EFFECTS OF THE COMPONENTS OF THE GET PATHWAY ON PRION PROPAGATION

A Thesis
Presented to
The Academic Faculty

by

Bhawana Bariar

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in the
School of Biology

Georgia Institute of Technology
December 2007
EFFECTS OF THE COMPONENTS OF THE GET PATHWAY ON PRION PROPAGATION

Approved by:

Dr. Yury Chernoff, Advisor
School of Biology
Georgia Institute of Technology

Dr. John Cairney
School of Biology
Georgia Institute of Technology

Dr. Kirill Lobachev
School of Biology
Georgia Institute of Technology

Dr. Donald Doyle
School of Chemistry
Georgia Institute of Technology

Dr. Jung Choi
School of Biology
Georgia Institute of Technology

Date Approved: November 12, 2007
I dedicate this work to my dad Prof. (Dr.) Lalit Mohan Bariar, my mom Mrs. Deepali Bariar, my husband Dr. Nilabh Srivastava, and my younger brother Vineet Bariar. This work would not have been possible without their perpetual love, faith, support, and encouragement.
ACKNOWLEDGEMENTS

I am very thankful to my advisor Dr. Yury Chernoff for guiding me throughout my research project and helping me develop my research skills.

I am also very grateful to my thesis committee members, Dr. Kirill Lobachev, Dr. Donald Doyle, Dr. Jung Choi and Dr. John Cairney for their support, encouragement, feedback and suggestions that helped me in my research.

I am grateful to our research technician, Gary Newnam, for always being willing to provide me with valuable suggestions and technical assistance. I am also thankful to Dr. Andrey Romanyuk for guiding me with the SDD-AGE experiments. I would like to thank Yuri Nishida for assisting me with the Get2 and Sup35 colocalization experiments. I am also thankful to my laboratory friends Nina Romanova, Buxin Chen, Meng Sun, He Gong and Stefka Gyoneva for their collegiality. I would also like to thank Jesse Patterson for helping me with the incipient theoretical and experimental phases of this project. I am also grateful to the departmental and university staff for rendering services cordially and efficiently.

I owe a huge debt of gratitude to my mom, my dad, my younger brother, and my extended family members for their constant love, support, and banter that allow me to take every challenge with confidence.

Last but not least, I am very thankful to my husband, Nilabh, for his constant support, encouragement, vast academic experience and love that helped me at every step of my graduate school experience and in accomplishing this task.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENTS</th>
<th>iv</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
</tbody>
</table>

## CHAPTER

1. **INTRODUCTION**
   - Prions 1
   - Heat Shock Proteins 3
   - The GET complex 7
   - Objectives 8

2. **MATERIALS AND METHODS** 9
   - Strains 9
   - Plasmids 11
   - Antibodies 12
   - Molecular biology techniques 12
   - E. coli plasmid DNA isolation 12
   - Yeast and *E. coli* transformation procedures 13
   - Standard yeast media and growth conditions 14
   - Yeast DNA isolation 14
   - Yeast protein isolation and analysis 15
   - Nonsense suppression assay for presence of $[\psi^+]$ 15
   - Assay to monitor for presence of $[\text{PIN}^+]$ 15
   - Thermotolerance assay 16
SDD-AGE
Pringle method for gene deletion and tagging
Fluorescence microscopy

3 EFFECT OF THE OTHER COMPONENTS OF THE GET COMPLEX (GET1 AND GET3) ON [PSI+] STABILITY
Curing defect in the presence of excess Hsp104
Spontaneous loss of [PSI+] on synthetic media in the get1 and get3 deletion strains
Conclusion

4 EFFECT OF GET2 ON PRIONS OTHER THAN [PSI+]
Spontaneous loss of [PIN+] in the get2 deletion strain
De novo induction of [PSI+]
Effects of get2 and get3 deletions in a weak [PSI+] background
Conclusion

5 DOES GET2 OPERATE BY ALTERING/MODULATING HSP104 LEVELS AND/OR ACTIVITY?
Hsp104 levels
Hsp104 activity analyzed by thermotolerance assay
Conclusion

6 COMPARISON OF THE [PSI+] AGGREGATE PATTERNS AND SIZES BETWEEN GET2 Δ MUTANT AND WILD TYPE
Analysis of aggregate sizes using SDD-AGE
Analysis of aggregates using fluorescence microscopy
Conclusion

7 MODULATION OF THE EFFECTS OF GET2 DEFICIENCY ON PRION BY CALCIUM CONCENTRATIONS
Effect of calcium on [PSI+] instability
Effect of calcium on $[PSI^+]$ curing 45

Conclusion 46

8 DISCUSSION 47

CONCLUSIONS 50

REFERENCES 51
LIST OF TABLES

Table 1.1: The role of Hsp104 in yeast prion propagation ........................ 5
Table 2.1: List of Saccharomyces cerevisiae strains used in this study .......... 9
Table 2.2: List and description of plasmids used in this study ...................... 11
Table 2.3: List and description of primers used in this study ....................... 18
Table 3.1: Effect of get1A and get3A on [PSI+] stability .......................... 24
Table 4.1: Get2 deletion strain exhibits spontaneous loss of [PIN+] prion ... 26
Table 4.2: Effects of get2A and get3A on [PSI+] stability in weak [PSI+] background ...................................................... 28
Table 6.1: Analysis of [PSI+] aggregate pattern in solid medium in get2-473 mutant as compared to wild type .................................................. 40
Table 6.2: Analysis of [PSI+] aggregate pattern in solid medium in get2-473 mutant as compared to wild type .................................................. 40
Table 6.3: Analysis of [PSI+] aggregate pattern in liquid medium in get2-473 mutant as compared to wild type .................................................. 41
Table 6.4: Analysis of [PSI+] aggregate pattern in liquid medium in get2-473 mutant as compared to wild type .................................................. 41
Table 7.1: Effect of calcium on [PSI+] instability in get2A through incubation on complete synthetic medium ..................................................... 44
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Nonsense Suppressor Assay for the $[PSI^+]$ prion</td>
<td>2</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Structural organization of Sup35 and Rnq1</td>
<td>3</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>The normal cellular function of Hsp104 and its role in $[PSI^+]$ maintenance and propagation</td>
<td>4</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Function of Get Complex and the effect of the $get2$-$473$ mutation on the structure of Get2 protein</td>
<td>6</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Effect of $get2$ on $[PSI^+]$ curing and stability</td>
<td>7</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Pringle method for making $GET$ gene deletions</td>
<td>20</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>The confirmation of $get1$ and $get3$ deletion constructions in strong $[PSI^+]$ background by PCR</td>
<td>21</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>$[PSI^+]$ curing defect by excess Hsp104 is observed in $get3\Delta$ and $get1\Delta$</td>
<td>22</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Effect of $get1\Delta$ and $get3\Delta$ on $[PSI^+]$ stability</td>
<td>23</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>$Get2$ deletion strain exhibits spontaneous loss of $[PIN^+]$ prion</td>
<td>25</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>$De novo$ induction of $[PSI^+]$ is not affected in $get2\Delta$</td>
<td>26</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>The confirmation of $get2$ and $get3$ deletion constructions in weak $[PSI^+]$ background by PCR</td>
<td>27</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>$Get2$ and $get3$ deletions do not have an effect in weak $[PSI^+]$ background</td>
<td>28</td>
</tr>
<tr>
<td>Figure 5.1</td>
<td>Growth curves for wild type and $get2$ mutants</td>
<td>30</td>
</tr>
<tr>
<td>Figure 5.2</td>
<td>The $[PSI^+]$ curing and maintenance defects in the $get2$ deletion and mutant strains are not due to a decrease in the Hsp104 levels</td>
<td>32</td>
</tr>
<tr>
<td>Figure 5.3</td>
<td>The experimental scheme for thermotolerance assay</td>
<td>33</td>
</tr>
<tr>
<td>Figure 5.4</td>
<td>The $[PSI^+]$ curing and maintenance defects in the $get2$ deletion and mutant strains are not due to a decrease in the activity of Hsp104</td>
<td>34</td>
</tr>
<tr>
<td>Figure 6.1</td>
<td>The $[PSI^+]$ polymer size is increased in the $get2\Delta$ strain as compared to the wild type</td>
<td>37</td>
</tr>
<tr>
<td>Figure 6.2</td>
<td>The bigger aggregates of the $get2\Delta$ strain are not reconstituted in trans</td>
<td>39</td>
</tr>
</tbody>
</table>
Figure 7.1: The experimental scheme for studying the effect of calcium on \([PSI^+]\) instability in \(get2\Delta\) through serial passages on complete synthetic medium 43

Figure 7.2: Effect of calcium on \([PSI^+]\) instability in \(get2\Delta\) through incubation on complete synthetic medium 44

Figure 7.3: Effect of calcium on \([PSI^+]\) curing in \(get2\Delta\) 45

Figure 8.1: Possible mechanism for the effect of the Get complex on prion propagation48
CHAPTER 1
INTRODUCTION

Prions

One of the most important discoveries of modern molecular biology is that information can be transmitted not only by nucleic acids, but also by infectious proteins called Prions. Prions are self-perpetuating protein isoforms that propagate by converting the normal form of the protein of the same amino acid sequence into the infectious form (27). Prions cause fatal neurodegenerative diseases in mammals e.g. Bovine Spongiform Encephalopathy (Mad Cow disease) and Creutzfeldt-Jacob disease in humans (1). Mammalian prions form fiber-like aggregates rich in \( \beta \)-sheet structures known as amyloid fibers. Amyloid fibers are associated with neurodegenerative diseases such as Alzheimer’s disease (AD), Huntington’s disease (HD) etc.

Prions control heritable traits in yeast. \([PSI^+]\) is a self-polymerized isoform of the yeast \textit{Saccharomyces cerevisiae} eukaryotic release factor (translation termination factor eRF3) Sup35. During translation the soluble (non-prion) form of \([PSI^+]\) along with Sup45 recognizes stop codons. An altered protein conformation of Sup35 creates the \([PSI^+]\) prion. \([PSI^+]\) alters translational fidelity and creates a nonsense suppressor phenotype (2) and allows the growth on medium lacking adenine in strains containing the \textit{adel-14} reporter. (Fig. 1.1B). On the other hand, in cells containing soluble Sup35 protein (Fig 1.1A), there is no growth on medium lacking adenine due the efficient termination at stop codon of \textit{adel-14} reporter.
Figure 1.1 Nonsense Suppressor Assay for the [PSI+] prion
The ade1-14UGA reporter system is used to detect the presence of [PSI+] prion. As shown in A, in [psi+] cells the Sup35 protein is in its soluble form and efficiently terminates protein synthesis at the UGA stop codon in the ade1-14UGA mRNA. In this case enough Ade1 product is not formed causing lack of growth on –Ade media and red color on YPD. On the other hand when the Sup35 protein is aggregated to form the [PSI+] prion (B), the translation termination function is impaired and it does not efficiently recognize the stop codon. This leads to enough production of Ade1 which enables the cells to grow on –Ade media. The color in this case is white or pink on YPD.

