 6000 widefield microscope.

2.2.21 Protein purification from E. coli

Ynm3, Ynm3S236C and Ynm3S236A were expressed from the constructs pME3460, pME3461 and pME3462 respectively and Fpr3 from pME3463 (Table 2). Recombinant proteins were expressed as GST fusions containing a PRESCISSON protease site in the RosettaII strain (NOVAGEN, Darmstadt, Germany) of E. coli for about 12 h at 20°C after induction with 250 µl 1mM IPTG in a total culture volume of 1l 2X-YT medium (16 g Tryptone, 10 g Yeast extract and 5 g NaCl) containing 2% glucose with 100 µg/ml Ampicillin and 100 µg/ml Chloramphenicol. Cells were pelleted after centrifugation at 4000 g for 30 minutes at 4°C. The cell pellets were resuspended in 30 mM PBS, pH 7.5. Cells were disrupted by passing the suspension through a microfluidizer five times at 80 psi and then
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centrifuged at 10,000 g for 15 min. The supernatant containing the GST fusion protein was applied onto GSH-Sepharose, washed with PBS and eluted with a buffer containing 20 mM Tris, 100 mM NaCl and 25 mM GSH, pH 7.5. The fusion proteins were cleaved overnight with PREScission protease (molar ratio 1:50) (GE HEALTHCARE, Munich, Germany) at 4°C. After concentration using the Vivaspin 20 (MWCO 30,000) concentrator (SARTORIUS, Goettingen, Germany), the samples were passed through either Superdex 200 26/60 (GE HEALTHCARE, Munich, Germany) for the Ynm3 variants or Superdex 200 16/60 (GE HEALTHCARE, Munich, Germany) for purifying Fpr3 and eluted in a buffer containing 20 mM Tris, 100 mM NaCl, pH 7.5. For the Ynm3 variants, an additional purification step using GSH-Sepharose matrix was performed.

2.2.22 Proteolysis assay

Reaction mixtures containing 1 µM purified Ynm3, Ynm3S236C or Ynm3S236A were incubated with 1.6 µM heat denatured β-casein in a total volume of 250 µl of 50 mM Heps-KOH, pH 7.3. 20 µl samples were withdrawn at the beginning of the reaction and after overnight incubation (16 h) at 37°C which were boiled immediately after the addition of 4 µl of 6X loading dye. The samples were separated by 15% SDS-PAGE and the protein bands were visualized by staining with Brilliant Blue G-Colloidal Coomasie (SIGMA-ALDRICH, Steinheim, Germany) and quantified using the Kodak 1D Image Analysis Software (EASTMAN KODAK, Rochester, NY).

2.2.23 Chaperone activity assay

2.2.23.1 Preparation of substrate (citrate synthase)

An ammonium sulfate suspension of citrate synthase (CS) from porcine heart mitochondria (BOEHRINGER INGELHEIM, Heidelberg, Germany) was dialyzed using Spectra/Por® dialysis membrane (MWCO 25,000) (ROTH, Karlsruhe, Germany) against TE buffer (50 mM Tris, 2mM EDTA, pH 8.0) overnight at 4°C and concentrated to about 17
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mg/ml using Vivaspin 20 (MWCO 30,000) concentrator (SARTORIUS, Goettingen, Germany). To remove precipitated proteins, the solution was then centrifuged at 13,000 rpm in a microcentrifuge (Biofuge fresco, HERAEUS, Hanau, Germany) for 30 minutes at 4°C. The exact concentration of the supernatant was determined using the published extinction coefficient of 1.78 for a 1 mg/ml solution in a 1 cm quartz cuvette (West et al., 1990). Aliquots were prepared and stored at -20°C. For each set of experiment, appropriate volume of TE buffer was added to an aliquot of CS to yield a final concentration of 30 µM CS monomer.

2.2.23.2 Thermal aggregation assay

The assay is based on the thermal aggregation of citrate synthase (CS) (Buchner et al., 1998). A fluorescence spectrophotometer (HITACHI F-4500) equipped with a stirrable, thermostatted cell holder was used in this assay. The reaction mixture containing 0.15 µM CS in the presence or absence of the indicated amounts of chaperones or a negative control protein like chymotrypsinogen in a total volume of 1 ml of 40 mM HEPES-KOH, pH 7.5 was subjected to 43°C temperature with constant stirring in a 3 ml quartz cuvette placed in the thermostatted cell holder. Aggregation was monitored using the spectrofluorometer with both excitation and emission wavelengths set to 500 nm at a spectral bandwidth of 2.5 nm. Data points were recorded every 0.5 s for 20 minutes. As a further step to prove that the observed suppression of light scattering was not due to proteolysis of CS, 20 µl samples were withdrawn at several time points during the course of the reaction and subjected to 15% SDS-PAGE followed by silver staining.

2.2.23.3 Thermal inactivation assay

The assay for thermal inactivation of CS was also performed as described (Buchner et al., 1998). In brief, thermal denaturation of CS at 43°C was performed as described for the thermal aggregation assay in the presence or absence of chaperone or a negative control
(chymotrypsinogen) in a total volume of 1 ml of 40 mM HEPES-KOH, pH7.5. 20 µl of the reaction mixture was withdrawn at several time points and the specific activity of CS was determined using an assay based on the first step of the citric acid cycle. For each activity assay, the reaction mixture consists of 930 µl of TE buffer (50 mM Tris, 2 mM EDTA, pH8.0), 10 µl of 10 mM oxaloacetic acid (SIGMA-ALDRICH, Steinheim, Germany) 10 µl of 10 mM DTNB (PIERCE, Rockford, IL, USA) in TE buffer and 30 µl of 5 mM acetyl-CoA (SIGMA-ALDRICH, Steinheim, Germany) in TE buffer. This reaction mixture was prepared in advance for at least 20 test assays and used within 1 hour of preparation. The reaction mixture was preincubated at 25°C. Before the activity test, 980 µl of the reaction mixture was pipetted into 1.5 ml plastic cuvettes and the reaction was started by the addition of 20 µl of 0.15 µM CS monomer. The coenzyme A formed in this assay stoichiometrically reduces the Ellmann’s reagent dithio-1,4-nitrobenzoic acid (DTNB) resulting in an increased absorbance at 412 nm. The linear slope of the initial increase in absorbance recorded online spectrophotometrically (UV-1601, SHIMADZU) for 1 minute, was used to calculate specific activity. The specific activity obtained before the start of the thermal inactivation was considered as 100% specific activity. The calculated specific activities during the course of each reaction were expressed as percentage of this value.
3. Results

3.1 The *Saccharomyces cerevisiae* strain YB322 requires Ynm3 for survival under heat stress

The bacterial periplasmic HtrA/DegP is required for survival of *E.coli* at temperatures above 40°C (Lipinska *et al.*, 1990). It functions by degrading denatured cell envelope proteins when subjected to heat stress and is therefore an indispensable quality control factor in the bacterial periplasm. The budding yeast HtrA-like protein, Ynm3 is localized to the nucleus (Fahrenkrog *et al.*, 2004). To test if Ynm3 has a similar role in cell survival at higher temperatures, the *YNM3* coding sequence was deleted in the yeast strain YB322 by homologous recombination of a kanamycin (G418) resistance cassette containing sequences homologous to the 5’ upstream and 3’ downstream regions of the gene. The deletion was verified by PCR.

Growth of wild type YB322 yeast strain and Δynm3 was compared at both ambient growth temperature (30°C) and at a sub-lethal higher temperature (38°C) on solid medium. At 30°C, growth of the wild type strain and Δynm3 was comparable, indicating that *YNM3* is not an essential gene. At 38°C, growth of Δynm3 was reduced compared to that of the wild type strain (Figure 3B). The DNA encoding *YNM3* amplified by PCR from the genomic DNA of the wild type YB322 yeast strain was cloned into a single copy (*CEN*) yeast vector under the control of the *MET25* promoter (Figure 3A). To prove that the heat sensitivity of Δynm3 was indeed due to the lack of Ynm3, growth of the Δynm3 strain transformed with this construct was compared with that of both the wild type YB322 strain and the Δynm3 strain transformed with empty vector at both 30°C and 38°C. As shown in Figure 3B, the growth impairment of Δynm3 at 38°C could be rescued by the construct carrying wild type *YNM3*. The difference in the growth pattern of wild type YB322 and Δynm3 in liquid culture was more pronounced at 39°C (Figure 3C).
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Figure 3: Deletion of \textit{YNM3} in the YB322 yeast strain results in reduced growth under sublethal heat stress. (A) The \textit{YNM3} coding sequence was cloned in a single copy vector under the control of the \textit{MET25} promoter. (B) The growth impairment of a \(\Delta\text{ynm3}\) strain at 38°C was rescued by the single copy plasmid carrying \textit{YNM3} under the control of the \textit{MET25} promoter. (C) Growth of the indicated strains was also assessed in liquid SC-Met-Ura medium at 39°C. (D) The endogenous promoter of the chromosomal copy of \textit{YNM3} was replaced with a module containing a \textit{GAL1} promoter and yeGFP encoding sequence with a kanamycin resistance cassette as a selectable marker. The module was PCR amplified using primers containing sequences homologous to the regions flanking the 5’ end of \textit{YNM3} as shown. The PCR product was transformed into the YB322 yeast strain for homologous recombination, which resulted in its insertion immediately upstream and inframe with the coding sequence of \textit{YNM3}. (E) The resulting yeast strain \textit{GAL1\textsubscript{UAS-yeGFP::YNM3}}, in which \textit{YNM3} is under the control of the inducible-repressible \textit{GAL1} promoter, exhibited impaired growth on YPD at 38°C due to complete repression of the \textit{GAL1} promoter in the presence of glucose. This growth impairment was alleviated on YPGal due to the induction of the \textit{GAL1} promoter.

