Hsp31 Is a Stress Response Chaperone That Intervenes in the Protein Misfolding Process

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Background: Hsp31 is a multifunctional cellular stress response protein.

Results: Hsp31 is a stress-inducible chaperone that has substoichiometric activity on a wide spectrum of substrates, including prions and the non-physiological client, α-synuclein.

Conclusion: Hsp31 protects cells from α-synuclein-mediated toxicity via chaperone activity and independently from enzymatic activity and autophagy.

Significance: This is the first characterization of the early acting and wide ranging chaperone activity of Hsp31.

The Saccharomyces cerevisiae heat shock protein Hsp31 is a stress-inducible homodimeric protein that is involved in diauxic shift reprogramming and has glyoxalase activity. We show that substoichiometric concentrations of Hsp31 can abrogate aggregation of a broad array of substrates in vitro. Hsp31 also modulates the aggregation of α-synuclein (αSyn), a target of the chaperone activity of human DJ-1, an Hsp31 homolog. We demonstrate that Hsp31 is able to suppress the in vitro fibrillization or aggregation of αSyn, citrate synthase and insulin. Chaperone activity was also observed in vivo because constitutive overexpression of Hsp31 reduced the incidence of αSyn cytoplasmic foci, and yeast cells were rescued from αSyn-generated proteotoxicity upon Hsp31 overexpression. Moreover, we showed that Hsp31 protein levels are increased by H2O2 in the diauxic phase of normal growth conditions, and in cells under αSyn-mediated proteotoxic stress. We show that Hsp31 chaperone activity and not the methylglyoxalase activity or the autophagy pathway drives the protective effects. We also demonstrate reduced aggregation of the Sup35 prion domain, PrD-Sup35, as visualized by fluorescent protein fusions. In addition, Hsp31 acts on its substrates prior to the formation of large aggregates because Hsp31 does not mutually localize with prion aggregates, and it prevents the formation of detectable in vitro αSyn fibrils. These studies establish that the protective role of Hsp31 against cellular stress is achieved by chaperone activity that intervenes early in the protein misfolding process and is effective on a wide spectrum of substrate proteins, including αSyn and prion proteins.

Heat shock proteins (HSPs) protect living cells against environmental stress and promote cell survival. This function is evolutionarily conserved throughout different organisms. HSPs interact with misfolded proteins and process them using several mechanisms, including chaperone functions to prevent misfolded protein aggregation, unraveling protein aggregates, or carrying out protease functions to eliminate irreversibly damaged proteins. The effect of HSPs in maintaining cellular homeostasis throughout the protein folding process is essential to preventing diseases associated with protein misfolding and aggregation, such as Alzheimer disease, Parkinson disease (PD), and Huntington disease. HSPs involved in counteracting these protein inclusions are currently under intensive study.

Among the neurodegenerative diseases, PD is the second most common neurodegenerative disorder that occurs in elderly individuals 50 years or older, and no cure is currently known. High levels of reactive oxygen species (ROS), dysfunctional mitochondria, and the aggregation of αSyn are observed in PD. DJ-1, a gene linked to familial forms of PD, has been found to be involved in oxidative insults, proteasome degradation, and protein folding and is the focus of extensive studies to understand its functions in regulating levels of αSyn aggregation.

Human DJ-1, yeast Hsp31 (YDR533c), and Escherichia coli Hsp31 (hchA or yedLI) are members of the DJ-1/Pffl/ThiI superfamily. Crystal structures of Hsp31 show that it contains a cysteine catalytic triad that is conserved in the DJ-1/Pffl/ThiI family. E. coli hchA is a close ortholog with yeast Hsp31, and they share superimposable active sites in each monomer, but their quaternary structures appear to be different. This protein family is of biomedical importance due to the role these proteins play in chaperone-like and

*This work was supported, in whole or in part, by National Institutes of Health, National Center for Advancing Translational Sciences, Clinical and Translational Science Award UL1TR001108 and National Institutes of Health Grant R01 GM087461 (to the Indiana Clinical and Translational Sciences Institute). This publication also supported by a Shoemaker Foundation grant (to J-C. R. and T. H.). The authors declare that they have no conflicts of interest with the contents of this article.