There are three broad regions in the Sup35 protein structure- the N-terminal region which is the prion domain which is not required for the normal cellular function of the protein, but required for prion formation and propagation. It is QN-rich and contains multiple imperfect repeats. Then there is the middle M region which is rich in charged residues and has some role in [PSI+] stabilization. The C-terminal region is required for the cellular function of the protein but not for the [PSI+] phenotype (3) (Fig 1.2 A).

There are other prions in yeast e.g. [URE3] and [PIN+] / [RNQ+]. Yeast prion proteins have prion domains that are responsible for prion formation. These domains are not required for the normal function of the protein within the cell. [PIN+] is the prion form of the protein Rnq1 (Fig. 1.2B). The function of this protein is not known. Its prion
domain is towards the C-terminus and is QN-rich. In the presence of $[PIN^+]$ prion, overexpression of Sup35 can induce de novo $[PSI^+]$ (4).

**Sup35**

![Sup35 Diagram]

- **N**: Prion domain
- **M**: Middle domain
- **C**: Functional domain

**Rnq1**

Prion domain

- **N**: Prion domain towards the C-terminus which comprises QN-rich sequences.

Figure 1.2 *Structural organization of Sup35 and Rnq1*

**A.** There are three structural domains in Sup35. There are a total of 685 amino acids in this protein. The N-terminal region (Sup35N) is the prion domain required for prion formation and propagation. It is rich in Q (Q, Asn (N), Gly, and Pro residues and contains multiple imperfect repeats. The end of N domain is not clearly known. It is generally placed between amino acid positions 113 and 137. In this figure the end of the N domain is shown at position 123. The middle domain (Sup35M) is rich in charged residues and has some role in $[PSI^+]$ stabilization. The C-terminal region (Sup35C) is required for the normal cellular function of the protein. **B.** Rnq1 protein has the prion domain towards the C-terminus which comprises of QN-rich sequences. The function of this protein is not known. Its prion form is $[PIN^+]$.

**Heat Shock Proteins**

Heat shock proteins (Hsp’s) act like molecular chaperones ensuring that the cell’s proteins are folded properly. They are induced when a cell undergoes various types of environmental stresses like heat, cold and oxygen deprivation. There are different families of the cytoplasmic molecular chaperones in yeast e.g. Hsp100, Hsp70 and Hsp40. Proteins which are damaged under conditions of stress are disaggregated and properly refolded by the action of these chaperone proteins (5). Propagation of yeast prions requires components of this cellular stress response system. Hsp104 is the first
cellular protein shown to affect $[\text{PSI}^+]$ propagation (8). It is a member of the Hsp100 family. It acts as a homohexamer and is an ATPase. It acts on aggregated stress-damaged proteins and disaggregates them (5). It is thus responsible for induced thermotolerance and response to other environmental stresses in yeast (Fig 1.3A). Induced thermotolerance is the phenomenon where cells acquire tolerance to heat when given a mild pre-heat treatment during which Hsp104 is induced (6). Basal thermotolerance measures the basal activity of Hsp104 through cell viability after a heat shock without any pre-heat treatment. Moderate levels of Hsp104 are required to break the $[\text{PSI}^+]$ aggregates into smaller seeds which initiate new rounds of propagation (7) (Fig. 1.3B).

Figure 1.3 The normal cellular function of Hsp104 and its role in $[\text{PSI}^+]$ maintenance and propagation

A. The normal cellular function of Hsp104 is disaggregation of stress-damaged proteins. As a result of this ability, it produces thermotolerance in the cells (discussed in chapter 5). Figure taken from http://biochemistry.utoronto.ca/glover/lab/research.html

B. Moderate levels of the Hsp104 chaperone are required for $[\text{PSI}^+]$ propagation through the production of seeds which initiate new rounds of propagation, whereas it has been hypothesized that Hsp104 overproduction cures yeast cells of $[\text{PSI}^+]$ by breaking down the aggregates into monomers. In the case of hsp104 deletion, the aggregates become too big to be efficiently passed on to the daughter cells which are then cured of the prion.
One model suggests that excess Hsp104 cures the cells of $[\text{PSI}^+]$ by possibly breaking it down into monomers (8). Alternatively it has been proposed that overexpression of Hsp104 may impair segregation of prions in cell divisions (36). In the absence of Hsp104 aggregates grow too big to be efficiently transmitted to daughter cells. Hsp104 also plays a role in the propagation of the other prions $[\text{PIN}^+]$ and $[\text{URE3}]$. While an Hsp104 deletion cures the cell of all the three prions: $[\text{PSI}^+]$, $[\text{PIN}^+]$ and $[\text{URE3}]$; over expression of Hsp104 only cures the cell of $[\text{PSI}^+]$. The other two prions are stable in the presence of excess Hsp104 (4, 9) (Table 1.1).

Table 1.1 The role of Hsp104 in yeast prion propagation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Function</th>
<th>Prion</th>
<th>Excess Hsp104</th>
<th>hsp104D</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUP35</td>
<td>Sup35</td>
<td>Translation termination</td>
<td>$[\text{PSI}^+]$</td>
<td>cures</td>
<td>Cures</td>
</tr>
<tr>
<td>RNQ1</td>
<td>Rnq1</td>
<td>Unknown</td>
<td>$[\text{RNQ}^+]/[\text{PIN}^+]$</td>
<td>stable</td>
<td>Cures</td>
</tr>
<tr>
<td>URE2</td>
<td>Ure2</td>
<td>Nitrogen catabolism</td>
<td>$[\text{URE3}]$</td>
<td>stable</td>
<td>Cures</td>
</tr>
</tbody>
</table>

A study was carried out in this laboratory aiming at the identification of additional factors that cause this difference in curing of prions by excess Hsp104 and are responsible for the curing of $[\text{PSI}^+]$ by over expression of this chaperone (S. Muller, J. Patterson and Y. Chernoff, unpublished data; J. Patterson Honors Thesis [23]). EMS mutagenesis was performed to generate mutants with decreased $[\text{PSI}^+]$ curing by Hsp104 over expression. 13 mutants were phenotypically characterized and library screen was performed to identify the mutated gene in one of the mutants with the strongest phenotype. This mutant was found to have a point mutation in the $\text{GET2}$ gene resulting in a premature stop codon at position 473, which leads to the formation of a truncated protein lacking the transmembrane domains. This is referred to as mutant $\text{get2-473}$ (Fig. 1.4A). Get2 (Rmd7) is a component of the Get Complex which is required for the retrieval of HDEL proteins from the Golgi to the Endoplasmic reticulum (ER) (retrograde
vesicle-mediated transport) in an Erd2 dependent fashion (10) (Fig 1.4B). The other two components of the Get complex are Get1 (Mdm39) and Get3 (Arr4).

**Figure 1.4 Function of Get complex and the effect of the get2-473 mutation on the structure of Get2 protein**

A. A truncated Get2 protein is formed in get2-473 mutant due to the generation of a premature stop codon at position 473. The truncated protein lacks transmembrane domains since it has only 157 amino acids as compared to 285 in wild type. Transmembrane domains may be necessary for the correct localization of Get2 and possibly for its proper function. B. Get2 (Rmd7) is a component of the Get Complex which is required for the retrieval of HDEL proteins from the Golgi to the Endoplasmic reticulum (ER) (retrograde vesicle-mediated transport) in an Erd2 dependent fashion (10). The other two components of the Get complex are Get1 (Mdm39) and Get3 (Arr4). The schematic is shown here with modifications from (10).

A deletion was made in the *GET2* gene in the strong [*PSI*⁺] strain OT56 (Table 2.1). Strong [*PSI*⁺] strains usually have a strong nonsense suppression phenotype compared to weak strains. Also, the aggregates are smaller in size in the strong strains which leads to efficient propagation (2). The get2Δ strain exhibits a [*PSI*⁺] curing defect in the presence of excess Hsp104 as well as spontaneous loss of [*PSI*⁺] prion when grown on synthetic medium, indicated by the spontaneous appearance of red and pink colonies (S. Muller, J. Patterson and Y. Chernoff, unpublished data, and 23) (Fig. 1.5).
Figure 1.5 Effect of get2 on [PSI+]

**A.** The wild type strain is efficiently cured of [PSI+] in the presence of pLH105 (PGPD-HSP104) and appears red on YPD. On the other hand get2-473 shows a [PSI+] curing defect by excess Hsp104 produced by pLH105 as seen by white color on YPD (S. Muller, J. Patterson, and Y. Chernoff, unpublished data; and 23).

**B.** Growth of get2Δ strain on synthetic medium causes [PSI+] instability. The strain is streaked out on YPD after growth on complete synthetic medium (~3-4 days) and the instability can be seen by the appearance of many [psi-] colonies which become red on YPD.

The Get Complex

The Get (Golgi-to-ER traffic) complex is required for the ATP-dependent retrieval of HDEL motif-containing proteins from the Golgi to the Endoplasmic reticulum (retrograde vesicle-mediated transport) in an Erd2 dependent fashion (10) (Fig. 1.4B). This complex comprises of three proteins namely, Get1, also known as Mdm39; Get2, also known as Rmd7; and Get3, also known as Arr4. These three components of the Get complex show physical and genetic interactions with each other (10, 11). These proteins also have some additional functions. Get1 is also required for normal mitochondrial morphology and inheritance (12). Get2 was originally discovered in a large-scale screen for genes that are necessary for meiosis (13). It is involved in cell wall organization and biogenesis and might be involved in cell wall function. get2Δ exhibits defects in growth on non-fermentable media, meiotic cell division, and cell wall biogenesis. (13, 14). Get3 is the ATPase subunit of the Get complex. It is also involved in resistance to heat and metal stress (arsenite transport) (15). Get3-GFP forms dot-like
structures in conditions of stress (15). Also, it has been shown that most of the Get complex shifts from the ER to dot-like structures under conditions of stress e.g. shift from rich to minimal medium (10). This suggests that the Get complex might have a role in stress response and its major functions have to be explored.

**Objectives**

Based on the above background, the following are the main objectives of this work:

1. Since *get2* mutation has been shown to affect \([PSI^+]\) stability (S. Muller, J. Patterson and Y. Chernoff, unpublished data; and 23), the question arises whether the other components of the Get complex i.e. *GET1* and *GET3* affect \([PSI^+]\) stability.