The aforementioned phenotype was also confirmed by an alternate approach. The endogenous promoter of the chromosomal copy of the \textit{YNM3} gene was replaced with the \textit{GAL1} repressible-inducible promoter. This was achieved by the PCR based epitope tagging method using the cassette \textit{natNT2-GAL1\textsubscript{UAS-yeGFP}} (Janke \textit{et al.}, 2004) as depicted in Figure 3D. Growth of the resultant strain \textit{GAL1\textsubscript{UAS-yeGFP::YNM3}} was compared with that of the
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wild type on solid rich medium containing either glucose (YPD), which completely represses the *GAL1* promoter or galactose (YPGal), which induces the *GAL1* promoter. As expected, growth of the above strain was reduced compared with that of the wild type YB322 strain at 38°C on YPD. This was due to repression of the *GAL1* promoter in the presence of glucose (Figure 3E). The observed growth defect was absent on YPGal as galactose present in the medium led to the induction of the *GAL1* promoter and therefore expression of the yeGFP-Ynm3 fusion protein (Figure 3E).

![Image](image_url)

**Figure 4:** The BY4741 yeast strain is significantly more resistant to heat stress than the YB322 yeast strain. (A) After just a single day of incubation, the BY4741 wild type strain exhibited much better growth compared to the YB322 wild type yeast strain at 38°C. Deletion of *YNM3* in the BY4741 strain background did not result in enhanced heat sensitivity. (B) Only when subjected to prior heat shock at 50°C for the indicated durations, the Δynm3 strain in the BY4741 background exhibited poor viability at 30°C indicating that the penetrance of the phenotype in this strain is lower compared to that in the YB322 strain.

Although both the wild type YB322 and the wild type BY4741 yeast strains are descendents of the same ancestral strain S288C, the former is more sensitive to heat compared to the latter as observed after just 24 hours of incubation on YPD at 38°C (Figure 4A). Consequently, the Δynm3 strain in the BY4741 background (EUROSCARF) was not more sensitive to growth at higher temperatures compared to the wild type strain (Figure 4A). This indicates that the genome of the YB322 wild type strain has undergone changes during evolution after its divergence from the ancestral strain. However, the BY4741 Δynm3 strain
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displayed poor viability at 30°C after subjection to heat shock at 50°C for the indicated durations (Figure 4B). Thus the penetrance of the phenotype in the BY4741 background is much lower compared to that in the YB322 background.

3.2 The serine protease activity of Ynm3 conferred by Ser236 mediates its thermoprotective function

The serine protease activity of the bacterial HtrA/DegP executes its protective function in response to heat stress by degrading irreversibly unfolded proteins in the periplasm (Lipinska et al., 1990; Skorko-Glonek et al., 1995; Spiess et al., 1999). Ynm3 has an HtrA-like serine protease domain and two PDZ domains (Apweiler et al., 2000). Ynm3 is a chymotrypsin A-like serine peptidase belonging to the clan PA with a serine nucleophile and catalytic residues in the order His, Asp, Ser (http://merops.sanger.ac.uk/). To determine whether the serine protease activity of Ynm3 is crucial to execute its thermoprotective function, site directed mutagenesis was performed to mutate the codons for two putative active site serine residues, namely Ser235 and Ser236, to those encoding alanine. Earlier reports published by the same group proposed Ser235 to be the catalytic serine residue (Fahrenkrog et al., 2004; Walter et al., 2006). The adjacent serine at position 236 was however, predicted to be the catalytic serine residue by an earlier version of the MEROPs peptidase database.

Wildtype YNM3 and the mutants, YNM3S235A and YNM3S236A were cloned into a single copy (CEN) vector, which enables expression of the variants as fusion proteins with a C-terminal GFP tag (Figure 5A). The GFP-tag enabled verification of the level of protein expressed by immunoblotting. The Δynm3 strain was transformed with these constructs for further experiments. An assay was performed to test growth of strains carrying these constructs in comparison with the wild type YB322 and Δynm3 encoding GFP alone. The growth assays were carried out at both 30°C and 38°C on plates containing SC-Met-Ura to
select for the *URA3* auxotrophy marker in the expression vectors and because the *MET25* promoter is induced only in the absence of the amino acid methionine.

![Diagram](image)

**Figure 5:** The serine protease activity of Ynm3, mediated by the catalytic serine residue at position 236 is required to execute its thermoprotective function. (A) The GFP fusion proteins in this set of experiments are encoded by a single copy (*CEN*) vector under the control of the *MET25* promoter, which is induced in the absence of the amino acid methionine. (B) The reduced growth of Δynm3 strain at higher temperatures was completely rescued by either Ynm3-GFP or Ynm3S235A-GFP. Ynm3S236A-GFP could not confer thermoprotection. (C) All these variants were expressed to similar levels as seen in the Western blot probed with mouse monoclonal anti-GFP antibody. The blots were stripped and reprobed with rabbit antiserum raised against Cdc28 to ensure equal loading of protein.

The growth defect of Δynm3 at 38°C was completely rescued by the construct encoding Ynm3-GFP and Ynm3S235A-GFP (Figure 5B). However, the construct encoding Ynm3S236A-GFP could not rescue the growth defect of the Δynm3 strain (Figure 5B).
Immunoblotting with the antibody targeted against GFP revealed comparable levels of expression of the variants showing that the mutations do not affect the stability of the variants. Equal loading of protein extracts was ensured by probing with the antiserum against Cdc28. As shown in Figure 5C, there were no marked differences in the amounts of the Ynm3 variants expressed by the strains used in the growth assay. Therefore, it is evident that the serine protease activity of the budding yeast Ynm3 plays an important role in executing its protective function at higher temperatures and the serine residue at position 236 is the catalytic serine.

3.3 Multi copy expression of Ynm3 is deleterious

Expression of Ynm3 from a single copy vector was beneficial, being sufficient to rescue the heat sensitivity of Δynm3. In order to assess the consequence of overexpression, YNM3 and the mutants YNM3S235A and YNM3S236A were cloned into multi copy (2µ) vectors enabling overexpression of these variants as GFP fusion proteins (Figure 6A). These constructs were transformed into the Δynm3 strain. The growth of the resultant strains were compared with the wild type YB322 strain and the Δynm3 strain transformed with vector encoding GFP alone at both ambient growth temperature 30°C and under elevated temperature (38°C) on SC-Met-Ura as described before. Whereas a single copy of Ynm3-GFP or Ynm3S235A-GFP rescued the heat sensitivity of Δynm3 (Figure 5B), multi copy expression of these variants resulted in growth impairment which was even more acute at 38°C. Multi copy expression of Ynm3-GFP or Ynm3S235A-GFP could therefore not relieve the stress experienced by Δynm3 at elevated temperatures (Figure 6B). On the contrary, multi copy expression of Ynm3S236A-GFP did not lead to growth impairment (Figure 6B). This suggests that toxicity due to overexpression of Ynm3 was solely due to excessive serine protease activity, which was abolished simply by the modification of the active site serine. Again, all these variants were expressed equally as shown by immunoblotting using anti-GFP
antibody. The blot was again probed with antiserum targeted against Cdc 28 to ensure equal loading of cell extracts (Figure 6C).

![Diagram of multi copy vector](image)

Figure 6: Overexpression of Ynm3 is deleterious. (A) The GFP fusion proteins in this set of experiments are encoded by a multi copy (2µ) vector under the control of the MET25 promoter, which is induced in the absence of the amino acid methionine. (A) Ynm3-GFP or its S235A variant, when overexpressed led to growth impairment even at 30°C and therefore could not confer thermoprotection at 38°C. The overexpression of the S236A variant did not lead to growth impairment. (B) The fusion proteins were expressed at similar levels as seen in the immunoblot probed with mouse monoclonal anti-GFP antibody. The blots were stripped and reprobed with rabbit antiserum raised against Cdc28 to ensure equal loading of protein.