1 Both authors contributed equally to this work.
2 Supported in part by a Purdue Research Foundation fellowship.
3 Supported by a Fulbright Foundation fellowship, United States Education Foundation - Pakistan.
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The abbreviations used are: HSP, heat shock protein; αSyn, α-synuclein; PD, Parkinson disease; ROS, reactive oxygen species; MGO, methylglyoxal; SD, synthetic dextrose; SC, synthetic complete; SDD-AGE, semidenaturing detergent-agarose gel electrophoresis; PrD, prion-forming domain; CFP, cyan fluorescence protein; EYFP, enhanced yellow fluorescence protein; DsRed, red fluorescence protein from Discosoma sp.; MORF, yeast movable ORF tag.
cytoprotective activities (15, 19). Despite the shared structural similarity, this family can be further subcategorized into three classes according to the structural and functional properties of the proteins or into five subclasses by protein sequence alignments (15, 19). The cellular functions of DJ-1 and E. coli hchA have been characterized, and their multifunctional roles in mediating oxidative stress, chaperone-like activity, methylglyoxalase activity, and cytoprotection have been described (10, 15, 16, 19). There has been limited characterization of the cellular functions of Hsp31, but several recent studies have demonstrated that it has glyoxylase activity (20), chaperone activity (21), and a role in autophagy (22).

Yeast PD models have been used to study the mechanism of the sporadic and familial forms of PD (23–25). We are extending the utility of these yeast models by investigating the biological activities of yeast Hsp31. Hsp31 appears to be a stress-inducible 26-kDa protein based on several large scale studies indicating that yeast Hsp31 is up-regulated when cells are exposed to environmental stress (26–29). For example, Hsp31 was implicated to be protective against ROS because an hsp31Δ strain was more sensitive to oxidative stress (26). HchA has structural similarity to Hsp31 and is established as having chaperone activities, suggesting that Hsp31 could act similarly; however, this has yet to be confirmed. The evidence for a role in stress response, possible chaperone activity, methylglyoxalase activity, and cross-talk with the autophagy pathway led us to investigate these activities of Hsp31 in modulating αSyn cytotoxicity. We also demonstrate that Hsp31 has methylglyoxalase activity that converts methylglyoxal (MGO) substrate to n-lactate, but this enzyme activity is not essential for protecting against αSyn-mediated cytotoxicity.

**Experimental Procedures**

**Yeast Cell Growth Conditions**

Yeast media were prepared according to Amberg et al. (30). Liquid yeast extract/pepetone dextrose medium contained Bacto yeast extract (1%; Fisher), Bacto peptone (2%; Fisher), and glucose (2%; Fisher). Synthetic dextrose (SD) minimum liquid medium was made of 0.17% Difco yeast nitrogen base (without amino acids) and 2% glucose and supplemented with necessary amino acids for auxotrophic strains needed at concentrations described previously (30). Solid medium plates were made with the same components of liquid medium plus 2% agar (Fisher). To express galactose-inducible proteins, 2% raffinose glucose or galactose. Plates were incubated at 30 °C for 2–3 days.

**Yeast Strain Construction**

Yeast strains used in this study are listed in Table 1. The hsp31Δ and atg8Δ deletions were generated in W303-1A or in a strain expressing one or two copies αSyn fused to fluorescent proteins (CFP and YFP) using the primers listed in Table 2. The nat (nourseothricin N-acetyltransferase) gene flanked by Hsp31 homology regions was obtained by PCR with 70-nucleotide-long forward primer and reverse primer. The primers consisted of 20 nucleotides for amplifying the nat gene from pFA6α-natNT2 (Euroscarf) and 50 nucleotides immediately preceding the HSP31 or ATG8 start codon or after the stop codon. The amplified product was integrated into W303 αSyn-expressing strains at ChrIV:1502160 to 1501447, as described previously (31). For double knock-out hsp31Δ atg8Δ strain, the kanMX4 resistance cassette was used to delete Hsp31, and then the natMX4 resistance cassette was used for deletion of ATG8.

**Hsp31–9myc Tagging**

We endogenously tagged HSP31 with the 9myc epitope using a PCR-based integration (12). The pYM20 plasmid was used as a template, and primers were used to obtain PCR product with HSP31 genomic flanking followed by transformation into W303 and W303 αSyn-CFP + αSyn-YFP strains. The transformants were selected on media containing hygromycin B (300 mg/liter), and correct integration was verified by PCR using primers spanning the integration junctions and by DNA sequence analysis.