2. *Get2* mutation has been shown to affect the maintenance of \([PSI^+]\) prion. We have addressed the question whether *get2* mutation has this effect on prions other than \([PSI^+]\).

3. Since the molecular chaperone Hsp104 plays an important role in the propagation of yeast prions, it will be studied whether *get2* mutation operates by altering/modulating the levels and/or activity of Hsp104.

4. Since *get2Δ* causes a defect in prion maintenance, it will be investigated whether the \([PSI^+]\) prion instability is reflected in the size of the polymers in the *get2Δ* strain.

5. Other possible mechanisms for the effect of *get2* on \([PSI^+]\) will be investigated.
CHAPTER 2
MATERIALS AND METHODS

MATERIALS

Strains

All yeast strains used in this study are listed in Table 2.1.

Table 2.1 List of *Saccharomyces cerevisiae* strains used in this study

<table>
<thead>
<tr>
<th>Name (synonym)</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1-74-D694 (OT55)</td>
<td>[PSI+] MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52</td>
<td>2</td>
</tr>
<tr>
<td>7-74-D694 (OT56)</td>
<td>[PSI+] MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52</td>
<td>8</td>
</tr>
<tr>
<td>74-D694 (OT60)</td>
<td>[psi- PIN+] MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52</td>
<td>8</td>
</tr>
<tr>
<td>GT17</td>
<td>[psi- pin+] MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52</td>
<td>32</td>
</tr>
<tr>
<td>GT982</td>
<td>[PSI+] MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get2::HIS3</td>
<td>23</td>
</tr>
<tr>
<td>GT905</td>
<td>[PSI+] MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52</td>
<td>23</td>
</tr>
<tr>
<td>GT1060</td>
<td>[psi- pin+] MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get2::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>GT1072</td>
<td>[PSI+] MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get2::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>GT1074</td>
<td>[PSI+] MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get3::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>GT1105</td>
<td>[PSI+] MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get1:: KAN&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>GT1107</td>
<td>[PSI+] MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get3:: HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>GT234</td>
<td>[psi- pin+] MATa ade1-14 leu2-3, 112 his3-Δ200(or -11, 15) lys2 trp1-Δ ura3-52</td>
<td>32</td>
</tr>
<tr>
<td>GT1078</td>
<td>[psi- PIN+] MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get2::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>GT1080</td>
<td>[psi- pin+] MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get2::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>BY4741(OT352)</td>
<td>get1 (YGL020C)::KAN&lt;sup&gt;R&lt;/sup&gt;</td>
<td>24 (obtained from Open Biosystems)</td>
</tr>
</tbody>
</table>
The most commonly used strains in this work are isogenic derivatives of yeast strain 74-D694, which differ only in their prion status. Strain 74-D694 ([psi PIN]) is also known as OT60. The weak [PSI' PIN+] strain OT55 and the strong [PSI' PIN+] strain OT56 were independently induced by overexpression of SUP35 in strain OT60 (2). These strains can be distinguished by their respective phenotypes. Nonsense suppression of the ade1-14UGA mutation is more efficient in strain OT56, resulting in growth on –Ade medium after 3-4 days and a white/light pink color on YPD, compared to the 7-8 day incubation time for OT55 cells on –Ade, and a pink color on YPD. Strain GT17 is [psi pin+] and was acquired by GuHCl treatment of strain OT56 as described previously (4). A second set of isogenic strains are derivatives of the strong [PSI' PIN+] diploid parent GT81 (16). GT234 (17) is a meiotic segregant of GT81, cured of [PSI+] and [PIN+] by GuHCl. GT982 was obtained by deleting GET2 gene in the OT56 background (23). GT905 was obtained by the EMS Mutagenesis of OT56 and is resistant to [PSI+] curing by Hsp104 overexpression (23). It has a point mutation in the GET2 gene at codon 473 and is hereafter referred to as get2-473. GT1060 is a [psii pin+] derivative of GT982. [PSI+] was lost spontaneously as a result of a transformation. It was tested for [PIN+] and found to be [pin-]. GT1072 was obtained by deleting the GET2 gene in the OT55 strain using the template plasmid pFA6a-His3MX6 (Table 2.2). GT1074 was obtained by deleting the GET3 gene in the OT55 strain using the template plasmid pFA6a-His3MX6. GT1105 was obtained by deleting the GET1 gene in the OT56 strain. This deletion was made by transforming OT56 with the PCR product obtained by amplifying the genomic DNA of the yeast strain BY4741 containing deletion in the GET1 gene (ORF YGL020C) (24). GT1107 was obtained by deleting the GET3 gene in the OT56 strain using the template plasmid pFA6a-His3MX6. GT1078 is a [psii PIN+] derivative of GT982. [PSI+] was lost spontaneously through incubation on complete synthetic medium and [PIN+] status was determined through [PSI+] induction test. GT1080 is a [psii pin+] derivative of GT982. [PSI+] was lost spontaneously through incubation on complete synthetic medium and [PIN+] status was
determined through \([PSI^+]\) induction test. Strain BY4741 containing a deletion in the GET1 gene (ORF YGL020C) was obtained from the Saccharomyces Genome Deletion Consortium (24).

**Plasmids**

All plasmids used in this study are listed in Table 2.2.

Table 2.2 **List and description of plasmids used in this study**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Markers (E. coli/ S. cerevisiae)</th>
<th>Description</th>
<th>Plasmid type</th>
<th>Reference (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YEp13</td>
<td>AMP(^r)/ LEU2</td>
<td>Basic cloning vector</td>
<td>YEp</td>
<td>17</td>
</tr>
<tr>
<td>pSTR7</td>
<td>AMP(^r)/ LEU2</td>
<td>(P_{SUP35-35}) SUP35</td>
<td>YEp</td>
<td>17</td>
</tr>
<tr>
<td>pLH105</td>
<td>AMP(^r)/ LEU2</td>
<td>(P_{GFP-HSP104})</td>
<td>YCp</td>
<td>17</td>
</tr>
<tr>
<td>pFA6a-His3MX6</td>
<td>AMP(^r)/ HIS3</td>
<td>Template plasmid used for making gene deletion constructs.</td>
<td>YEp</td>
<td>22</td>
</tr>
<tr>
<td>pMCUP1</td>
<td>AMP(^r)/ URA3</td>
<td>Base vector for copper induction.</td>
<td>YCp</td>
<td>Lindquist lab</td>
</tr>
<tr>
<td>pU-CUP-SUP35</td>
<td>AMP(^r)/ URA3</td>
<td>Full length SUP35 gene under (P_{CUP})</td>
<td>YCp</td>
<td>Eugene</td>
</tr>
<tr>
<td>pL-Sp-SUPNM-GFP</td>
<td>AMP(^r)/ LEU2</td>
<td>(P_{SUP35-35NM}) fused to GFP</td>
<td>YCp</td>
<td>26</td>
</tr>
<tr>
<td>pMCUP-Hsp104</td>
<td>AMP(^r)/ URA3</td>
<td>HSP104 under (P_{CUP})</td>
<td>YCp</td>
<td>Kavita</td>
</tr>
<tr>
<td>pRS316-GAL</td>
<td>AMP(^r)/ URA3</td>
<td>(P_{GAL}) inserted in polylinker of pRS316</td>
<td>YCp</td>
<td>17</td>
</tr>
<tr>
<td>pGAL104-URA3</td>
<td>AMP(^r)/ URA3</td>
<td>HSP104 fused to (P_{GAL})</td>
<td>YCp</td>
<td>35</td>
</tr>
<tr>
<td>pYS104</td>
<td>AMP(^r)/ URA3</td>
<td>(P_{HSP104-HSP104}) inserted into pRS316</td>
<td>YCp</td>
<td>6</td>
</tr>
<tr>
<td>pRS316</td>
<td>AMP(^r)/ URA3</td>
<td>Basic cloning vector</td>
<td>YCp</td>
<td>33</td>
</tr>
<tr>
<td>pmCUPNMsGFP</td>
<td>AMP(^r)/ URA3</td>
<td>SUP35-NM-GFP under (P_{CUP})</td>
<td>YCp</td>
<td>Lindquist lab</td>
</tr>
</tbody>
</table>

YEp- yeast episomal plasmid (2\(\mu\)) (high copy number)
YCp- yeast centromeric plasmid (1-3 copies per cell)

The multicopy 2\(\mu\) DNA-based LEU2 plasmid pSTR7 bearing the SUP35 gene under its own promoter, (and matching and empty vector control YEp13) have been described previously (17). pLH105 plasmid contains the HSP104 gene under the constitutively
active GPD promoter (17). pFA6a-His3MX6 is the template plasmid used for making gene deletion constructs. His3MX6 is the HIS5+ gene of Schizosaccharomyces pombe, it complements his3 mutants in S. cerevisiae (22). pmCUP1 is a base vector for copper induction. pU-CUP-SUP35 contains the full length SUP35 gene under the copper-inducible promoter. pL-Sp-SUPNM-GFP contains the NM region of SUP35 fused to GFP under the endogenous SUP35 promoter (26). pmCUP-Hsp104 contains the HSP104 gene under the copper-inducible promoter. pRS316-GAL (17) contains the galactose-inducible promoter in the basic cloning vector pRS316 (33). pGAL104-URA3 contains the HSP104 gene under the galactose-inducible promoter (35). pYS104 contains the HSP104 gene under the endogenous HSP104 promoter (6).

Antibodies

Antibody to Hsp104 was obtained from Dr. Susan Lindquist and used in 1:5000 dilution. Secondary antibody (anti-rabbit) was obtained from Sigma-Aldridge and used in 1:5000 dilution. Antibody to Sup35C was obtained from Dr. David Bedwell and used in 1:2000 dilution. Secondary antibody (anti-rabbit) was obtained from Sigma-Aldridge and used in 1:6000 dilution.

METHODS

Molecular biology techniques

Standard protocols were used for DNA electrophoresis and bacterial transformation (18).