Cell culture experiments that involve the overexpression of HtrA2/Omi in mammalian cells has led to the conclusion that it has a pro-apoptotic function (Verhagen et al., 2002). Yeast has an apoptotic pathway showing characteristic features of apoptosis such as accumulation of reactive oxygen species, membrane blebbing or nuclear fragmentation (Madeo et al., 1997; Madeo et al., 2002a). I therefore analysed whether the overexpression of Ynm3 in yeast enhanced apoptosis. For this purpose, YNM3 was cloned into a multi copy
vector that enables expression of the protein from the stronger GAL1 promoter. This construct was transformed into the wild type YB322 yeast strain. Spot dilution assays were performed under both inducing and repressing conditions, i.e, in the presence of galactose or glucose respectively. As shown in Figure 7A, overexpression of Ynm3 from a multi copy vector under the control of the GAL1 promoter led to a drastic impairment in growth. The growth impairment was visibly strong even when Ynm3 was expressed from a multi copy vector under the control of the MET25 promoter (Figure 6B). The deletion of the gene encoding the yeast metacaspase (YCA1) has been shown to result in increased cell survival (Madeo et al., 2002b). However, the deletion of the yeast metacaspase locus YCA1 was unable to rescue the growth defect that resulted from Ynm3 overexpression (Figure 7A). This suggests that the observed growth impairment was not a result of a caspase mediated cell death pathway. To rule out the existence of any caspase independent mechanisms of apoptosis that may be operative, the YB322 strain overexpressing Ynm3 or the mammalian HtrA2/Omi were subjected to mild oxidative stress (1mM H2O2 for 4hr) and the presence of reactive oxygen species (ROS), the key regulators of apoptosis in yeast (Madeo et al., 1999), was assessed using the dihydrorhodamine (DHR) staining protocol. DHR stains ROS and emits a red fluorescence. Neither overexpression of Ynm3 nor the mammalian HtrA2/Omi led to an elevation in the number of cells stained for ROS compared with the control strain transformed with empty vector (Figure 7B), ruling out the mediation of caspase independent apoptosis.

Apoptosis is not known in bacteria. Therefore, one would not expect the bacterial HtrA/DegP to possess a programmed cell-death promoting activity. Yet, overexpression of the bacterial HtrA/DegP led to an even more drastic growth impairment of yeast. The overexpression of the mammalian HtrA2/Omi in yeast also led to growth impairment, albeit mild (Figure 7C). It is notable that excessive amounts of quality control factors can be toxic due to unsupervised elimination of cellular proteins (Wickner et al., 1999) which could explain why the overexpression of all these HtrA members led to toxicity in yeast.
Figure 7: Overexpression of Ynm3 leads to growth impairment due to cytotoxicity and not apoptosis. (A) Overexpression of Ynm3 under the control of the GAL1 promoter from a multi copy vector led to growth impairment. This growth impairment could not be rescued by the deletion of the yeast metacaspase YCA1. (B) The wild type YB322 strains overexpressing Ynm3 or mammalian HtrA2/Omi were subjected to mild oxidative stress (1mM H\textsubscript{2}O\textsubscript{2}) for 4 hours and then stained to detect the presence of reactive oxygen species (ROS), the key regulators of apoptosis in yeast, using dihydroorhodamine (DHR). Cells overexpressing Ynm3 or HtrA2/Omi did not show an increase in the number of cells stained for ROS. Calculation from a single representative experimental set is shown here. (C) Overexpression of the bacterial HtrA/DegP from a multi copy vector from the GAL1 promoter also led to a drastic growth impairment of yeast. Overexpression of the mammalian HtrA homologue, HtrA2/ Omi also led to very mild growth impairment.
3.4 An *in vitro* autocalysis assay further corroborated the finding that Ser236 is the catalytic serine residue of Ynm3

Several attempts were made during the course of this work to find *in vivo* substrates or interaction partners of Ynm3 by both yeast two-hybrid assay using the *GAL4* system and the tandem affinity purification (TAP) method (data not shown). None of these attempts proved to be fruitful probably due to transient enzyme-substrate interactions *in vivo*. The bacterial HtrA/DegP cleaves a heterogenous group of unfolded proteins *in vitro* in addition to its native substrate MalS (Clausen et al., 2002; Spiess et al., 1999). Although purified Ynm3 had no effect on a non-native substrate like β-casein, it underwent slow autocatalysis, which could be monitored by SDS-PAGE (Figure 8A). The protein bands on the Coomassie stained gel were quantified and the net intensities plotted (Figure 8B). After 16 hours of incubation at 37°C in the presence of denatured β-casein, the amount of native Ynm3 in the reaction mixture was reduced to less than 17% as shown by the intensity of the protein band on the Coomassie stained gel, whereas the S236A variant was significantly stable with more than 86% of the protein remaining intact after 16 hours (Figure 8B). This clearly validates the *in vivo* finding that the serine at position 236 confers protease activity on Ynm3 since the modification of this serine residue led to stabilization of the protein. In some viral proteases, it has been documented that exchange of active site serines to cysteines does not necessarily abolish proteolytic activity completely which remains to varying degrees in different proteases (Hahn & Strauss, 1990; Tautz et al., 2000). In the case of Ynm3, exchanging Ser236 to alanine significantly reduced its autocalytic activity thereby enhancing its stability but exchanging to cysteine was not as effective with only 65% of the initial amount of protein left in the reaction after 16 hours (Figure 8B).
Figure 8: Ynm3 undergoes slow autocatalysis in vitro due to its serine protease activity. (A) Upon incubation of a reaction mixture containing Ynm3 in the presence of heat denatured β-casein at 37°C, it underwent slow autocatalysis which was dramatically reduced in the S236A variant. (B) The protein bands were quantified and the net intensities plotted. The percentage intensity that remained after 16 h of incubation is shown. Exchange of the catalytic serine of Ynm3 to cysteine was not so effective in reducing autocatalytic activity when compared to exchange with alanine. (C) A single copy of Ynm3S236C could not rescue the heat sensitivity of Δynm3 completely. However, multi copy expression of Ynm3S236C could do so. This confirms the in vitro observation that exchange of Ser236 to cysteine does not completely abrogate its catalytic activity.

A single copy of Ynm3-GFP is sufficient to complement the heat sensitivity of Δynm3 (Figure 5B) whereas Ynm3S236A-GFP does not complement, even when present in multiple copies (Figure 6B). A single copy of Ynm3S236C-GFP also could not completely rescue the heat sensitivity of Δynm3 but multi copy expression of this variant provided sufficient
proteolytic activity to rescue the heat sensitivity of Δynm3 (Figure 8C). This again shows that the exchange of the catalytic Ser236 of Ynm3 to cysteine does not completely abrogate its proteolytic activity whereas exchange to alanine effectively abolishes its proteolytic activity.

3.5 Deletion of either of its PDZ domains destabilizes Ynm3

The HtrA family of proteins is characterized by the presence of a serine protease domain and at least one PDZ domain. PDZ domains are modular protein-protein interaction domains that possess unique structural features, which enable interaction with C-terminal residues of ligand proteins. The PDZ domains of the bacterial HtrAs and the mammalian HtrA2/Omi are important for regulating their protease activities and are also required for substrate binding or multimerization (Clausen et al., 2002; Krojer et al., 2002; Li et al., 2002). The budding yeast Ynm3 is unique in that its PDZ domain is duplicated. The functional significance of this duplication remains to be established.

The PDZ domain present immediately proximal to the protease domain (residues 290 to 375) was designated as PDZ1 and the other predicted PDZ domain at the C-terminal end of the protein as PDZ2 (779-868). To investigate whether the predicted PDZ domains of the budding yeast HtrA orthologue, Ynm3, have any aforementioned essential roles, either the PDZ1 or PDZ2 encoding regions were deleted using an overlap PCR method. The resultant products were cloned into single copy vectors enabling expression of these variants as C-terminal GFP fusion proteins (Figure 9A).

The Δynm3 strain was transformed with CEN vectors encoding either Ynm3ΔPDZ1-GFP or Ynm3ΔPDZ2-GFP fusion proteins. Growth assay of strains carrying these constructs in comparison to the wild type YB322 and Δynm3 encoding GFP alone were performed under inducing conditions in plates containing SC-Met-Ura at 30°C and 38°C. As shown in Figure 9B, only full length Ynm3 was able to completely rescue the heat sensitivity of the Δynm3 strain at higher temperatures. The lack of PDZ1 or PDZ2 domains drastically reduced its
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protective function. This was because the absence of one or the other PDZ domain dramatically reduced the stability of the protein as shown in the immunoblot probed with anti-GFP antibody (Figure 9C).