**DNA Manipulation**

The plasmids used in this study are listed in Table 3. Plasmid BG1805 was linearized with NdeI (New England Biolabs, Ipswich, MA) and cloned into pDONR221 (Invitrogen) with BP Clonase (Invitrogen) with the method provided by the manufacturer. Hsp31 was shuttled into pAG415–GPD–ccdB–DsRed...
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Table 2

| Gene/Description | Forward | Reverse |
|------------------|---------|---------|
| HSP31 deletion   | AGTTGCAATTCACCTGCTATTGAGGAGATATTTAATGACACATTCGTAACAAACATTCCAA|
| 9myc tag of HSP31 | AGTGGAGAATACCCATCTGACATGCAATCCCGGCGTTAGG|
| HSP31            | ACACTTTGCGCTCGCTGGACTGAG|
| ATG8 deletion    | TTTGCACAGCTCGCTGGACTGAG|
| HSP31 mutation C138D | GAGCGGAAAGGAGAACATCCCC|
| ATG8 deletion diagnostic | GAGCGGAAAGGAGAACATCCCC|
| HSP31 9myc tag diagnostic | GAGCGGAAAGGAGAACATCCCC|

Table 3

| Plasmids | Type of plasmid | Source/Reference |
|----------|-----------------|------------------|
| pAG415-GPD-HSP31-DsRed | Yeast, CEN | This study |
| pAG415-GPD-HSP31-DsRed | Yeast, CEN | Alberti et al. (39) |
| pAG424-GAL-PrD-Sup35-EYFP | Yeast, 2 μm | Alberti et al. (79) |

Antibodies and Immunoblotting

To determine protein expression, yeast cells were collected by centrifuging at 5,000 rpm, and pellets were resuspended in extraction buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 4 mM MgCl₂, 5 mM DTT). Glass beads (Sigma) were added into the mixture, which was vortexed 5 times for 10 s each, plus 1-min intervals on ice. Clear crude protein lysate was obtained by centrifugation, and pellets were resuspended in lysis buffer (25 mM KPi, pH 7.0, 200 mM KCl) containing protease inhibitor mixture set IV (Calbiochem). Crude protein was prepared by sonicating cells and clarifying the lysate by centrifugation at 10,000 × g for 10 min. GST-tagged DJ-1 was immobilized by glutathione-agarose resin (Thermo Scientific), and DJ-1 was eluted by cleaving from the GST tag with PreScission Protease (GE Healthcare). 2 units of protease for every 100 μg of tagged DJ-1, leaving a linker amino acid sequence: NPAFLYKVVDVSRHHHGRIFYPY-DVPDYAGLEVLFQ.

Sedimentation velocity ultracentrifugation was performed with two different Hsp31 concentrations (34 and 17 μM) and run at 50,000 rpm in a Beckman Coulter XLA centrifuge (Beckman Coulter, Fullerton, CA). The sedimentation coefficients and apparent molecular weights were calculated from size distribution analyses (c(s)) using SEDFIT (37).

Hsp31 was immobilized by His-Select® nickel affinity gel (Sigma-Aldrich) and eluted with elution buffer (Tris-Cl, pH 7.5, 250 μM imidazole). The purity of proteins was determined by SDS-PAGE followed by Coomassie Blue staining, and concentrations were determined by a Micro BCA protein assay kit (Thermo Scientific). The αSyn protein for in vitro fibrillation assays was purified by expressing the pT7-7 plasmid containing human αSyn cDNA (provided by R. Jakes and M. Goedert, Medical Research Council, Cambridge, UK) with isopropyl β-D-1-thiogalactopyranoside in BL21 (DE3) for 4 h of incubation at 37 °C. The cells were harvested and resuspended in 10 mM Tris-Cl, pH 8, 1 mM EDTA, 1 mM PMSF at 1/50 culture volume. The cell lysate was obtained by one round of freezing-thawing and French press treatment, and protein was partially purified and enriched by two rounds of ammonium sulfate precipitation (30% saturation first and then 50% saturation at 0°C). The pellets were resuspended and boiled in 10 mM Tris-HCl, pH 7.4, for 5 min. The insoluble protein was removed by centrifugation, and the supernatant was filtered using a 0.22-μm under high salt conditions, which minimizes contaminant co-purifying proteins. Human DJ-1 was encoded in pGEX 6p-1 (GE Healthcare), hchA was encoded in pNT-hchA, and αSyn was expressed from the p17 plasmid. The protein expression and purification was described previously (34–36). Briefly, constructs were transformed into BL21 (DE3) cells. The transformants were grown to an A₆₀₀ of 0.4–0.6 in LB medium supplemented with 100 μg/ml ampicillin at 37 °C, and isopropyl β-D-1-thiogalactopyranoside (1 mM final concentration) was added to induce protein expression. After 3 h of induction at 37 °C, cells were harvested by centrifugation at 2,000 × g and resuspended in lysis buffer (25 mM KPi, pH 7.0, 200 mM KCl) containing protease inhibitor mixture set IV (Calbiochem).