E. coli plasmid DNA isolation

For large scale/maxi prep isolation of plasmid DNA, standard laboratory protocols were used (18). Fresh bacterial colonies were suspended in 250 ml of Luria
broth (LB) (10g/l tryptone, 5g/l yeast extract, 10g/l NaCl, pH 7.0) contained in a 1 liter flask, plus an appropriate antibiotic (i.e. Ampicillin) for plasmid selection. Cells were grown to an optical density (OD) reading of 0.8 at 550 nm (OD\textsubscript{550}). Cells were transferred to sterile plastic bottles, and pellets were collected at 7000 rpm. Pellets were suspended in 10 ml Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0), were transferred to sterile Oak Ridge tubes, and were respun at 7000 rpm. Pellets were resuspended in 4.5 ml of Solution I plus lysozyme (0.5 ml of 20mg/ml lysozyme), incubated at room temperature for 10 minutes, then were placed on ice for 20 minutes, after which 10 ml of freshly prepared Solution II (0.2N NaOH, 1% SDS) was added, and cells were returned to ice for an additional 15 minutes. 7.5 ml of sodium acetate (3 M, pH 5.0) was added and cells were incubated on ice for 1 hour. Cell debris was pelleted at 14,000 rpm at 4°C for 20 minutes. Supernatants were collected into sterile tubes, and 20 ml isopropanol was added and samples were incubated for 20 minutes at room temperature, followed by 12,000 rpm spin for 15 minutes, and a wash with 70% ethanol. Dried pellets were resuspended in 4.0 ml TE (pH 8.0) and after DNA was completely dissolved, 4 ml LiCl (9 M) was added and the samples were transferred to -20°C for at least 20 minutes. The suspension was separated by centrifugation at 12,000 rpm for 20 minutes, and supernatants were transferred to sterile tubes to which 8 ml of 95% ethanol was added. Tubes were placed on ice for 1 hour, followed by collection of pellets at 12,000 rpm for 15 minutes. DNA pellets were washed with 70% ethanol, were dried, and resuspended in TE (pH 7.4).

**Yeast and E. coli transformation procedures**

All yeast transformations were performed according to lithium-treatment procedure described previously (19, 20). All E. coli transformations were performed using chemically competent E. coli cells according to standard laboratory protocols (18).
Standard yeast media and growth conditions

Yeast cultures were grown at 30°C unless otherwise noted. Standard yeast media and standard procedures for yeast cultivation and transformation were used (20). Cells were counted using a hemacytometer (Brightline). Synthetic media lacking adenine, histidine, leucine, or uracil are designated as –Ade, -His, -Leu, and -Ura, respectively. In all cases when the carbon source is not specifically indicated, 2% glucose (Glu) was used. 2% galactose (Gal) or 2% galactose and 2% raffinose (Gal+Raf) instead of glucose was used to induce GAL promoter. Liquid cultures were grown with at least a 1/5 liquid/flask volume ratio in a shaking incubator (200-250 rpm).

Yeast DNA isolation

Plasmid and genomic DNA from yeast cultures was collected according to standard laboratory protocols (20). Briefly, cells from late log phase cultures were centrifuged at 2000 rpm, and cell pellets were resuspended in 500 ul of 1M sorbitol, 0.1 M EDTA, pH 7.5 containing 4% of a 4 mg/ml lyticase solution and were incubated at 37°C overnight. Cells were briefly spun down at 14,000 rpm, and pellets were resuspended in 500 ul of a 50 mM Tris-HCl (pH 7.4), 20 mM EDTA solution. SDS was added to a final concentration of 10%, and the samples were incubated at 65°C for 30 minutes. 0.2 ml of 5 M potassium acetate was added and samples were placed on ice for 1 hour. Following 14,000 rpm centrifugation, 0.75 ml isopropanol was added to the supernatants, samples were centrifuged at 14,000 rpm for 5 minutes. Supernatants were discarded and pellets were washed with 70% ethanol, dried, resuspended in 0.4 ml TE (pH 7.4) plus 22 µl of a 1 mg/ml solution of RNAse A, and incubated at 37°C for 30 minutes. An equal volume of chloroform: isoamylalcohol (24:1) was added, and after centrifugation at 14,000 rpm for 1 min, 1/10th volume of 3M sodium acetate (pH 5.0) was added to the top aqueous layer. DNA was precipitated with 2 volumes of 95% ethanol. Samples were
centrifuged at 14,000 rpm for 15 minutes, and pellets were washed with 70% ethanol. DNA pellets were dried and resuspended in 50 µl TE (pH 7.4) or water (if used for PCR).

**Yeast protein isolation and analysis**

Collection and analysis of yeast total protein lysates was conducted using standard yeast laboratory procedures (20, 18). Analysis of yeast total lysates was done according to previously published procedures (21), with slight modification. Yeast cells suspended in lysis buffer (100 mM Tris-HCl, pH7.5 + 200 mM NaCl + 1 mM EDTA + 5% glycerol + 0.5 mM DTT + 20 mM PMSF) were lysed with glass beads on a vortex mixer. Cell debris was removed by centrifugation at 3,000 rpm to produce a “total lysate” fraction. Resulting samples were heated at 95°C for 10 min and run on the standard SDS-polyacrylamide gel. For performing the protein assays, gels were transferred onto Hybond ECL nitrocellulose membranes and reacted to the appropriate antibodies. Densitometry measurements were obtained from exposed films using the program Alphaimager 2000, Alpha Innotech Corporation.

**Nonsense suppression assay for presence of \([PSI^+]\)**

Presence of \([PSI^+]\) was detected by its ability to suppress the \(ade1-14_{UGA}\) mutant allele, as described previously (8). The \([\psi i]\) \(ade1-14_{UGA}\) strains are not able to grow on medium lacking adenine (-Ade) and exhibit dark red color on rich (YPD) medium, while \([PSI^+]\) \(ade1-14_{UGA}\) strains are able to grow on –Ade and exhibit white or light-pink color on YPD.

**Assay to monitor for presence of \([PIN^+]\)**

The \([PIN^+]\) prion induces de novo formation of \([PSI^+]\) in the presence of Sup35 overproduction (4). \([PIN^+]\) is the prion form of the Rnq1 protein. The presence of \([PIN^+]\) was monitored by the following assay: \([\psi i]\) strains were mated to the strain \([\psi i\ psi^- pin^-]\)
GT234 bearing the multicopy SUP35 plasmid pSTR7 (or YEpl3 empty vector control). The \([PIN^+]\) diploids, in contrast to \([pin^-]\) diploids, grew on medium lacking adenine after 10-14 days of incubation due to \textit{de novo} induction of \([PSI^+]\).

**Thermotolerance assay**

Thermotolerance assay was done as described previously (6). 20 ml cultures were grown in 125 ml flasks in SD+13 or YPD media at 25°C for two hours to reach an OD of 0.8-1.0. The control set was directly kept on ice, the basal thermotolerance set was grown at 25°C for another 30 min, and the induced thermotolerance set was incubated at 37°C for 30 min. 500 µl aliquots each of both the thermotolerance sets were then incubated in 50°C water bath for 5, 10, 15 and 20 min and immediately moved to ice. 4 µl of cells from all the sets were then spotted on YPD plates in serial dilutions as \(10^0\) (undiluted), \(10^{-1}\) (diluted 10 times), \(10^{-2}\) and \(10^{-3}\). Image was taken after 2 days of growth at 30°C.

**SDD-AGE**

Semi-Denaturing Detergent Agarose Gel Electrophoresis (SDD-AGE) for the analysis of prion polymer sizes was performed as described previously (25) with slight modifications. 2-3 ml precultures were grown overnight in Oakridge tubes and diluted to \(10^6\) cells/ml cultures in flasks maintaining a 1:5 ratio between culture volume and the flask volume. The cultures were grown at 30°C with shaking at 200rpm or higher till the OD reached the exponential phase specific to the strain (refer to growth curves in Fig. 5.1). The cells were collected at 3000 rpm for 10 min and washed in 300 µl of complete buffer (1 Roche complete tablet in 2ml of sterile H₂O). The cells were frozen at -80°C for 20 min to break the cell wall and then resuspended in an appropriate volume of lysis buffer (25 mM Tris- HCl pH 7.5, 100 mM NaCl, 1 mM DTT, 10mM EDTA, 2 mM PMSF and proteinase inhibitors) depending on the size of the pellet. After adding 1/3 volume of acid-washed glass beads, the cells were vortexed 9 times at high speed for 20
sec each time (total of 3 min) with 60 sec on ice between vortexing. This was followed by centrifugation at 3000 rpm for 2 min. The supernatant was taken and protein concentration was measured using Bradford assay. ~120μg of protein was mixed with 1/3 vol. of 4X sample buffer (0.5x TAE (40 mM Tris-acetate, 1 mM EDTA), 2% SDS, 5% Glycerol and Bromophenol blue) and incubated at 37°C for 10 min. After incubation, the protein sample was loaded on the SDD-AGE gel (horizontal 1.8% agarose gels in 1X Tris-acetate-EDTA buffer with 0.1% SDS). Electrophoresis was performed using the running buffer (1X Tris-acetate-EDTA, 0.1% SDS) at 70-80 volts for 1 hour. Then, the gel was run at 90 volts until the dye reached the bottom. Western blotting was done using PVDF membrane using the tank electroblotting system (Biorad). Before transfer, the PVDF was incubated for 2-5 min in methanol and then in transfer buffer (15% methanol, 0.08% SDS, 5.8 g/L Tris, and 2.8 g/L Glycine) two times for 5 min each. Also, the gel was incubated in transfer buffer for 2 X 15 min on the shaker before the transfer. The electrophoresis and transfer were performed at 4°C.

**Pringle method for gene deletion and tagging**

For making gene deletions with a *HIS* marker, plasmid pFA6a-His3MX6 was used as described previously (22). His3MX6 is the *HIS5*+ gene of *Schizosaccharomyces pombe*, it complements his3 mutants in *Saccharomyces cerevisiae*. A construct for making gene deletion was prepared by using a template DNA. PCR was performed using this template and a pair of primers which contain regions which are homologous to the flanking regions of the gene of interest (Fig. 3.1). After transformation, gene deletion takes place due to homologous recombination with this construct. As shown in Fig. 3.2, gene deletion was identified on 0.8% agarose gel after doing PCR using another set of primers. The sequences of primers for making and checking *GET* gene deletions are shown in Table 2.3:
<table>
<thead>
<tr>
<th>Table 2.3 List and description of primers used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GET1</strong></td>
</tr>
<tr>
<td>Deletion* F: 5’AGTACTCGTGACCAAATCTG3’</td>
</tr>
<tr>
<td>R: 5’GGCTAGATCATCCACTCTTTT3’</td>
</tr>
<tr>
<td>Checking F: 5’GCAATCCTTTGAACTACGTCT3’</td>
</tr>
<tr>
<td>R: 5’AAAAGATTTGGAGACGGAGGA3’</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>GET2</strong></td>
</tr>
<tr>
<td>Deletion F: 5’CTCTTCCATGTTTGTAGCATCAGCAACGTAGCTCTAGGAACGGATCC</td>
</tr>
<tr>
<td>CCGGGTTAATTA3’</td>
</tr>
<tr>
<td>R: 5’CCTG AAAAGAAAGCCGGGAATAATGTCGGGTTATGAGAAACGAATTCC</td>
</tr>
<tr>
<td>GAGCTCGTTAAAC3’</td>
</tr>
<tr>
<td>Checking F: 5’CGTTTGCTGGGACAAAAAGAA3’</td>
</tr>
<tr>
<td>R: 5’GATTTTTTCACGAACTCATCGC3’</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>GET3</strong></td>
</tr>
<tr>
<td>Deletion F: 5’AAACGTACGACAAGAACAAGAAGATCATCACATTGTAATTCCGGATC</td>
</tr>
<tr>
<td>CCGGGTTAATTA3’</td>
</tr>
<tr>
<td>R: 5’TATATATGTCGTATGTATCTATTTTATGGTATTGCAGGGGCTTGAAATTC</td>
</tr>
<tr>
<td>GCTCGTTAAAC3’</td>
</tr>
<tr>
<td>Checking F: 5’AAACGTACGACAAGAACAAG3’</td>
</tr>
<tr>
<td>R: 5’CACACACATACCATCGTATT3’</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>GET2</strong></td>
</tr>
<tr>
<td>Tagging F: 5’TTCGTTTGTGCTAATTGTCTTAGCTACTTAGGAGCTTACGGAGATCC</td>
</tr>
<tr>
<td>CCGGGTTAATTA3’</td>
</tr>
<tr>
<td>R: 5’TTATGAAACAAATGTATTATAATCTGAACATTATCTAGGAATTCGAGCTCGTTAAAC3’</td>
</tr>
<tr>
<td>Checking F: 5’GAGGCGGAAAAGCGCAGGTT3’</td>
</tr>
<tr>
<td>R: 5’GCCGGGAATAATGTCGGGTT3’</td>
</tr>
</tbody>
</table>
*: The template DNA for making the deletion construct was the genomic DNA of strain BY4741 which contains a deletion in the \textit{GET1} gene.