Figure 9: The lack of either of its two PDZ domains destabilizes Ynm3. (A) The GFP fusion proteins in this set of experiments are encoded by a single copy (CEN) vector under the control of the MET25 promoter, which is induced in the absence of the amino acid methionine. (B) Deletion of either of the two PDZ domains of Ynm3 drastically reduced its function. (C) The lack of either PDZ domain led to drastic destabilization of Ynm3 as seen in the immunoblot probed with mouse monoclonal anti-GFP antibody. The blots were stripped and reprobed with rabbit antiserum raised against yeast Cdc28 to ensure equal loading of protein. (D) Lack of PDZ domains did not mislocalize the protein. Ynm3ΔPDZ1-GFP and Ynm3ΔPDZ2-GFP were predominantly localized in the nucleus.
It was speculated in an earlier report that the second PDZ domain of Ynm3 could be important for its nuclear localization as it was observed that the variant lacking it was not present exclusively in the nucleus (Fahrenkrog et al., 2004). However, in this study, the Ynm3 variants lacking either of its PDZ domains were correctly localized to the nucleus (Figure 9D). Thus the lack of PDZ domains markedly reduced the stability of Ynm3 rendering it insufficient for conferring thermoprotection although it did not affect its nuclear localization.

3.6 The first 100 N-terminal amino acid stretch of Ynm3 contains its nuclear localization signal

In order to define the nuclear localization sequence (NLS) of Ynm3, constructs were made encoding GFP fused C-terminally to 100 amino acid stretches of Ynm3 spanning its entire length. These vectors were transformed into Δynm3 to determine the nuclear localization of these 100 amino acid stretches of Ynm3 by fluorescence microscopy. The NLS of Ynm3 was narrowed down to its first 100 N-terminal amino acids because the variant lacking these residues (Ynm3ΔN100aa) fused with GFP was localized entirely outside the nucleus (Figure 10A). The first 100 N-terminal amino acid stretch of Ynm3 (N100aa) fused with GFP on its own was localized in the nucleus confirming that the NLS of Ynm3 lies in this region. The PDZ domains of Ynm3 are therefore not important for its nuclear localization. It was interesting to determine if Ynm3ΔN100, which lies entirely outside the nucleus could still rescue the heat sensitivity of a Δynm3 strain. For this purpose, both single and multi copy vectors encoding GFP tagged fusions of Ynm3ΔN100 were transformed into the Δynm3 strain. Growth tests were performed as described in the previous section at both 30°C and 38°C on plates containing SC-Met-Ura. As shown in Figure 10B, Ynm3ΔN100 in either single or multiple copies could not confer thermoprotection although multi copy
expression of Ynm3ΔN100 led to growth impairment indicating that it is a functional protease.

Figure 10: The nuclear localization signal in Ynm3 lies in its first 100 N-terminal amino acid residues. (A) GFP tagged Ynm3ΔN100aa lacking the first 100 N-terminal amino acids was localized entirely outside the nucleus whereas the first 100 amino acid stretch of Ynm3 (N100aa) tagged with GFP on its own was entirely localized in the nucleus. This indicates that the nuclear localization signal of Ynm3 lies in the region containing the first 100 N-terminal amino acids. (B) Ynm3ΔN100 could not rescue the heat sensitivity of a Δynm3 strain when expressed from either a single or a multi copy vector under the control of the MET25 promoter. Multi copy expression of Ynm3ΔN100 resulted in growth impairment indicating that it retains protease activity. (C) Ynm3ΔN100 expressed from a single copy vector was only half as stable as native Ynm3 as indicated by the immunoblot probed with anti-GFP antibody. The blot was stripped and reprobed with anti-Cdc28 antiserum. The bands were quantified and the relative intensity plotted.
This suggests that the nuclear localization of Ynm3 is important to confer its protective function under higher temperatures. Immunoblotting with anti-GFP antibody revealed that Ynm3ΔN100 expressed from a single copy vector was only half as stable as native Ynm3 (Figure 10C).

**3.7 Ynm3 exhibits chaperone activity, *in vitro.***

The prokaryotic HtrA members are dual chaperone-proteases. Chaperone activity in any eukaryotic HtrA has not been recognised so far. I analyzed if the budding yeast Ynm3 exhibits chaperone activity employing a widely used assay based on the thermal aggregation of citrate synthase (CS), a non-native substrate (Buchner *et al.*, 1998). CS when subjected to heat, denatures and forms aggregates, which renders the solution turbid because these aggregates scatter light that increases with time. The increase in the amount of light scattered with time is due to both increase in the number and also the size of aggregates formed by thermally denaturing CS (Buchner *et al.*, 1998). As shown in Figure 11A, in the absence of chaperones, the reaction mixture containing 0.15 µM CS scattered light at 500 nm which increased with time when subjected to a temperature of 43°C. Presence of equimolar amounts of Ynm3 in the reaction mixture completely suppressed this light scattering whereas the negative control protein, chymotrypsinogen, did not (Figure 11A). This indicates that Ynm3 possesses ATP-independent general chaperone activity due to its ability to bind unfolding intermediates of a non-native substrate like CS at 43°C thereby preventing its aggregation without any energy requirement. Several known chaperones like GroEL, Hsp70, Hsp90 and small heat shock proteins have been shown to possess the ability to suppress the aggregation of thermally denatured CS (Buchner *et al.*, 1998).
Figure 11: **Ynm3 exhibits chaperone activity, in vitro.** (A) In the absence of chaperones, the reaction mixture containing 0.15 µM citrate synthase (CS) scattered light in an increasing manner at 43°C due to thermal aggregation of CS. In the presence of equimolar amounts of Ynm3 or Ynm3S236A, this light scattering was completely suppressed. (B) Presence of Ynm3 did not affect the thermal inactivation kinetics of CS.

The *E.coli* HtrA/DegP is a chaperone at lower temperatures and switches to being a protease only at higher temperatures (Spiess et al., 1999). In contrast, Ynm3 acts as a chaperone at 43°C *in vitro*, a condition similar to heat shock temperatures *in vivo*. But the protease dead S210A variant of bacterial HtrA/DegP retains chaperoning ability even at higher temperatures which is sufficient to rescue the heat sensitivity of a *degP* mutant when
overexpressed (Spiess et al., 1999). This does not seem to be the case with the budding yeast Ynm3. Although the protease dead variant Ynm3S236A exhibited chaperone activity at 43°C (Figure 11A), even its overexpression could not compensate for the lack of its proteolytic activity (Figure 6B), which seems to be indispensable for protection against heat stress.

Ynm3 could prevent the aggregation of CS, yet it had no significant influence on the thermal inactivation of CS at 43°C (Figure 11B) suggesting that Ynm3 tightly binds those unfolding intermediates of CS which are no more in equilibrium with their native state.

3.8 Lack of Ynm3 may lead to compromised mitochondrial function in older yeast colonies

There is increasing evidence that the mammalian HtrA2/Omi might play an important role in maintaining mitochondrial homeostasis. Although, Ynm3 is predominantly localized in the nucleus, its absence in the budding yeast YB322 strain was also reported to result in respiratory deficiency (Tong et al., 2006). The Δynm3 strains generated in this study did not show such a strong respiratory deficient phenotype although a small fraction of the Δynm3 transformants was respiration deficient. Nevertheless, a distinct difference between the colour of YB322 wild type and the Δynm3 colonies was repeatedly noticed upon prolonged incubation for about 15-20 days on synthetic complete (SC) agar containing glucose and 100 mg/l adenine at 30°C (Figure 12A). Strains like YB322 that have an ade2 mutation accumulate an intermediate of the adenine biosynthetic pathway, which is converted into a red pigment by a series of covalent and oxidative reactions. This pigment formation pathway is defective due to the lack of OXPHOS enzymes in strains that have damaged mitochondrial DNA which therefore remain white (Shadel, 1999). The colonies of the wild type YB322 strain developed a deep red colour upon prolonged incubation whereas the Δynm3 colonies remained light pink (Figure 12A). The deep red colour was completely restored in the Δynm3 strain by introducing single copy (CEN) plasmid carrying the gene encoding native Ynm3
under the control of the \textit{MET25} promoter (Figure 12A). The pink coloration of the \textit{Δynm3} colonies in contrast to the deep red coloration of the wild type YB322 colonies on the same plate at ambient growth temperature (30°C) is indicative of compromised oxidative growth in old \textit{Δynm3} colonies, which is restored upon re-introduction of a copy of \textit{YNM3}. As aging is often associated with compromised protein quality control, these observations hint at the possibility that in addition to its primary function in the nucleus, Ynm3 may also become important for mitochondrial homeostasis in aging yeast cells.