Protein Purification

BY4741 harboring yeast Hsp31 expression plasmid from the yeast movable ORF collection (33) (Thermo Fisher Scientific) was used to express and purify the protein (33). We did not see any evidence of co-purifying proteins on SDS-polyacrylamide gels and observed similar high activity from protein purified

(Adgene, Cambridge, MA) (32) from pDONR221 with LR Clonase (Invitrogen) to produce pAG415-GPD-HSP31-DsRed. Both DJ-1 and Hsp31 were cloned into BamH1/XhoI sites of pGEX 6p-1. pESC-Leu myc-HSP31 was constructed by cloning myc-HSP31 into yeast genomic DNA. The Hsp31 C138D mutant was prepared by PCR amplification using pAG415-ccdB-DsRed Yeast, CEN Alberti et al. (79) constructs were transformed into BL21 (DE3) cells. The transformants were grown to an A₆₀₀ of 0.4–0.6 in LB medium supplemented with 100 μg/ml ampicillin at 37 °C, and isopropyl β-D-1-thiogalactopyranoside (1 mM final concentration) was added to induce protein expression. After 3 h of induction at 37 °C, cells were harvested by centrifugation at 2,000 × g and resuspended in lysis buffer (25 mM KPi, pH 7.0, 200 mM KCl) containing protease inhibitor mixture set IV (Calbiochem). Crude protein was prepared by sonicating cells and clarifying the lysate by centrifugation at 10,000 × g for 10 min. GST-tagged DJ-1 was immobilized by glutathione-agarose resin (Thermo Scientific), and DJ-1 was eluted by cleaving from the GST tag with PreScission Protease (GE Healthcare) (2 units of protease for every 100 μg of tagged DJ-1), leaving a linker amino acid sequence: NPAFLYKVVDVSRHHHGRIFYPY-DVPDYAGLEVLFQ.

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filter. The filtered fraction of the protein was applied to a DEAE-Sepharose FF column (GE Healthcare) with 10 mM Tris-HCl, pH 7.4, and eluted with 0–500 mM NaCl over 100 min. The fractions containing αSyn were dialyzed against 10 mM NH₄HCO₃ and lyophilized. The protein was ~95% pure based on Coomassie Blue staining with SDS-PAGE.

**Chaperone Activity Aggregation Suppression Assays**

**Thermal Aggregation Assay of Citrate Synthase (CS)—** CS aggregation suppression assay was performed according to the procedures described previously (38). Briefly, CS (porcine CS, Sigma), Hsp31, DJ-1, and hchA were buffer-exchanged into 40 mM Hepes-KOH (pH 7.5). Proteins were diluted to concentrations ranging from 0.1 to 0.6 μM in a 500-μl reaction. Samples were loaded into a quartz cuvette and placed into the FluoroMax-3 spectrofluorometer (Horiba, John Yobin) heated to 43 °C. Aggregation of CS, with and without Hsp31, was measured by light scattering with the excitation and emission set to 360 nm and a slit width of 2 nm. BSA fraction V and hchA served as negative and positive controls. Measurements were taken every second for 2,000 s. Data were plotted using GraphPad Prism version 5.0.