**: For making tagged \textit{GET2} constructs, plasmids pFA6a-GFP(S65T)-HIS3MX6 and pFA6a-3HA-His3MX6 were used for placing GFP and HA tags respectively in frame at the 3’ end of the \textit{GET2} gene as described previously (22).

\textbf{Fluorescence microscopy}

Cells from liquid cultures or plates were taken directly and applied to slides for immediate visualization. Cells on slides were sealed with a coverslip using clear nail polish. The samples were scanned using an Olympus BX41 fluorescence microscope and image analysis was conducted using the Olympus DP71 camera.

The behavior of Sup35-GFP fusion proteins is closely linked to the existing prion state of the cell. 100 μm CuSO₄ was used to induce expression of \textit{CUP1}-regulated constructs. Whenever the frequency of cells containing GFP aggregates was tabulated, at least 100 cells were counted in each case.
CHAPTER 3

EFFECT OF THE OTHER COMPONENTS OF THE GET COMPLEX (\textit{GET1} AND \textit{GET3}) ON $[\text{PSI}^+]$ STABILITY

Since \textit{get2} was shown to have an effect on $[\text{PSI}^+]$ stability (S. Muller, J. Patterson and Y. Chernoff, unpublished data), deletions were made in the other components of the Get complex i.e. \textit{GET1} and \textit{GET3} to investigate whether they too influence the stability of $[\text{PSI}^+]$ prion. Deletions were made in the OT56 background using the Pringle method (22) as explained in Chapter 2. The sequences of primers are given in Table 2.3. A schematic description is given in Fig. 3.1.

![Pringle method for making GET gene deletions](image)

**Figure 3.1 Pringle method for making GET gene deletions**
The procedure for making gene deletions is carried out in two steps. A. A construct for making gene deletion is prepared by using a template plasmid pFA6a-His3MX6 (Table 2.2) or genomic DNA from BY4741 (Table 2.1). PCR is performed using this template and a pair of primers which are homologous to the plasmid or genomic DNA (both of which contain a marker) and contain regions which are homologous to the flanking regions of the gene of interest. These primers are listed in Table 2.3 as deletion primers for different genes. B. This PCR product is transformed into the wild type strain. Homologous recombination takes place with the flanking regions of the gene of interest as a result replacing it with the marker. The disruptants are selected on an appropriate selective medium. The presence of gene deletion can be confirmed by doing PCR with primers listed in Table 2.3 as checking primers for different genes.
A construct for making gene deletion is prepared by using a template DNA. PCR is performed using this template and a pair of primers which contain regions which are homologous to the flanking regions of the gene of interest. After transformation, gene deletion takes place due to homologous recombination with this construct. As shown in Fig. 3.2, gene deletion can be identified on 0.8% agarose gel after doing PCR using another set of primers (chapter 2). Using primers to detect \textit{get1} gene deletion (Fig. 3.2A), wild type shows a 0.9 kb band whereas a deletion shows 1.88 kb band. As a control, genomic DNA from the collection strain used for creating the gene deletion construct (chapter 2) was also checked by PCR. It is an aneuploid and shows both wild type and deletion bands. For detecting \textit{get3} gene deletion using plasmid pFA6a-His3MX6 (Fig. 3.2B) another set of primers were used showing wild type (1.18 kb) and deletion (1.43 kb) bands of different sizes.

![Figure 3.2](image)

\textit{Figure 3.2} The confirmation of \textit{get1} and \textit{get3} deletion constructions in strong [\textit{PSI}^+] background by PCR

The strong [\textit{PSI}^+] strain (OT56) was transformed with PCR products having homologous regions to the genes of interest. These products were prepared by amplifying \textit{GET1} gene from the genome of the \textit{get1}\Delta strain found in the collection of yeast gene deletion strains (see materials and methods and fig. 3.1) in (A), and pFA6a-His3MX6 plasmid (see materials and methods and Fig. 3.1) in (B). Disruption of genes was checked using another set of primers and confirmed by the fragment sizes on 0.8% agarose gel.
Curing defect in the presence of excess Hsp104

First, it was studied whether there was any curing defect in these strains in the case of Hsp104 over expression. The get1Δ and get3Δ strains along with the wild type control i.e. OT56 were transformed with plasmid pLH105 (Table 2.2). This plasmid contains HSP104 gene under the constitutively active P_{GPD} promoter. The transformants were selected and patched on –Leu media. These plates were then replica-plated to –Ade and –Leu-Ade media to detect the curing of [PSI⁺] prion by lack of growth on medium lacking adenine (as explained in chapter 2).

As seen in Fig. 3.3, in the case of both get3 and get1 gene deletions [PSI⁺] loss is decreased in the presence of excess Hsp104. This effect is similar to the effect previously observed for get2 deletion. This experiment was also done with another plasmid where HSP104 is under the Galactose inducible promoter (pGAL104-URA3) and similar results were obtained (not shown here).

Figure 3.3 [PSI⁺] curing defect by excess Hsp104 is observed in get3Δ and get1Δ
A. Both wild type and get3Δ strains were transformed with pLH105 (P_{GPD}-HSP104) or control plasmids. The transformants were patched on –Leu and replica plated to –Leu-Ade media. Image was taken after ~10 days of incubation. Wild type strain is cured of the [PSI⁺] prion by Hsp104 overexpression as seen by the lack of growth on media lacking Ađenine. On the other hand, the isogenic get3Δ strain is not cured by excess Hsp104 and shows a [PSI⁺] curing defect. B. The experiment described in A was repeated for wild type and get1Δ strains. Curing is observed in wild type whereas in some of the transformants of get1Δ, a curing defect is observed.
Spontaneous loss of \([PSI^+]\) on synthetic media in the get1 and get3 deletion strains

Experiments were also performed to analyze \([PSI^+]\) maintenance in the get1Δ and get3Δ strains through incubation on complete synthetic medium. The strains were streaked out on complete synthetic medium and several colonies were patched on another synthetic medium plate. This plate was then replica-plated to another synthetic medium plate and to YPD. Also, several colonies from the replica-plated synthetic medium plate were streaked out on YPD. In parallel, serial passages were also performed on YPD as a control. After such serial passages on synthetic media, the number of \([psi^-]\) colonies was estimated based on red or dark pink color on YPD. Preliminary results suggest that some \([PSI^+]\) instability is observed in the get3Δ strain as well, but not at the same level as seen in the get2Δ. However, get1Δ does not cause any \([PSI^+]\) instability (Fig. 3.4 and table 3.1). More sensitive experiments are needed to analyze the effect of deletion of GET1 and GET3 genes on \([PSI^+]\) stability.

Figure 3.4 Effect of get1Δ and get3Δ on \([PSI^+]\) stability
The strains were streaked out on YPD after growth on complete synthetic medium (~3-4 days). Image was taken after 2 days of incubation on YPD. \([PSI^+]\) loss was studied by the appearance of red colonies on YPD which spontaneously lose \([PSI^+]\). get1Δ does not cause any \([PSI^+]\) instability whereas get3Δ causes some instability on synthetic medium. See table 3.1.
Table 3.1 Effect of get1Δ and get3Δ on [PSI+] stability

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ade+</th>
<th>Ade-</th>
<th>Total</th>
<th>% Ade-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>get1Δ</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>get3Δ</td>
<td>100</td>
<td>14</td>
<td>114</td>
<td>12.3</td>
</tr>
</tbody>
</table>

See Fig. 3.4

Conclusion

Deletion of get3 or get1 exhibits a defect in [PSI+] curing by excess Hsp104 similar to that in get2 mutants. The above data suggests that the Get complex affects prion maintenance not only through Get2 but through a common function of the Get complex. There might be some effect of get3 on [PSI+] stability as well, as suggested by preliminary data. However, the instability observed is not as strong and more sensitive experiments are needed to study the same.
CHAPTER 4

EFFECT OF GET2 ON PRIONS OTHER THAN [PSI⁺]

Since get2 affects [PSI⁺] maintenance (S. Muller, J. Patterson and Y. Chernoff, unpublished data), it was interesting to study whether it has an effect on the stability of other prions as well.

Spontaneous loss of [PIN⁺] in the get2 deletion strain

Experiments were conducted to study the maintenance of [PIN⁺] prion in get2Δ strain through incubation on complete synthetic medium as explained in Chapter 3. After incubation on synthetic medium, the number of [psi⁻] colonies was estimated based on red or dark pink color on YPD. These [psi⁻] colonies were saved on complete medium for [PSI⁺] induction test as described in Chapter 2 and Fig. 4.1A. As shown in the Table 4.1 and Fig 4.1B, the proportion of [pin⁻] colonies was more as compared to those that were [PIN⁺].