The mammalian HtrA2/Omi is predominantly localized in the mitochondria. It was observed that Ynm3-GFP, encoded by its endogenous promoter, was localized mainly in the nucleus. Since the lack of Ynm3 hints at mitochondrial dysfunction, I wondered if Ynm3, like its mammalian homologue, is at least partially localized to the mitochondria. Interestingly, a subpopulation of cells from overnight cultures of the BY4741 strain background, expressing Ynm3-GFP from its endogenous promoter grown in SC medium showed a distinct dotted pattern of GFP fluorescence in the cytoplasm. This was also described in a different strain background in a previous report (Fahrenkrog \textit{et al.}, 2004). Considering the predominant localization of the mammalian HtrA homologue, HtrA2/Omi, in the mitochondrial intermembrane space, I suspected that this dotted GFP fluorescence pattern represented mitochondria. The BY4741 Ynm3-GFP encoding strain was transformed with a plasmid encoding mitochondria-targeted blue fluorescent protein (BFP). Fluorescence microscopy of overnight cultures of this strain revealed colocalization of Ynm3-GFP with the mitochondria-targeted BFP signal in a subset of cells (Figure 12B), thus indicating that a fraction of Ynm3 is associated with mitochondria. To address the possibility that the BFP signal could have bled through the GFP channel, snapshots of the same cells were obtained after completely bleaching the BFP signal by overexposure to UV light. The mitochondrial GFP signal in these cells, however, remained intact, making it clear that there was no bleed through of the BFP signal in the GFP channel.
Figure 12: Lack of Ynm3 leads to reduced oxidative growth in older yeast cells. (A) Prolonged incubation of the indicated strains for about 20 days revealed a difference in the intensity of red pigment formation characteristic of ade2 yeast strains growing oxidatively. The lighter colour attained by the Δynm3 strain could be restored to wild type levels (deep red) by a single copy plasmid expressing Ynm3 (B) In addition to nuclear localization, Ynm3-GFP expressed under the control of its endogenous promoter from its chromosomal locus, in the strain BY4741 was found to be colocalized with mitochondria targeted BFP in a subpopulation of cells (indicated by arrow) in overnight cultures. Images of GFP were also taken after photobleaching of the BFP signal in order to rule out any bleed through between the channels. (C) The number of cells exhibiting either nuclear or mitochondrial localization of Ynm3-GFP or both were counted from a set of eleven snapshots. About 25% of the cells counted from a total of 88 showed colocalization of Ynm3-GFP with mito-BFP.

3.9 A genetic screen identified FPR3 as a suppressor of the heat sensitivity of Δynm3

A suppressor screen was performed in this study by transforming a yeast genomic DNA library (Connelly and Hieter, unpublished, June 1990) into Δynm3 to identify other genes that could suppress its heat sensitivity. Plasmids were isolated from those Δynm3 colonies that
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appeared larger at 38°C. From the screen, only one candidate plasmid was found to contain complete coding sequences and was able to partially rescue the heat sensitivity of Δynm3 even upon isolation and retransformation. Upon blasting the insert sequence of the plasmid against the yeast genome database (http://seq.yeastgenome.org/cgi-bin/blast-sgd.pl), complete coding sequences of adjacent genes on chromosome XIII namely FPR3, encoding a PPIase and RPL6A, encoding a ribosomal subunit, along with their upstream and downstream sequences were identified. The two genes were individually cloned and transformed into Δynm3 to identify, which one of these was responsible for conferring better growth for Δynm3 at elevated temperatures. An additional copy of FPR3 but not RPL6A was sufficient to partly rescue the heat sensitivity of Δynm3 as observed in the genetic screen (Figure 13).

![Image](image.png)

**Figure 13:** *FPR3* is a partial knockout suppressor of Δynm3. (A) A single copy plasmid carrying FPR3 under the control of the MET25 promoter partially rescued the heat sensitivity of a Δynm3 strain under inducing conditions whereas (B) that carrying RPL6A, whose coding sequence is present adjacent to FPR3 on the chromosome, could not.

*FPR3* encodes a peptidyl-prolyl cis-trans isomerase (PPIase) localized to the nucleolus of the budding yeast (Shan et al., 1994). Surprisingly, in *E. coli* too, a functional relationship exists between HtrA/DegP and the periplasmic PPIase, SurA, which is mainly responsible for
the maturation of outer membrane proteins (OMPs). HtrA/DegP is not essential for bacteria under normal conditions. Its role is only to rescue OMPs that have escaped SurA mediated folding and maturation. However it’s role is amplified in the absence of SurA or under stressful conditions. HtrA/DegP along with another periplasmic chaperone Skp can substitute for the absence of SurA (Sklar et al., 2007) and therefore surA/degP double deletion is synthetic lethal (Rizzitello et al., 2001).

To test if such a genetic interaction exists between Ynm3 and the PPIase, Fpr3, I deleted FPR3 in the wild type YB322 strain and also in the Δynm3 strain using a cassette conferring nourseothricin (cloNAT) resistance. Genomic DNA was isolated from colonies that could grow on plates containing YPD+cloNAT. The presence of the drug resistance cassette disrupting the FPR3 locus was verified by PCR. As shown in Figure 14, the double deletion of FPR3 and YNM3 was not synthetically lethal and deletion of FPR3 alone did not lead to discernable heat sensitivity. I speculated that Fpr3 like some other PPIases of yeast like Cpr6 and Cpr7 might in addition to its PPIase activity, have chaperone activities that would gain prominence only when other quality control mechanisms fail.

![Figure 14](image.png)

**Figure 14: In the presence of Ynm3, Fpr3’s thermoprotective function is dispensable.** Lack of Fpr3 alone does not lead to noticeable heat sensitivity in the YB322 strain. Moreover, double deletion of YNM3 and FPR3 was not synthetically lethal.
4. Discussion

A variety of intracellular and extracellular factors can threaten the quality of proteins in the cell. Gene mutations or biosynthetic errors lead to accumulation of aberrant proteins, which are unable to attain their native conformations. External factors like high temperatures can affect the tertiary structure of proteins resulting in unfolding or misfolding which can lead to aggregate formation. To prevent such an occurring, cells have developed an elaborate machinery of quality control factors: chaperones and proteases, which ensure that proteins are maintained in their natively folded and active forms, thereby safeguarding cell viability and function. Mutations in genes encoding chaperones and proteases can lead to failure of quality control and accumulation of aberrant proteins, which is deleterious to the cell as this might interfere with efficient functioning of cellular pathways or lead to sequestration of proteins into aggregates due to the inherent stickiness of the exposed hydrophobic surfaces of misfolded proteins. Toxic gain-of-function mutations acquired by proteins as in neurodegenerative disorders such as Parkinson’s disease that enable them to escape quality control can lead to massive aggregation. Efficiency of the cellular quality control system also dwindles with age. This is the reason for the late onset of most neurodegenerative disorders where failure of protein quality control may be both the cause and the consequence.

Mitochondrial dysfunction is a common feature of neurodegenerative diseases like Parkinson’s disease (PD). Mutations in genes encoding proteins that directly or indirectly affect mitochondrial homeostasis have been associated with PD. One such factor is the serine protease HtrA2/Omi. Mammalian HtrA2/Omi was first identified as a homologue of the bacterial HtrA proteins that are stress responsive serine proteases in the periplasmic space required for survival at higher temperatures (Faccio et al., 2000; Gray et al., 2000). HtrA2/Omi was originally described as an apoptosis inducer in cell culture. According to
various reports, HtrA2/Omi, a resident of the mitochondrial intermembrane space, exits the organelle and enters the cytoplasm when cells receive apoptotic stimuli. In the cytoplasm, it binds and cleaves a group of proteins called the inhibitor of apoptosis (IAP) proteins thereby relieving their inhibitory effect towards caspaces (Hegde et al., 2002; Martins et al., 2002; Suzuki et al., 2001; Verhagen et al., 2000). In the mean time, studies using mutant mice have revealed a contradictory physiological function for HtrA2/Omi. The mnd2 (motor neuron degeneration 2) mutant mice, in which the corresponding gene encodes an intact IAP binding domain but carries a protease inactivating point mutation (S276C), suffer neurodegeneration leading to juvenile death (Jones et al., 2003). Interestingly, the HtrA2 knockout mice also show a similar phenotype underscoring the physiological relevance of the serine protease activity of this protein (Jones et al., 2003; Martins et al., 2004). Cells from these mice are more prone to apoptosis rather than being resistant. A certain percentage of cells from HtrA2/Omi knockout mice exhibit abnormal mitochondrial morphology combined with a decreased mitochondrial density (Martins et al., 2004). Moreover, mutations affecting its protease activity resulting in increased sensitivity to cell death and compromised mitochondrial complex I activity have been identified as susceptibility factors in PD (Strauss et al., 2005). Since HtrA2/Omi closely resembles the bacterial HtrAs, which aid cell survival at elevated temperatures, it is indeed debatable whether a close homologue of a bacterial cell survival protein has switched functions to becoming a cell death-promoting factor in higher organisms. Recent reports have provided hints further strengthening the view that HtrA2/Omi could have a more protective role in the physiological milieu. It has been shown that HtrA2/Omi phosphorylation in a PINK1 dependent manner (another factor associated with PD), which increases its proteolytic activity is involved in mitochondrial stress response (Plun-Favreau et al., 2007). A second checkpoint function for HtrA/Omi in the mitochondria following proteasomal inhibition in the cytoplasm has also been reported (Radke et al., 2008). Considering that mitochondria are evolutionary derivatives of ancestral α-proteobacteria, it is
tempting to suspect that HtrA2/Omi and other eukaryotic HtrAs have conserved their original bacterial function i.e., protection against unfolding stresses. The possibility that the mammalian HtrA2/Omi might also act as a dual chaperone-protease like the bacterial HtrA/DegP is an immensely attractive hypothesis though as yet untested. So far, no such definitive function has been ascribed to a eukaryotic HtrA member. In this study, this issue was addressed for the budding yeast HtrA representative, Ynm3, a eukaryotic HtrA member whose cellular function is not yet clear since contradictory ideas regarding its function has been put forth leading to a contention similar to that of the mammalian homologue (Fahrenkrog et al., 2004; Tong et al., 2006).