**Reducing Agent-induced Aggregation of Insulin (38, 39)—** Briefly, Hsp31 was buffer-exchanged into PBS (pH 7.2). Insulin was diluted with PBS to 26 μM, and 4 or 8 μM of Hsp31 was used in a 500-μl reaction. Samples were loaded into a quartz cuvette and placed into the FluoroMax-3 spectrofluorometer. Aggregation of insulin induced by 20 mM DTT, with and without Hsp31, was measured by light scattering with the excitation and emission set to 360 nm and a slit width of 1 nm. Measurements were taken every second for 2,000 s. Data were plotted using GraphPad Prism version 5.0.

**Spontaneously Fibrillizing αSyn Assay—** The procedure for performing the Thioflavin T aggregation assay was described previously (40). The αSyn was prepared by centrifuging the recombinant protein through a 100 kDa spin filter to remove aggregates. The filtered fraction of the protein was dialyzed against 10 mM Tris /H₉₂₅₁ and 2% galactose (induced expression) with 5-fold serial dilutions. The plates were incubated at 30 °C for 3 days.

**Fluorescence Imaging of αSyn Localization**

pESC-Leu myc-Hsp31 and control empty vector were transformed into two copies of αSyn-expressing strain. The αSyn expression was induced by growing cells in synthetic minimum medium plus 2% raffinose and 2% galactose for 8 h. The live cells were visualized using a Nikon TE2000-U inverted fluorescence microscope with Nikon Plan apochromat ×60 (numerical aperture 1.4) oil immersion objective and YFP filters plus ×300 magnification. In order to differentiate the localization of protein, ~50 individual cells were randomly counted in multiple regions of interest for each set of experiments. The ratio of membrane to cytoplasmic localization of αSyn fluorescent fusion protein was presented as the mean from three independent sets of experiments.

**MGO Addition and Microscopy**

αSyn-expressing strains with or without the HSP31 gene deleted were grown in liquid yeast extract/peptone dextrose medium and transferred into medium containing 2% galactose and MGO (0.5–2 mM) for 6 and 12 h. Cells were washed and subjected to confocal microscopy to observe αSyn foci formation. The presence of MGO inhibited the growth of cells for the initial 12 h and resulted in an equal growth rate between strains regardless of the HSP31 gene deletion. Strains could not be grown in the presence of high concentrations of MGO (20 mM), so αSyn was induced for 10 h, and then MGO was added and incubated for another 2 h before confocal microscopy analysis.

**Assessment of Intracellular ROS**

Cells were harvested after the induction of αSyn expression for 12 h, and 5 × 10⁶ cells were prepared for staining with dihydroethidium. Cells were suspended in 250 μl of 2.5 μg/ml dihydroethidium in PBS and incubated in the dark for 10 min. Cells were washed in the PBS and were subjected to fluorescence microscopy (excitation at 485 nm and emission at 520 nm) and flow cytometry with the FL-2 channel. FlowJo software was used to calculate median fluorescence intensity.

**Prion Expression Experiments**

W303 wild type strain was co-transformed with pAG424-GAL-PrD-Sup35-EYFP and pAG415-GPD-HSP31-DsRed. Single transformations were also performed using the same constructs and their corresponding control vector. Cells were grown at 30 °C in appropriate medium overnight (SD–Trp for pAG424, SD–Leu for pAG415, and SD–Trp–Leu for co-transformants), and protein expression was induced for 24 h in SC 2% raffinose and 2% galactose-containing medium at 30 °C. After induction, cells were subjected to fluorescence microscopy using a Nikon A1 confocal microscope with a Nikon Plan apochromat ×60 (numerical aperture 1.4) oil immersion objective to acquire fluorescence and differential interference contrast images and were analyzed using ImageJ. Cultures identical to those used in microscopy were used to prepare samples for flow cytometry, and cells were washed and resuspended in PBS at a density of ~10 × 10⁶ cells/ml. Cells were filtered and ana-
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Lyzed for EYFP fluorescence intensity using a flow cytometer with the FL-1 channel. A total of 10,000 events were acquired for each sample, and data were analyzed using FlowJo software to calculate median fluorescence intensity after three biological replicates.