Figure 4.1 Get2 deletion strain exhibits spontaneous loss of [PIN⁺] prion

A. The scheme of the [PSI⁺] induction test used to determine the presence of [PIN⁺] prion in the [psi⁻] get2 deletion strain. This strain picked up from various time points of the serial passage experiment, is mated on rich media (YPD) with a [psi⁻ pin⁻] strain containing pSTR7 (multicopy SUP35) plasmid. After selecting for diploids on synthetic medium selective for diploids, the plate was replica plated to synthetic medium without adenine selective for diploids. Image was taken after ~10 days and the presence of [PIN⁺] was detected through the de novo induction of [PSI⁺] which leads to growth on media lacking adenine. B. Get2 deletion strain was incubated on complete synthetic media for several passages. From each time point several [psi⁻] colonies were picked up and used in [PSI⁺] induction test to determine their [PIN⁺] status. More [psi⁻] colonies were [pin⁻] as compared to [PIN⁺]. This was determined by the lack of growth of the diploid on media lacking adenine indicating that no [PSI⁺] induction took place. Image was taken after ~10 days.
Table 4.1 Get2 deletion strain exhibits spontaneous loss of \([PIN^+]\) prion

<table>
<thead>
<tr>
<th>Round of incubation</th>
<th>([psi-]) ([PIN^+]) colonies tested</th>
<th>([PIN^+])</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>9</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>6</td>
<td>15</td>
</tr>
</tbody>
</table>

See figure 4.1 for experimental scheme.

**De novo induction of \([PSI^+]\)**

Also, *de novo* induction of \([PSI^+]\) in the \([psi- PIN^+]\) get2Δ colonies was studied. \([psi- PIN^+]\) wild type and \([psi PIN^+]\) get2Δ were transformed with pU-CUP\(\cdot\)SUP35 (SUP35 under copper promoter) or control plasmids. Transformants were selected and patched on -Ura and incubated for ~4 days. This was followed by replica plating to -Ura plates with or without 100 µM CuSO4. After incubation for ~4 days, *de novo* induction of \([PSI^+]\) was analyzed by growth on medium lacking adenine. *De novo* \([PSI^+]\) induction was not affected in get2Δ as compared to wild type (Fig. 4.2).

![Image of growth plates showing induction of \([PSI^+]\)]

**Figure 4.2 De novo induction of \([PSI^+]\) is not affected in get2Δ**

\([psi PIN^+]\) wild type and \([psi PIN^+]\) get2Δ were transformed with pU-CUP\(\cdot\)SUP35 (SUP35 under copper promoter) or control plasmids. Transformants were selected and patched on -Ura and incubated for ~4 days. This was followed by replica plating to -Ura plates with or without 100 µM CuSO4. After incubation for ~4 days, these plates were then replica plated to synthetic medium selective for the plasmids and lacking adenine. Images were taken after ~10 days of incubation. *De novo* induction of \([PSI^+]\) is seen by growth on medium lacking adenine. Induction is not affected in get2Δ as compared to wild type.
Effects of get2 and get3 deletions in a weak \([PSI^+]\) background

Get2 and get3 genes were also deleted in the weak \([PSI^+]\) background i.e. OT55. OT55 was transformed with PCR products having homologous regions to the genes of interest. This product was prepared by using pFA6a-His3MX6 template plasmid and primers (see materials and methods and Fig. 3.1). Disruption of genes was checked using another set of primers and confirmed by the fragment sizes on 0.8% agarose gel. The fragment sizes for get2Δ were 1.1 kb (wild type) and 1.53 kb (deletion) whereas the fragment sizes for get3Δ were 1.18 kb (wild type) and 1.44 kb (deletion) (Fig. 4.3).

These deletions were analyzed for the stability of \([PSI^+]\) through incubation on complete synthetic medium as described in chapter 3. However, get2 and get3 deletions do not have any effect on \([PSI^+]\) stability in a weak \([PSI^+]\) background (Fig. 4.4 and table 4.2).
Figure 4.4 Get2 and get3 deletions do not have an effect in weak [PSI+] background
The strains were streaked out on YPD after incubation on complete synthetic medium (~3-4 days). Images were taken after 2 days of incubation on YPD. [PSI+] is stably maintained in both get2 and get3 deletions in a weak [PSI+] background as seen by the absence of red colonies which depict loss of [PSI+] prion. See table 4.2

Table 4.2 Effects of get2Δ and get3Δ on [PSI+] stability in weak [PSI+] background

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pink (Ade+) colonies</th>
<th>Red (Ade-) colonies</th>
<th>Total colonies</th>
<th>% Ade-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (weak [PSI+])</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>get2Δ</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>get3Δ</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ade+</th>
<th>Ade-</th>
<th>Total</th>
<th>% Ade-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (strong [PSI+])</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>get2Δ</td>
<td>50</td>
<td>46</td>
<td>96</td>
<td>47.9</td>
</tr>
<tr>
<td>get3Δ</td>
<td>100</td>
<td>14</td>
<td>114</td>
<td>12.3</td>
</tr>
</tbody>
</table>

See Fig. 4.4

As shown in table 4.2, get2 and get3 deletions do not have any effect on [PSI+] stability in a weak [PSI+] background. However, as discussed in chapters 1 and 3 respectively, get2 and get3 deletions affect [PSI+] stability in a strong [PSI+] background.

Conclusion

Get2 causes instability of both [PSI+] and [PIN+] prions on synthetic medium in a strong [PSI+] background. De novo [PSI+] induction is not affected in get2Δ. Also, get2...
and get3 deletions do not have any effect on $[PST^+]$ stability in a weak $[PST^+]$ background.
CHAPTER 5

DOES GET2 OPERATE BY ALTERING/MODULATING HSP104 LEVELS AND/OR ACTIVITY?

Hsp104 levels

Since Hsp104 plays a role in prion propagation (Fig. 1.3), it was studied whether the effect of get2 on prion maintenance is due to an alteration in the levels of the molecular chaperone Hsp104. Total protein extracts were prepared from wild type OT56, get2Δ and get2-473 strains grown to the exponential and stationary phases of growth. Growth curves of these strains were obtained first by preparing 10^6 cells/ml cultures in complete synthetic medium and recording the OD readings approximately every two hours. (Fig. 5.1).

Figure 5.1 Growth curves for wild type and get2 mutants
10^6 cells/ml cultures of wild type, get2-473 and get2Δ strains were made in complete synthetic medium and growth curves were obtained by recording OD values approximately every two hours. At 16-hour time point, all three cultures were in exponential phase and protein extracts for Western blotting were prepared at this time point.
As seen in the figure, at the 16-hour time point all the strains were in the exponential phase of growth and reached the stationary phase at around 48 hours. After running the samples in polyacrylamide gel electrophoresis, Western Blotting was performed and the levels of Hsp104 were detected using antibody to Hsp104 as described in Materials and Methods. Fig. 5.2A shows the protein levels among the three strains. Endogenous Hsp104 levels show an increase in the get2-473 and get2Δ strains as compared to those found in wild type. Ade 2 was used as a loading control.

After normalizing with the levels of Ade2 protein, it was found that the Hsp104 levels in the get2 mutant strains were not decreased as compared to the wild type strain in the stationary phase either (not shown here).

Experiments were also done to compare the Hsp104 levels among the three strains in case of overexpression of this protein in the cell. All three strains were transformed with Hsp104 overexpression (pLH105) or control (YEp13) plasmids as described in table 2.2. Total protein extracts were prepared and the levels of Hsp104 were detected after Western blotting. Fig. 5.2B shows the Hsp104 levels in all the combinations. The fold induction of Hsp104 as compared to control for each strain are shown. Get2Δ strain shows significant increase in induction levels of Hsp104 as compared to wild type. Get2-473 strain also shows an increase in Hsp104 induction levels.
Figure 5.2 The [PSI+] curing and maintenance defects in the get2 deletion and mutant strains are not due to a decrease in the Hsp104 levels

A. Wild type, get2-473 and get2Δ strains were grown in complete synthetic medium (SD+13) and total protein extracts were prepared from 10⁶ cells/ml cultures grown to exponential phase (~16 hours) as shown in figure 5.1. The protein samples were run in polyacrylamide gel electrophoresis and Western blotting was done using Hsp104 antibody. Endogenous Hsp104 levels show an increase in the get2-473 and get2Δ strains as compared to those found in wild type. Ade 2 was used as a loading control. The relative levels as shown by numbers below the blots were determined by densitometry.

B. Wild type, get2-473 and get2Δ strains were transformed with pLH105 (PGPD-HSP104) or control plasmids and grown in –Leu media. Total protein extracts were prepared from cells grown to exponential phase and run in polyacrylamide gel electrophoresis. Western blotting was done using Hsp104 antibody. Get2Δ shows a significant increase in over expressed Hsp104 levels as compared to wild type.

Hsp104 activity analyzed by thermotolerance assay

Experiments were performed to analyze the activity of Hsp104 through Thermotolerance assay as described in Chapter 2. The mutants get2-473 and get2Δ were analyzed along with the wild type strain in the Thermotolerance assay as described in Fig. 5.3. The strains were grown at 37°C for 30 min as a mild pre-heat treatment. After this they were given heat shock at 50°C for 5, 10, 15 and 20 min and spotted on complete medium plates to assess their viability.
Figure 5.3 The experimental scheme for thermotolerance assay

Wild type, get2-473 and get2Δ strains were grown in complete synthetic medium or YPD at 25°C for two hours to reach an OD of 0.8-1.0. The control set (a) was directly kept on ice, the basal thermotolerance set (b) was grown at 25°C for another 30 min, and the induced thermotolerance set (c) was incubated at 37°C for 30 min. Sets b and c were then incubated at 50°C for 10 min. 4 µl of cells from all the sets were then spotted on YPD in serial dilutions as 10^0 (undiluted), 10^-1 (diluted 10 times) and so on. Image was taken after 2 days of growth at 30°C.

Since the molecular chaperone Hsp104 is induced during the pre-heat treatment, it helps in the disaggregation of aggregated proteins during the heat shock treatment and thus increases cell survival. This is a measure of induced thermotolerance. As a measure of basal thermotolerance, no pre-heat treatment was given before the heat shock at 50°C. As seen in Fig. 5.4, in the complete synthetic medium, basal thermotolerance (partly Hsp104 dependent) is increased in the mutants as compared to wild type. Induced thermotolerance (strictly Hsp104 dependent) in the get2-473 and get2Δ strains is comparable to the wild type. In the rich medium, induced thermotolerance in the get2-473 and get2Δ stains is comparable to the wild type. Basal thermotolerance is comparable in all the three strains but is lower as compared to that in complete synthetic medium.
The [PSI+] curing and maintenance defects in the get2 deletion and mutant strains are not due to a decrease in the activity of Hsp104. Thermotolerance assay was done in complete synthetic medium as described in Fig. 5.3. Image was taken after 2 days of growth at 30°C. Basal thermotolerance (partly Hsp104 dependent) is increased in the mutants as compared to wild type (b). Induced thermotolerance (strictly Hsp104 dependent) in the get2-473 and get2Δ strains is comparable to the wild type as seen in (c).