4.1 The serine protease activity of Ynm3 mediates cell survival under heat stress

Mutational loss of quality control factors often lead to growth arrest especially under heat stress due to structural damage to proteins and accumulation of protein inclusions. If Ynm3 were a quality control protease like HtrA/DegP in bacteria, the lack of it would be manifested as retarded growth of yeast under stressful conditions. Here, the growth of the wild type YB322 yeast strain was compared with the Δynm3 strain at a higher temperature and indeed the loss of Ynm3 led to visible growth impairment, which could be rescued by just a single copy of the wild type protein. As mentioned earlier, the E. coli HtrA/DegP is a serine protease that protects the bacterial cell at elevated temperatures by eliminating unfolded proteins in the periplasm by its serine protease activity (Lipinska et al., 1990; Spiess et al., 1999). Ynm3 is a chymotrypsin A-like serine peptidase with a serine nucleophile and catalytic residues in the order His, Asp, Ser (http://merops.sanger.ac.uk/). Two adjacent serine residues were selected, namely Ser235 and S236, for exchange to alanine. Ser235 was proposed as the catalytic serine by the same group in two of their reports (Fahrenkrog et al., 2004; Walter et al., 2006). The adjacent serine at position 236 was predicted to be the catalytic serine residue by the
2007 version of the MEROPs peptidase database. Expression of the Ynm3S235A variant could completely rescue the heat sensitivity of Δynm3 whereas the Ynm3S236A variant could not do so indicating the importance of the serine protease activity of Ynm3 conferred by the serine residue at position 236, in executing its protective function in response to heat stress. Therefore, it is very likely that Ynm3, like its bacterial counterpart HtrA/DegP in the periplasm, binds and proteolytically cleaves toxic aberrant proteins in the nucleus of the budding yeast. A range of specialized ATP dependent proteolytic machines mediate proteolysis in bacteria and organelles such as mitochondria of eukaryotes whereas the proteasome is the only proteolytic machinery involved in protein quality control in the nucleus and cytoplasm of eukaryotes. The existence of a second proteolytic machinery, Ynm3, mediating protein quality control in the budding yeast nucleus is therefore a novel finding.

In vitro, HtrA/DegP cleaves a heterogenous group of unfolded proteins (Clausen et al., 2002). Ynm3 had no effect on heat denatured non-native substrates like β-casein. Nevertheless it underwent slow autocatalysis in vitro. It is possible that a specific modification triggers its proteolytic activity, which is available only in the physiological milieu, or on the other hand Ynm3 may target only a specific class of proteins. Several attempts made during the course of this work to find in vivo substrates of Ynm3 using yeast two-hybrid assay and tandem affinity purification method failed probably due to transient enzyme-substrate interactions in vivo.

An interesting observation emerged from the in vitro autocatalysis assay of Ynm3. The amount of purified wild type Ynm3 in the reaction had reduced to less than 17% after 16 hours of incubation as observed by Coomassie staining after SDS-PAGE. As expected, exchange of its catalytic serine residue to alanine dramatically reduced the autocatalytic activity of Ynm3 with more than 86% of the initial amount of protein remaining in the reaction mixture. This again validates the in vivo finding that the protease activity of Ynm3 is
conferred by the serine at position 236. Although the exchange of the active site serine to cysteine also greatly reduced the autocatalytic activity of Ynm3, it was not as effective as in the case of the Ynm3S236A variant with only 65% of Ynm3S236C left in the reaction after 16 hours. This indicates that Ynm3S236C retains some residual catalytic activity. Consequently, the overexpression but not single copy expression of Ynm3S236C could provide sufficient proteolytic activity to execute its protective function upon heat stress. This was not the case with the overexpression of Ynm3S236A suggesting that it is completely protease dead. It has been documented for some viral proteases that exchange of active site serines to cysteines does not necessarily abolish proteolytic activity completely which remains to varying degrees in different proteases (Hahn & Strauss, 1990; Tautz et al., 2000). Here, this seems to be the case with the budding yeast Ynm3 also.

### 4.2 Over expression of Ynm3 is deleterious to yeast

Whereas an optimal amount of Ynm3 is beneficial to yeast, especially at elevated temperatures, overexpression of Ynm3 led to growth impairment, which was enhanced under heat stress. As mentioned in the previous section, Ynm3 expressed form a single copy vector could completely rescue the heat sensitivity of a Δynm3 strain. In contrast, multi copy expression of Ynm3 proved to be deleterious. However, multi copy expression of Ynm3S236A did not lead to any growth impairment unlike the case with the Ynm3S235A variant. Thus abrogating the serine protease activity of Ynm3 by exchanging the serine at position 236 to alanine could abolish its overexpression induced toxicity. This is an additional confirmation that the catalytic activity of the Ynm3 protease is conferred by the serine residue present at position 236 of the polypeptide and not the one present at position 235 as proposed earlier (Fahrenkrog et al., 2004).

The cellular quality control mechanism works efficiently because it has devised a strategy to target only misfolded or unfolded proteins by recognizing exposed hydrophobic
patches on their surface thus avoiding native proteins since they have buried hydrophobic residues. Overexpression can disturb the balance between the amount of quality control proteases and the load of unfolded proteins present in the cell. Thus at optimal levels, quality control proteases aid cells by eliminating dysfunctional proteins but forced overproduction of these otherwise beneficial proteases can create havoc in the cell as they may also target native functional proteins for degradation. This would deny the cell of essential proteins leading to disruption of the normal functioning of cellular pathways resulting in growth impairment.

It was proposed in the first report (Fahrenkrog et al., 2004) that overexpression of Ynm3 resulted in apoptosis in yeast similar to the overexpression of mammalian HtrA2/Omi in cell culture. Hence the protein was termed nuclear mediator of apoptosis, 111kDa protein (Nma111). As there was no indication for either caspase mediated cell death or increased ROS production that could trigger a caspase independent cell death pathway, I conclude that the observed growth impairment was solely due to the cytotoxicity mediated by Ynm3’s serine protease activity when present in excess.

4.3 The PDZ domains of Ynm3 are critical for its stability

The HtrA family of proteins is characterized by the presence of a serine protease domain in combination with at least one PDZ domain. PDZ domains represent a common protein–protein interaction motif, and their name was derived from the first three proteins in which such domains were identified, namely PSD-95, Drosophila melanogaster Disc large protein, and zonula occludens protein 1. The PDZ domains of the HtrA family members are important for substrate binding and/or regulating the proteolytic activity of the enzyme (Krojer et al., 2002; Li et al., 2002; Wilken et al., 2004). The PDZ domains of the thermophillic bacterium Thermotoga maritima HtrA (TmHtrA), which is a DegQ homologue, is important for dimerization of individual trimers (Kim & Kim, 2005). Interestingly, the eukaryotic budding yeast Ynm3 has two PDZ domains, one present immediately proximal to the protease domain
and the other at the C-terminus of the protein. The functional significance of this duplication is not known. In this study, Ynm3 lacking either of the two PDZ domains could not confer thermoprotection to the Δynm3 strain although these variants were localized correctly to the nucleus. Immunoblotting revealed that these variants were highly unstable in comparison to the wild type protein. The instability of these variants could be due to disruption of their native tertiary structure because of the absence of a PDZ domain. This could in turn lead to their elimination by the quality control apparatus of the cell. Most quality control proteases studied so far exists as oligomeric complexes forming a compartmentalized structure in which their catalytic chambers are hidden from the environment. As in Tm HtrA, it is plausible that Ynm3 may exist as a stable oligomeric complex via interactions mediated by the PDZ domains. The oligomerization of Ynm3 may in turn be important for its function. Structural studies are necessary to probe into the intra and intermolecular interactions within or between the monomers and to explain why the lack of PDZ domains destabilizes the protein. The lack of either PDZ domain of Ynm3 although has a great impact on its stability, it does not effect the nuclear localization of the protein contrary to what was speculated in an earlier report (Fahrenkrog et al., 2004). The site of Ynm3 rather lies in its first 100 amino acids residues.