**Glutathione-independent Glyoxalase Biochemical Assay**

Purified recombinant Hsp31 and Hsp31 C138D with the GST tag removed were used to assay methylglyoxalase activities. The reaction was initiated by adding the specified concentration of proteins (1 \( \mu M \) Hsp31 and 5 \( \mu M \) Hsp31C138D) in reaction buffer (100 mM HEPES, pH 7.5, 50 mM KCl, 2 mM DTT) to a 6 mM initial concentration of MGO (Sigma; 40% solution), followed by incubation at 30 °C. The assay was performed by removing 50-\( \mu l \) aliquots of the reaction at fixed time points (15, 30, 45, and 120 s) after the addition of protein. The amount of D-lactic acid produced by Hsp31 as a result of MGO consumption was measured. The initial rate obtained was divided by the amount of protein in the reaction mixture to calculate the specific activity. Samples were also subjected to gas chromatography-mass spectroscopy analysis (Agilent 5975C MSD) to identify the presence of D-lactic acid. Samples were derivatized with \( N,O \)-bis(trimethylsilyl) trifluoroacetamide and trimethylchlorosilane and heated to 50 °C for 10 min and run in the electron impact mode with scanning from 42 to 400 atomic mass units. A lactic acid standard displayed a peak at 6.09 min identical to the peak detected in the Hsp31-treated sample, whereas Hsp31C138D did not display this peak. The spectral scan was visualized and displayed using the OpenChrom software (41). To obtain the enzymatic parameters, the reaction was initiated as described above by adding 1 \( \mu M \) Hsp31 or 5 \( \mu M \) Hsp31C138D protein into reaction buffer, and reactions were stopped after 1 min by heating at 85 °C for 30 s. A range of MGO substrate from 50 \( \mu M \) to 2 \( \mu M \) and the EnzyFluo D-lactate assay kit (Bioassay Systems) were used to determine the amount of D-lactic acid produced in each reaction. Each rate was determined in triplicate, and mean values were plotted to obtain enzymatic parameters (\( V_{\text{max}} \) and \( K_m \)) by fitting to a Michaelis-Menten model using GraphPad Prism.

**Semidenaturing Detergent-Agarose Gel Electrophoresis (SDD-AGE)**

W303 cells harboring pAG424-GAL-cPrDSup35-EYFP and pAG415-GPD-HSP31-DsRed plasmids were used, and aggregates were produced by inducing for 24 h in SC 2% raffinose + 2% galactose medium. Prion aggregates were analyzed using SDD-AGE (42). Briefly, to prepare lysates, cells were harvested by centrifugation at 3,000 \( \times g \) for 2 min and resuspended in spheroplast solution (1.2 M D-sorbitol, 0.5 mM MgCl\(_2\), 20 mM Tris (pH 7.5), 50 mM \( \beta \)-mercaptoethanol, and 0.5 mg/ml Zymolyase) and incubated at 30 °C for 30 min with shaking. After spheroplast formation, the samples were centrifuged at 800 rpm for 5 min at room temperature, and supernatant was removed. The pelleted spheroplasts were resuspended into 100 \( \mu l \) of lysis buffer (100 mM Tris, pH 7.5, 50 mM NaCl, 10 mM \( \beta \)-mercaptoethanol, and protease inhibitor) and vortexed at high speed for 2 min. The lysates were collected and mixed with 4 \( \times \) sample buffer (2 \( \times \) TAE, 20% glycerol, 4% SDS, 0.01% bromophenol blue). The samples were incubated at room temperature for 15 min and loaded onto a 1.8% agarose gel containing 1\( \times \) TAE and 0.1% SDS and run at 50 V, followed by transfer onto nitrocellulose membrane using the capillary transfer method. The nitrocellulose membrane was subjected to Western blot analysis using anti-GFP antibody (Roche Applied Science). A strain harboring p2HG-Hsp104 plasmid was included as a positive control.