B. Thermotolerance assay was done in YPD as described in Fig. 5.3. Image was taken after 2 days of growth at 30°C. As seen in c, induced thermotolerance in the get2-473 and get2Δ stains is comparable to the wild type. Basal thermotolerance is comparable in all the three strains (b) but is lower as compared to that in complete synthetic medium (A-b).

Conclusion

Since the Hsp104 levels are not decreased in the mutants as compared to wild type, the curing and maintenance defects in the get2 mutants is not due to a decrease in Hsp104 levels. Also, as seen in the thermotolerance assay, the activity of Hsp104 is not decreased in the get2 mutants as compared to the wild type. Thus the curing and maintenance defects are not due to a decrease in the activity of Hsp104 either. On the
other hand, basal thermotolerance in synthetic medium is increased in the mutants as compared to wild type. This corresponds to the results for the Hsp104 protein levels as seen by Western Blotting. The Hsp104 protein levels are higher in the mutants as compared to wild type.
CHAPTER 6

COMPARISON OF THE $[\text{PSI}^+]$ AGGREGATE PATTERNS AND SIZES BETWEEN $\text{GET2A}$ MUTANT AND WILD TYPE

Analysis of aggregate sizes using SDD-AGE

Since neither Hsp104 levels nor its activity are seen to be affected in the $\text{get2A}$ strain, the next question to be asked was regarding the $[\text{PSI}^+]$ aggregate size in the $\text{get2A}$ strain. As the $\text{get2A}$ strain shows some defects in prion maintenance, it was worth checking whether the $[\text{PSI}^+]$ aggregates show any difference with respect to size as compared to that in wild type. Total protein extracts were prepared from the wild type and $\text{get2A}$ strains and SDD-AGE was performed as described in materials and methods. Fig. 6.1A shows that the range of aggregate sizes is wider, and there are more of aggregates of larger sizes in $\text{get2A}$ strain as compared to wild type. This experiment was repeated four times and similar results were obtained. Also, the result was unchanged when an additional high speed centrifugation (100,000g) was performed on the protein samples. The supernatant was then run on SDD-AGE. The huge aggregates of the $\text{get2A}$ strain are not pulled down at high speed of centrifugation (Fig. 6.1B).
Figure 6.1 The $[\text{PSI}^+]$ polymer size is increased in the get2Δ strain as compared to the wild type

A. Wild type and get2Δ strains were grown in complete synthetic medium till the late exponential phase and total protein extracts were prepared as described in Materials and Methods. After normalizing by Bradford assay, ~120µg of protein with 2% SDS was loaded on SDD-AGE. After transferring the proteins to PVDF membrane, immunoblotting was performed using Sup35C antibody. The range of aggregate sizes is wider, and there is more of aggregates of larger sizes in get2Δ strain as compared to wild type. This experiment was repeated four times and similar results were obtained. B. The result was unchanged when an additional high speed centrifugation (100,000g) was performed on the lysates after adding 2% SDS to them. This increased the final concentration of SDS to 4% after adding the loading buffer, as compared to only 2% in A. The supernatant was then run on SDD-AGE. The huge aggregates of the get2Δ strain are not pulled down at high speed. C. Wild type, get2Δ and get2-473 strains were transformed with plasmid containing HSP104 gene under endogenous promoter or control plasmid. 10^6 cells/ml cultures were grown in –Ura media for ~24 hours and total protein extracts were prepared. SDD-AGE was performed using the protein lysates and immunoblotting was done using Sup35C antibody. The aggregates are bigger in wild type expressing excess Hsp104 as reported previously (Kryndushkin et al. 2003) and as discussed in chapter 6. The effect in get2Δ appears to be in the same direction. The variability in the experiment is discussed in chapter 6.

The aggregate sizes were also compared in the presence of excess Hsp104. Wild type, get2Δ and get2-473 strains were transformed with HSP104 under its endogenous promoter (pYS104) or control (pRS316) plasmids. The cultures were grown in selective medium and SDD-AGE was performed on total protein extracts (Fig. 6.1C). Wild type strain over expressing Hsp104 showed bigger aggregates as compared to wild type strain containing the control plasmid. This result agrees with that reported previously (25) that when Hsp104 is over expressed, the bigger aggregates resistant to Hsp104 action are selected for since they grow faster with an increasing pool of monomers. Get2Δ strain showed results in the same direction as wild type indicating that the difference in aggregate size is not due to ineffective action of Hsp104. In get2-473 mutant, clear difference from wild type was not seen. Also, decreased range of polymer distribution in
get2Δ in Fig. 6.1C, compared to 6.1A and B, is probably due to less amount of protein loaded. However, the results were not completely reproducible due to the inherent variation in the experiment. This is possible due to the loss of plasmid from some cells during growth. These cells contribute to the total protein extract and cause variation in the experiment. Another reason could be the loss of [PSI+] prion from some of the cells. More experiments are needed, possibly using galactose- inducible promoter for HSP104 expression so that over expression of Hsp104 can be regulated for specific time-points to see the effect on aggregate sizes.

To study whether combining the wild type and mutant samples had any effect, another SDD-AGE experiment was performed (scheme described in Fig. 6.2A). 2% SDS was added to the protein extract from wild type which was then centrifuged at 100,000g and the supernatant was run on SDD-AGE. The protein extract from the [psi] get2Δ strain was centrifuged at 100,000g and the pellet was combined with the supernatant of the wild type strain. This combination was run on SDD-AGE and western blotting was done using the Sup35C antibody. As seen in Fig. 6.2B, the bigger aggregates of the get2Δ strain are not reconstituted in trans; the combination shows the same [PSI+] aggregate size as wild type.
Figure 6.2 The bigger aggregates of the get2Δ strain are not reconstituted in trans
A. 2% SDS was added to the protein extract from wild type which was then centrifuged at 100,000g and the supernatant was run on SDD-AGE. The protein extract from the [psi] get2Δ strain was centrifuged at 100,000g and the pellet was combined with the supernatant of the wild type strain. This combination was run on SDD-AGE and western blotting was done using the Sup35C antibody. B. The experiment was performed as described in A. The bigger aggregates of the get2Δ strain are not reconstituted in trans; the combination shows the same [PSI+] aggregate size as the wild type.

Analysis of aggregates using fluorescence microscopy

Also, fluorescence microscopy experiment was performed to compare the pattern of aggregates between wild type and get2-473 mutant. The strains were transformed with pmCUPNMsGFP plasmid (SUP35NM-GFP under copper inducible promoter) and high Hsp104 (pGAL104-URA3) or control plasmids. Transformants were selected on selective medium and replica plated to -Ura-His/Gal + CuSO4 medium. Cells were taken directly from plates and observed under fluorescence microscope after 5 and 10 days of induction. In general, the percentage of cells containing visible aggregates was much lower in get2-473 strain as compared to wild type (Tables 6.1 and 6.2). Also, more than 50% of get2-473 aggregates in the presence of excess Hsp104 were rings/bars, which is significantly higher than that observed in wild type.
Table 6.1 Analysis of [\textit{PSI}^+] aggregate pattern in solid medium in \textit{get2-473} mutant as compared to wild type

<table>
<thead>
<tr>
<th>Strain + Plasmid</th>
<th>No. of fluorescing cells</th>
<th>No. of cells with visible aggregates</th>
<th>% of cells with visible aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT+ CUP-Sup35-GFP + control</td>
<td>108</td>
<td>52</td>
<td>48.1</td>
</tr>
<tr>
<td>WT + CUP-Sup35-GFP + high Hsp104</td>
<td>130</td>
<td>48</td>
<td>36.9</td>
</tr>
<tr>
<td>\textit{get2-473}+ CUP-Sup35-GFP + control</td>
<td>40</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>\textit{get2-473}+ CUP-Sup35-GFP + high Hsp104</td>
<td>52</td>
<td>7</td>
<td>13.4</td>
</tr>
</tbody>
</table>

The data was recorded after 5 days of induction

Table 6.2 Analysis of [\textit{PSI}^+] aggregate pattern in solid medium in \textit{get2-473} mutant as compared to wild type

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>No. of fluorescing cells</th>
<th>No. of cells with visible aggregates</th>
<th>% of cells with visible aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT + CUP-Sup35-GFP + control</td>
<td>139</td>
<td>31</td>
<td>22.3</td>
</tr>
<tr>
<td>WT + CUP-Sup35-GFP + high Hsp104</td>
<td>100</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>\textit{get2-473}+ CUP-Sup35-GFP + control</td>
<td>116</td>
<td>12</td>
<td>10.3</td>
</tr>
<tr>
<td>\textit{get2-473}+ CUP-Sup35-GFP + high Hsp104</td>
<td>106</td>
<td>37</td>
<td>34.9</td>
</tr>
</tbody>
</table>

The data was recorded after 10 days of induction

This experiment was also performed in liquid medium repeating the transformations as above. 10^6 cells/ml cultures were prepared in –Ura-His/Gal-Raf + 100\mu M CuSO_4 and cells were observed under the fluorescence microscope after 24 and 48 hours of induction. In general, the percentage of cells containing visible aggregates was much lower in \textit{get2-473} strain as compared to wild type (Tables 6.3 and 6.4). Rings/bars were not observed in liquid medium.
Table 6.3 Analysis of [PSI⁺] aggregate pattern in liquid medium in get2-473 mutant as compared to wild type

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>No. of fluorescing cells</th>
<th>No. of cells with visible aggregates</th>
<th>% of cells with visible aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT + CUP-Sup35-GFP + high Hsp104</td>
<td>113</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>WT + CUP-Sup35-GFP + control</td>
<td>101</td>
<td>11</td>
<td>10.8</td>
</tr>
<tr>
<td>get2-473+ CUP-Sup35-GFP + high Hsp104</td>
<td>102</td>
<td>7</td>
<td>6.8</td>
</tr>
<tr>
<td>get2-473+ CUP-Sup35-GFP + control</td>
<td>100</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Two transformants were analyzed for each combination after 24 hours of induction.

Table 6.4 Analysis of [PSI⁺] aggregate pattern in liquid medium in get2-473 mutant as compared to wild type

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>No. of fluorescing cells</th>
<th>No. of cells with visible aggregates</th>
<th>% of cells with visible aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT + CUP- Sup35-GFP + high Hsp104</td>
<td>106</td>
<td>75</td>
<td>70.7</td>
</tr>
<tr>
<td>WT + CUP- Sup35-GFP + control</td>
<td>102</td>
<td>21</td>
<td>20.5</td>
</tr>
<tr>
<td>get2-473+ CUP- Sup35-GFP + high Hsp104</td>
<td>105</td>
<td>29</td>
<td>27.6</td>
</tr>
<tr>
<td>get2-473+ CUP- Sup35-GFP + control</td>
<td>103</td>
<td>17</td>
<td>16.5</td>
</tr>
</tbody>
</table>

Two transformants were analyzed for each combination after 48 hours of induction.