4.4 Ynm3 is a dual chaperone-protease like its bacterial HtrA/DegP counterpart

The idea that chaperone activity could be associated with HtrA/DegP emerged from studies in the intracellular parasite, Rickettsia where a DegP homologue lacking the catalytic triad and two others lacking one catalytic residue were discovered (Bass et al., 1996). Subsequently, the chaperone activity of E. coli HtrA/DegP towards its native substrate, the periplasmic MalS and a non-native substrate, citrate synthase was characterized (Spiess et al., 1999). Eukaryotic HtrA homologues have been characterized as serine proteases involved in
cell growth and development, stress response, apoptosis and ageing. The existence of chaperone activity in a eukaryotic HtrA has not been described so far.

I addressed this possibility for Ynm3, the HtrA counterpart of the simple eukaryotic model organism, the budding yeast and could indeed demonstrate the existence of general chaperone activity reminiscent of its prokaryotic homologues. In vitro, Ynm3 in equimolar amounts was able to completely prevent the thermal aggregation of a non-native substrate like citrate synthase in an ATP independent manner. Citrate synthase (CS) is a widely used substrate for assaying chaperone activity in vitro and its folding pathway has been well characterized (Buchner et al., 1998). In contrast to bacterial DegP, which is a chaperone only at ambient growth temperatures, Ynm3 exhibited chaperone activity even at higher temperatures. Ynm3 did not influence the thermal inactivation kinetics of CS suggesting that it functions by strongly sequestering and preventing the aggregation of those unfolding intermediates, which are unable to refold to their native state. The protease dead Ynm3S236A alone could not rescue the heat sensitivity of Δynm3 even in excessive amounts despite being a robust chaperone in vitro unlike the situation in bacteria where DegPS210A in excessive amounts is sufficient to do so (Spiess et al., 1999). This implies that the protease activity of Ynm3 is crucial for handling irreversibly unfolded proteins, which may otherwise continue to accumulate forming toxic aggregates, a non-productive side reaction that not only renders damaged proteins refractory to proteolysis but also disrupts cellular pathways by sequestering functional proteins. Moreover, in the in vitro assay, although Ynm3 prevented the thermal aggregation of CS, it could not prevent its thermal inactivation, which suggests that Ynm3 binds only to irreversibly unfolded proteins that cannot refold to their native conformations. Consequently, in the absence of its protease activity, the chaperone activity of Ynm3 alone was unable to tackle unfolding stresses.

The ATP dependent proteolytic machines in bacteria like the ClpXP or ClpAP and that of the eukaryotic mitochondria like the Lon and Ftsh homologues have associated ATP-
dependent chaperone activities either in the same or in physically associated subunits which aid in the unfolding of substrates facilitating their entry into the proteolytic chamber whose dimensions make it inaccessible for native or partially folded substrates to enter (Gottesman et al., 1997a). The ATPase subunits of the proteasomal lid also function in a similar manner (Benaroudj et al., 2003; Lee et al., 2001). Evolution has probably preserved the chaperone activity of Ynm3 for a similar purpose so that it recognizes heat-denatured proteins, preventing their aggregation and delivering them in a more soluble state to its protease domain thereby increasing the efficiency of proteolysis. The existence of a chaperone component in Ynm3 is probably meant only for effective recognition and sequestration of irreversible unfolded proteins and allowing their entry into the proteolytic center, which may not be able to accommodate folded or even partially folded conformers like in the classic proteases of bacteria and mitochondria, thereby avoiding unsupervised degradation. The protease subcomponents of the classic proteolytic complexes of bacteria and mitochondria are totally dependent on their associated chaperone components for substrate unfolding and delivery and hence cannot function all alone separated from their associated chaperone subunits. It would be interesting to find out if this is the case for Ynm3, too. This would require identification and subsequent mutation or deletion of the domain of Ynm3 that confers its chaperone activity.

The essence of this work describing Ynm3’s role in protein quality control is summarized in the model (Figure 16) in which Ynm3 is a dual chaperone protease with the chaperone and protease activity residing in the same protein. Ynm3, by its chaperone activity is able to bind and sequester irreversibly unfolded proteins thereby preventing their aggregation. In addition, like the ATP dependent proteases of bacteria and the eukaryotic mitochondria, the chaperone activity of Ynm3 could help bind heat denatured irreversibly unfolded proteins to deliver them into its catalytic center in an ATP-independent manner for degradation. The only proteolytic machine in eukaryotes other than the ones present in
cytoplasmic organelles is the 26S ATP dependent proteasome. Structural studies will further our understanding of the organization of this novel ATP-independent chaperone-protease apparatus present in the nucleus of the budding yeast.

Figure 16: Model for Ynm3’s role as a dual chaperone protease. Ynm3 is a dual chaperone protease with the chaperone and protease activity residing in the same protein. Ynm3’s chaperone activity is able to sequester irreversibly unfolded proteins preventing their aggregation and maintaining them in a soluble state accessible for proteolytic attack. In addition, like the classic quality control proteases of bacteria and the mitochondria, the chaperone activity of Ynm3 could help maintain proteins in their unfolded state for delivery to its catalytic center for degradation in an ATP-independent manner.
4.5 **Ynm3 may be involved in mitochondrial homeostasis during ageing**

Ynm3’s protective role during heat stress is abolished when it is excluded from the nucleus, which indicates that it functions predominantly in the nucleus. However, observations made during this study have provided initial evidence pointing towards a possible mitochondrial function for the protein. The YB322 yeast strain is defective in adenine synthesis due to the *ade2* mutation. Upon prolonged incubation on synthetic complete (SC) agar, the colonies turn deep red due to the accumulation of a dark red pigment, which is derived by the covalent and oxidative modification of an adenine biosynthetic pathway intermediate that accumulates in *ade2* strains. *Δynm3* colonies display a marked decrease in the intensity of this red pigment formation compared with the wild type colonies upon prolonged incubation. The intensity of the red coloration was restored to wild type levels by reintroduction of a single copy of native *YNM3*. Since this red pigment formation in *ade2* strains like YB322 requires oxidative modifications of the accumulated adenine biosynthetic pathway intermediate by the mitochondrial oxidative phosphorylation system (OXPHOS) (Shadel, 1999), this phenotype is an indication that Ynm3 is either directly or indirectly involved in the proper functioning of mitochondrial respiration in the budding yeast which becomes apparent upon prolonged incubation.

The absence of the mammalian mitochondrial HtrA2/Omi or the protease deactivating mutation in the *mnd2* mouse results in decreased mitochondrial density in tissues (Martins *et al.*, 2004). In humans, mutations in HtrA2/Omi affecting its protease activity led to mitochondrial dysfunction and Parkinson’s disease (Strauss *et al.*, 2005). Recent reports suggest that HtrA2/Omi may be involved in mitochondrial quality control when the cytoplasmic proteasome is inhibited (Radke *et al.*, 2008). This study hints at a similar phenomenon of compromised mitochondrial function in the budding yeast due to the lack of Ynm3. Moreover, colocalization of endogenous Ynm3-GFP with mitochondrial markers was
observed in overnight cultures of the BY4741 yeast strain. Since the protein does not possess an obvious mitochondrial targeting sequence, it is possible that it is either passively imported into the mitochondria or may only bind to the mitochondrial surface. This observation raises the question whether Ynm3 plays a protective quality control function in the budding yeast mitochondria or its absence impairs another pathway leading to slowdown of respiration and mitochondrial function in aged yeast cells.

Ageing can result in a diminished ability for cells to maintain strict quality control which is the reason why neurodegenerative diseases like Parkinson’s have a late onset. Failure of mitochondrial protein quality control can in turn lead to accumulation of mutations in mtDNA. The observed phenotype indicative of poor respiratory growth became apparent only in older yeast colonies and therefore parallels the manifestation of symptoms related to mitochondrial dysfunction induced neurodegenerative disorders like Parkinson’s disease in aged individuals. Since the budding yeast is a valuable model to study oxidative damage and ageing, unraveling the molecular pathway underlying the observed phenotype might lead to a better understanding of the connection between HtrA2/Omi and mitochondrial homeostasis.