**Results**

**Hsp31 Has Chaperone Activity—**The S. cerevisiae open reading frame (YDR533C) has been assigned the name HSP31; however, direct functional studies demonstrating chaperone activity have not been conducted on this protein to support its designation within the HSP family. Hsp31, human DJ-1, and hchA are members of the ThiJ/DJ-1 superfamily based on similarity in structure, so we directly compared the in vitro chaperone activity between these proteins. The influence of Hsp31 on the aggregation of substrate proteins was measured using three different model substrates: CS, insulin, and aSyn. CS has been used as a classical substrate for in vitro chaperone assays, and both DJ-1 and hchA have demonstrated an ability to suppress CS aggregation (9, 43). To assess the chaperone activity of Hsp31, we purified the protein from yeast using the BG1805 plasmid-based system from the yeast movable ORF (MORF) collection (33). The C terminus of the protein retains a 4.8-kDa tag, as confirmed by mass spectrometry, and includes a His\(_6\) and HA tag (see the amino acid sequence under “Experimental Procedures”). We also expressed Hsp31 with a GST tag fused to its N terminus using the pGEX-6p1 plasmid in E. coli. The tag was removed by 3C protease, and only 3 amino acids (GPL) were retained on the recombinant Hsp31 protein, which was confirmed by mass spectrometry (data not shown). We determined that the purified proteins were folded correctly because they behaved as homodimers based on gel filtration chromatography (data not shown) and by analytical ultracentrifugation with a sedimentation coefficient of 3.7 S (Fig. 1A). The Hsp31-MORF, purified from yeast, can completely suppress the heat-induced aggregation of CS when present at a 1:1 molar ratio (Fig. 1B). BSA, which was added at a 6-fold molar excess concentration of CS, had a slight effect on suppressing CS aggregation in agreement with a previous study that reported BSA as having a mild chaperone effect in the CS aggregation assay (44). E. coli hchA suppressed CS aggregation in a dose-dependent manner, but the high concentration of 0.6 \( \mu M \) did not approach the considerably more efficient suppression of CS aggregation by Hsp31-MORF (Fig. 1B). In addition, recombinant yeast Hsp31 purified from E. coli did not have activity at a 1:1 molar ratio and was needed at high molar ratios similar to hchA (data not shown) (Fig. 1B). In conclusion, Hsp31 purified from yeast can suppress CS aggregation at a 1:1 molar ratio, whereas hchA is needed at a 6:1 molar ratio to generate an appreciable suppression of aggregation. These results are consistent with previous studies, where high molar ratios of hchA were required in CS assays (38, 45).

To further characterize the chaperone activity of Hsp31, additional protein substrates were studied. Hsp31-MORF suppressed insulin aggregation induced by reducing agent, when
using a 3–6-fold molar excess of insulin (Fig. 1C), further establishing its chaperone activity in another commonly used chaperone assay. The mechanism of αSyn aggregation is unclear, and DJ-1 has been reported to decrease αSyn aggregates or cytoplasmic foci in vitro and in vivo (9, 14, 46, 47). Hsp31 completely suppressed αSyn fibrillation despite the presence of an 8-fold molar excess of αSyn and maintained chaperone activity when αSyn was present at a 35:1 molar ratio (Fig. 1D). In agreement with this result, we observed that DJ-1 suppressed αSyn fibrillation although not to the same extent as Hsp31. The difference may be due to the oxidation state of DJ-1, which has been shown to affect its activity (46). Taken together, these results demonstrate that Hsp31 has the ability to act as a chaperone in preventing several types of protein aggregation at substoichiometric concentrations.

Hsp31 Protects against αSyn Toxicity and Reduces αSyn Foci Formation and ROS Levels—Outeiro and Lindquist (23) demonstrated that two genomic copies of GAL-αSyn in yeast decreased cellular fitness, which was correlated with αSyn cellular aggregates (46). DJ-1 has been reported to alleviate protein aggregation (9, 46), and αSyn has been implicated as a factor in the progression of PD (48–50). To determine the chaperone effect of Hsp31 with its anti-aggregation activity and effect on cellular fitness, we tested the rescue of yeast cells from αSyn-mediated toxicity in dilution growth assays. Overexpression of one copy of the αSyn gene from the GAL (galactose) promoter had negligible effects on cellular fitness, but GAL-driven overexpression of two αSyn gene copies resulted in severe toxicity, confirming previous findings of dose-dependent toxicity. The αSyn toxicity was markedly alleviated in the presence of constitutive expression of Hsp31 (pGPD-HSP31), as evidenced by the full rescue of cell viability (Fig. 2A). In addition, we expressed one copy of GAL-αSyn in an hsp31Δ strain to determine whether there was a synthetic genetic interaction between these genes. Dilution growth assays demonstrated that expression of a single copy of αSyn or knocking-out HSP31 alone was phenotypically similar to the wild-type strain with respect to viability. However, cellular fitness was significantly reduced when expressing a single copy of αSyn in the hsp31Δ strain (Fig. 2B). The synthetic lethal interaction of αSyn and deleted HSP31...
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**FIGURE 2.** Hsp31 rescues cells from αSyn mediated toxicity. A, Hsp31 co-expression in yeast reduced αSyn toxicity. A yeast strain containing one or two copies of genomically expressed GAL-αSyn was used in a growth dilution assay, in the presence of pGPD-HSP31-DsRed or empty vector, in media containing galactose/raffinose (left) or glucose (right). The strain harboring two copies of the αSyn gene had better fitness when transformed with the Hsp31-encoding construct compared with the empty vector. B, combination of hsp31Δ with single copy αSyn expression is toxic for yeast. The expression of a single copy of αSyn in hsp31Δ strain reduced cellular fitness in the presence of glucose (top) and raffinose/galactose (bottom). The overexpression of Hsp31 in the same strain was able to rescue viability on both types of media.