Another fluorescence microscopy experiment was performed on cells expressing SUP35-GFP under its endogenous promoter. However, no systematic difference was observed between the mutant and wild type (data not shown).
Conclusion

The range of aggregate sizes is wider, and there are more of aggregates of larger sizes in get2Δ strain as compared to wild type. Also, these large aggregates are not pulled down at the high centrifugation speed of 100,000g.
CHAPTER 7
MODULATION OF THE EFFECTS OF GET2 DEFICIENCY ON PRION BY CALCIUM CONCENTRATIONS

Effect of calcium on \([PSI^+]\) instability

To explore other mechanisms for the effect of get2 on prion propagation, we studied the effect of calcium on prion maintenance in get2Δ strain. Incubation of the get2Δ strain was done on three types of complete synthetic media. These were control, medium containing 10mM CaCl2, and medium containing 780µM EGTA. The master plate was replica plated to these three different media so that the same patches can be compared from all the media. At each time point, several colonies were streaked out on YPD to assess \([PSI^+]\) instability by color (Fig. 7.1).

Figure 7.1 The experimental scheme for studying the effect of calcium on \([PSI^+]\) instability in get2Δ through serial passages on complete synthetic medium
The level of \([PSI^+]\) instability is decreased in the presence of 10 mM CaCl₂ as compared to control since there are less red or \([psi^-]\) colonies. On the other hand, 780 µM EGTA which removes calcium, increases \([PSI^+]\) instability as seen by the appearance of more red colonies (Fig. 7.2 and Table 7.1).

Figure 7.2 Effect of calcium on \([PSI^+]\) instability in \(get2\Delta\) through incubation on complete synthetic medium

Get2Δ strain was incubated for several passages on three different types of synthetic media as shown in figure 7.1A. Colonies were picked at each time point and streaked out on YPD. The level of \([PSI^+]\) instability is decreased in the presence of 10 mM CaCl₂ (A) as compared to control (B) since there are less red or \([psi^-]\) colonies. On the other hand, 780 µM EGTA which removes calcium, increases \([PSI^+]\) instability as seen by the appearance of more red colonies (C). Levels of calcium ions modulate the effects of \(get2\Delta\) on \([PSI^+]\) propagation. See table 7.1 for tabulated data.

Table 7.1 Effect of calcium on \([PSI^+]\) instability in \(get2\Delta\) through incubation on complete synthetic medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control</th>
<th>+ Ca</th>
<th>- Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ade⁺</td>
<td>Ade⁻</td>
<td>Total</td>
</tr>
<tr>
<td>Wild type</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>(get2\Delta)</td>
<td>50</td>
<td>46</td>
<td>96</td>
</tr>
</tbody>
</table>

See figure 7.2 for experimental details.

Another version of this experiment was performed where the strains were streaked out on the three different media (control, +Ca and –Ca) and then patched on the
same media. Therefore, the origin of patches on the three plates was different in this case. However, similar effects of calcium and EGTA on \([PSI^+]\) instability in get2Δ strain were observed.

**Effect of calcium on \([PSI^+]\) curing**

Also, \([PSI^+]\) curing experiment was performed in get2Δ strain in the presence of calcium to see its effect. Wild type and get2Δ strains were transformed with high *HSP104* (pLH105) or control (YEp13) plasmids and the transformants were selected on −Leu medium. The colonies were patched on YPD to analyze the \([PSI^+]\) curing based on color. The presence of calcium restores some \([PSI^+]\) curing by excess Hsp104. \([PSI^+]\) loss can be detected by reddish color on edges in the get2Δ strain. Eight transformants were tested and similar result was observed in five. The wild type strain is completely cured by excess Hsp104 as seen by red color. (Fig. 7.3).

![Wild type and get2Δ strains were transformed with pLH105 (P_gpd-HSP104) or control plasmids, patched on -Leu media and incubated for ~4 days. The -Leu plate was replica plated to YPD (with or without 10mM CaCl₂) and incubated at 30°C for two days. This was followed by incubation at 4°C for two days. The presence of calcium restores some [PSI] curing by excess Hsp104. [PSI] loss can be detected by reddish color on edges in the get2Δ strain. Eight transformants were tested and similar result was observed in five. The wild type strain is completely cured by excess Hsp104 as seen by red color. The controls are shown for comparison. Note that there is no curing in get2Δ by excess Hsp104 in the absence of calcium as shown on right.](image)

Figure 7.3 Effect of calcium on \([PSI^+]\) curing in get2Δ

Wild type and get2Δ strains were transformed with pLH105 (P_gpd-HSP104) or control plasmids, patched on -Leu media and incubated for ~4 days. The -Leu plate was replica plated to YPD (with or without 10mM CaCl₂) and incubated at 30°C for two days. This was followed by incubation at 4°C for two days. The presence of calcium restores some [PSI] curing by excess Hsp104. [PSI] loss can be detected by reddish color on edges in the get2Δ strain. Eight transformants were tested and similar result was observed in five. The wild type strain is completely cured by excess Hsp104 as seen by red color. The controls are shown for comparison. Note that there is no curing in get2Δ by excess Hsp104 in the absence of calcium as shown on right.
Conclusion

$[\text{PSI}^+]$ mitotic stability and $[\text{PSI}^+]$ curing by excess Hsp104 are partially restored in the get2Δ strain by addition of CaCl₂. This shows that some effects of get2 on $[\text{PSI}^+]$ propagation are expressed due to changes in calcium ion levels.
CHAPTER 8

DISCUSSION

In this study we demonstrate a connection between the Get pathway and yeast prions. Get2 affects prion maintenance, and get1, get2 and get3 affect [PSI⁺] curing by excess Hsp104; thus there is a role played by the Get complex in prion maintenance and propagation. The other yeast prion, [PIN⁺], is also affected by the deletion of get2. These defects are not due to any decrease in the Hsp104 levels or activity.

However, the range of sizes of [PSI⁺] polymers is wider, and there are more of aggregates of larger sizes in get2Δ strain. It is possible that Get2 plays a role in the efficient action of Hsp104 on the [PSI⁺] aggregates. It could do this by presenting the aggregates to Hsp104 chaperone in a manner that allows their effective breakdown into smaller seeds. More specifically, vesicles containing Get complex might directly interact with aggregates and keep them from further growth or agglomeration into larger structures (Fig. 8.1). In the absence of this activity, some aggregates might grow much bigger in size and become unstable. It is known that larger size of aggregates impairs transmission and mitotic stability as reported previously (26, 28). This could explain the prion propagation defect in get2 mutants.

We may check the hypothesis proposed above by investigating whether Get complex colocalizes with the prion aggregates. We have generated wild type strain OT56 containing GET2-GFP and GET2-HA fusion constructs. On introduction of a plasmid having a SUP35-RFP fusion into the GET2-GFP strain, we may utilize fluorescence microscopy techniques to study localization of these two proteins with respect to each other. Based on the model proposed in Fig. 8.1, we expect that Get2 and Sup35 will colocalize with each other.
Also, get2Δ might affect the integrity of the Get complex which might lead to perturbations in calcium ion homeostasis. Get1 has been implicated in the regulation of calcium ion fluxes in the ER (29). Also, Get3 is known to have similarities to metal transporters (15). It has been reported previously (30) that metal ions influence aggregation of other prion or amyloid proteins e.g. copper affects PrP aggregation. Also, effects of metal ions on heat shock proteins have been reported (37). We have observed that the levels of calcium ions modulate the effects of get2Δ on \([PSI^+]\) propagation (Figs. 7.2 and 7.3 and table 7.1). It is possible that defects in Get complex and/or retrieval of ER proteins may influence the influx of calcium. Calcium may influence Sup35 aggregation or chaperone induction, and in this way counteract the effects of Get complex disruption. Therefore, we see that \([PSI^+]\) mitotic stability and \([PSI^+]\) curing by excess Hsp104 are partially restored in the get2Δ strain by addition of CaCl₂.
We have also observed that prion instability is seen only on synthetic medium. This may suggest that interaction with Get complex becomes important only in certain conditions. On synthetic medium yeast cells become partly stressed and levels of chaperones are higher. We know that some chaperones e.g. Hsp70-Ssa promote prion aggregation (31). This could suggest the importance of the Get complex activity of preventing further growth of aggregates in such conditions. In the absence of this function of the Get complex, the prions become unstable.

The effect of \textit{get2} is not seen in a weak \([PSI^+]\) background. The dependence of the \textit{get2} effect on the strength of the prion is worth checking further. There could be differences between the weak and strong prion variants. The aggregates of the weak prion variant may not interact with the Get complex effectively. This might lead to their larger size and lower mitotic stability as compared to the aggregates of the strong prion variant in a wild type cell. However, when there are any alterations in the Get complex, the sizes of the already bigger aggregates do not increase any further and thus we do not see the appearance of prion instability on making deletions in the Get complex genes in a weak \([PSI^+]\) background.
CONCLUSIONS

1) Deletions of get3 or get1 exhibit defects in \([PSI^+]\) curing by excess Hsp104 and deletion of get3 exhibits a defect in \([PSI^+]\) maintenance, that are in the same direction as defects caused by get2 mutants. This shows that some functions that are common for different components of Get complex are involved in prion maintenance.

2) Get2 deletion causes instability of \([PIN^+]\) prion on synthetic media in a strong \([PSI^+]\) background. This shows that effects of Get complex on prions are not specific only to \([PSI^+]\).

3) De novo induction of \([PSI^+]\) by Sup35 overproduction in the presence of \([PIN^+]\) is not significantly affected by get2Δ.

4) Get2 deletion does not affect mitotic stability of the weak \([PSI^+]\) variant.

5) Hsp104 levels are increased rather than decreased in the get2Δ and get2-473 strains, as compared to wild type. This shows that the prion curing and maintenance defects in the get2 mutants are not due to a decrease in Hsp104 levels.

6) Induced thermotolerance is not affected while basal thermotolerance in synthetic media is increased rather than decreased in the get2 mutants as compared to wild type. This shows that the prion curing and maintenance defects are not due to a decrease in the activity of Hsp104.

7) The range of sizes of \([PSI^+]\) polymers is wider, and there are more of aggregates of larger sizes in get2Δ strain.

8) \([PSI^+]\) mitotic stability and \([PSI^+]\) curing by excess Hsp104 in the get2Δ strain are partially restored by addition of CaCl2. This shows that some effects of get2 on \([PSI^+]\) propagation are expressed due to changes in calcium ion levels.
REFERENCES