4.6 A genetic screen identified the yeast nucleolar PPIase Fpr3 as a partial suppressor of the heat sensitivity of Δynm3

A genetic screen was performed using a budding yeast genomic DNA library to identify other candidates that could suppress the heat sensitivity of Δynm3. A plasmid containing the complete coding sequences of two adjacent genes encoding Fpr3, a nucleolar PPIase and Rpl6A, a ribosomal protein of the large subunit was found to enhance the growth of Δynm3 under elevated temperatures. Confirmatory tests were performed subsequently using constructs encoding either Fpr3 or Rpl6A, which led to the identification of the gene encoding Fpr3 as the candidate supressor. Fpr3 is a nucleolar peptidyl prolyl cis-trans isomerase (PPIase) implicated in maintaining meiotic recombination checkpoint activity (Hochwagen et
in a manner which requires its PPIase domain but not its PPIase activity. PPIases are ubiquitous enzymes whose cellular roles remain largely undefined although these enzymes exhibit PPIase activity \textit{in vitro}: catalysis of the cis-trans conversion of peptidyl prolyl bonds in polypeptides, which otherwise is a rate-limiting step in protein folding. Several PPIases in the budding yeast are known to be induced under heat stress and some of them are known to interact physically and functionally with heat shock proteins (Arevalo-Rodriguez \textit{et al.}, 2004). In addition to their PPIase activities, some of them like the budding yeast Cpr6, Cpr7 and the bacterial periplasmic SurA exhibit general chaperone activity \textit{in vitro} (Behrens \textit{et al.}, 2001; Buchner \textit{et al.}, 1998; Mayr \textit{et al.}, 2000).

A cooperative interaction between HtrA/DegP and a PPIase, SurA exists in \textit{E. coli}. The periplasmic PPIase, SurA, aids in the folding of cell envelope proteins (Rouviere & Gross, 1996). Cooperation between HtrA/DegP and SurA in the periplasm of \textit{E. coli} is exemplified by the synthetic lethality of a \textit{surA/degP} double deletant (Rizzitello \textit{et al.}, 2001). SurA is the major periplasmic chaperone required for the folding of outer membrane proteins (OMPs). Under normal conditions, HtrA/DegP and another general chaperone Skp rescue those OMPs that have fallen-off the assembly pathway. But the roles of these proteins are greatly amplified under stress or in the absence of SurA (Sklar \textit{et al.}, 2007). The discovery of a similar compensatory interaction between the budding yeast HtrA homologue, Ynm3 and a PPIase Fpr3, where an additional copy of Fpr3 could partly substitute for the lack of Ynm3, was indeed surprising and further confirms that the budding yeast Ynm3 has a role analogous to the bacterial HtrA/DegP.

4.7 Fpr3 exhibits strong general chaperone activity

\textit{In vitro}, the periplasmic PPIase SurA exhibits chaperone activity where a 64-fold molar excess of SurA could completely suppress the thermal aggregation of a non-native substrate like citrate synthase (Behrens \textit{et al.}, 2001). In this work, the possibility that Fpr3
might also possess chaperone activity was tested and indeed it was found that substoichiometric amounts of purified Fpr3 could completely suppress the thermal aggregation of a non-native substrate like citrate synthase (CS) indicating that Fpr3 has excellent ATP independent chaperoning ability. This most likely contributes to the suppression of the heat sensitivity of the Δynm3 strain mediated by Fpr3. Equimolar amounts of Fpr3 reduced the rate of thermal inactivation of CS which follows apparent first order kinetics (Buchner et al., 1998). In the experiment depicted in Figure 15, the presence of equimolar amounts of Fpr3 reduced the rate constant of thermal inactivation of CS by about 18 fold. This demonstrates that Fpr3 binds to CS conformers that are native-like or are able to refold to their native states. This is unlike the case with Ynm3, which tightly sequesters irreversibly unfolded proteins, preventing their aggregation and hence has no influence on the inactivation kinetics of CS.

In *E. coli*, lack of both SurA and HtrA/DegP is synthetically lethal. In the budding yeast, whereas just an additional copy of *FPR3* could suppress the heat sensitivity of a Δynm3 strain, deletion of *FPR3* alone did not lead to detectable heat sensitivity and double deletion of *YNM3* and *FPR3* was not synthetically lethal. This suggests that Fpr3 under normal conditions may have other physiological roles and is not indispensable for tackling heat-denatured substrates due to other redundant and probably more prominent protein quality control mechanisms like that conferred by Ynm3. The chaperoning activity of Fpr3 gains importance under heat stress only in the absence of Ynm3, probably by rescuing substrates common to both Ynm3 and Fpr3 from denaturation and aggregation. Thus, the chaperoning function of Fpr3 gains significance under conditions of heat stress only in the absence of Ynm3. The interaction between Ynm3 and Fpr3 observed in this study is depicted in Figure 17. Such a compensatory chaperoning mechanism in the budding yeast nucleus resembles that between HtrA/DegP, SurA and Skp in the bacterial periplasm (Sklar et al., 2007).
Figure 17: Fpr3 partly compensates for the lack of Ynm3 under heat stress. (A) In the presence of Ynm3, the chaperone activity of Fpr3 is dispensable to counter the unfolding stresses arising due to exposure of yeast to higher temperatures. Fpr3’s function is redundant with that of Ynm3. (B) In the second scenario, i.e., in the absence of Ynm3, the chaperone activity of Fpr3 gains prominence where it binds unfolding substrates, preventing their aggregation and also providing them a chance to refold to their native structures. The interrupted arrows are used here to show that Fpr3 only partly compensates for the absence of Ynm3.
5. Summary and Conclusions

This study has led to the functional characterization of Ynm3, the budding yeast orthologue of the widely conserved HtrA family of proteins whose prokaryotic members are implicated in protein quality control. The well-studied bacterial HtrA/DegP acts as a dual chaperone-protease in the periplasm. A role for the mammalian mitochondrial HtrA2/Omi as a quality control factor is also very likely but not explicit. This study shows that the budding yeast Ynm3 has retained through evolution the characteristic chaperone-protease function reminiscent of its prokaryotic homologues.

In the YB322 yeast strain, the proteolytic activity of Ynm3 is crucial for handling unfolding stresses arising at elevated temperatures. The lack of Ynm3 or its proteolytic activity renders the strain susceptible to heat stress resulting in drastic growth impairment. From this work, the active site serine of Ynm3 has been identified as the serine residue at position 236 of the polypeptide and not Ser235 as proposed in earlier reports.

Overexpression of this otherwise beneficial protease is toxic resulting in growth impairment. This toxicity could be abolished by exchanging the active site serine (Ser 236) to alanine. The observed growth impairment might be due to the mistargeting of functional native proteins for proteolytic cleavage, which are otherwise strategically avoided. This could exhaust components of essential cellular pathways since under conditions in which quality control factors are overexpressed, the “triage” mechanism of substrate recognition fails.

In addition to acting as a protease, Ynm3 exhibits a strong general chaperone activity in vitro, which is able to prevent the thermal aggregation of a non-native substrate in an ATP independent manner. The chaperone activity of Ynm3 alone is not sufficient to confer thermoprotection since the protease dead Ynm3S236A was unable to rescue the heat sensitivity of Δynm3 even when overexpressed. I propose a model in which the chaperone activity of Ynm3 helps bind irreversibly unfolded proteins to prevent their aggregation, which
could otherwise render them refractory to proteolytic attack. On the other hand, the chaperone activity might function to deliver substrates in an unfolded form into the proteolytic center like in the case of classic ATP-dependent proteases of bacteria and the eukaryotic mitochondria whose proteolytic chambers do not accommodate native or partially folded proteins.

In conclusion, this work has led to the identification of a novel ATP-independent chaperone-protease apparatus in the nucleus of the budding yeast. There is initial evidence that Ynm3 could also play a role in mitochondrial homeostasis during ageing since the Δynm3 strain shows signs of poor respiratory metabolism upon prolonged incubation. Further studies could lead to a better understanding of the function of the mammalian HtrA2/Omi with respect to protein quality control in the mitochondria.

During the course of the study, a nucleolar PPIase was identified as a knockout suppressor of Ynm3. Subsequent work led to the identification of a strong chaperone activity in Fpr3, which could prevent the thermal aggregation and also slow down the thermal inactivation of citrate synthase, a non-native substrate. Presumably, the chaperone activity of Fpr3 is able to partially compensate for the lack of Ynm3 in tackling unfolded proteins under heat stress. A similar phenomenon of compensatory chaperoning mechanism operates between HtrA/DegP and the PPIase, SurA in the periplasm of E. coli.
6. References


Publication

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