using this approach provided further evidence that Hsp31 is a molecular chaperone with the ability to protect cells from αSyn-mediated toxicity.

Previous studies demonstrated that the subcellular localization of αSyn changed from the plasma membrane to cytoplasmic foci with increased expression of αSyn in yeast cells (23). Using this relocalization property, we examined the effect of pGAL HSP31 overexpression in altering the distinctive subcellular localization profile of 2xGAL-αSyn. The same strains used in the dilution growth assay were used to monitor the effect of Hsp31 expression on αSyn localization. We quantified the number of cells with specific localization profiles at 8 h post-induction and observed that 60% of cells exhibited αSyn foci when only expressing αSyn, but only 33% of cells co-expressing αSyn and Hsp31 contained foci (Fig. 3, A and B). We observed that the suppression of αSyn foci by pGAL HSP31 was transient because 24 h after induction, the percentage of cells with foci increased and was similar to the percentage observed for cells expressing αSyn alone. This result is consistent with our rescue experiments, which were only successful when HSP31 was expressed from the GPD promoter (Fig. 2, A and B) and not when expressed from the GAL promoter (data not shown).

The expression of αSyn has been demonstrated to increase the level of ROS in yeast (51). The in vivo suppression of αSyn foci by Hsp31 and increased toxicity of one copy αSyn in the hsp31Δ strain prompted us to investigate the level of ROS in these different genetic contexts. We found that wild-type yeast did not have detectable superoxide radicals when treated with dihydroethidium using microscopy and a fluorescence level of 0.5 arbitrary units by flow cytometry (Fig. 3, C and D). ROS levels were detectable in a fraction of cells in the hsp31Δ strain (6 arbitrary units), indicating that the presence of Hsp31 in the cell can decrease ROS levels of normal cells not expressing αSyn. The remaining strains had increased ROS levels in the following order: αSyn-YFP strain (23 arbitrary units), αSyn-YFP hsp31Δ (41 arbitrary units), αSyn-YFP αSyn-CFP (64 arbitrary units), and αSyn-YFP αSyn-CFP hsp31Δ (133 arbitrary units). The latter three strains with the highest ROS levels exhibit reduced viability when αSyn is expressed and form increased foci, demonstrating a correlation between these phenotypes.

**Hsp31 Is an Integral Part of the Yeast Cellular Stress Response**—Multiple studies have shown that environmental stresses, such as oxidative stress and increasing temperature, increase the expression of Hsp31 (26, 52). A recent study demonstrated increased HSP31 mRNA levels upon treatment with H2O2, during the diauxic shift growth phase and stationary phase (22). However, the Hsp31 protein level was not characterized under these conditions. We generated a yeast strain with a genomically integrated 9myc epitope at the C terminus of the HSP31 locus to quantify protein expression. We examined Hsp31 protein expression levels during growth in rich media relative to optical density and demonstrated that Hsp31 expression could be divided into three phases. We found that Hsp31 expression decreased during log phase and then reached maximum expression during early stationary phase relative to the cell density of the culture and remained at approximately the same level in stationary phase when cell density was saturated after growth for 24 h (Fig. 4A). This distinctive expression pattern results in the initiation of elevated expression of Hsp31 corresponding to the diauxic shift, which occurs between log phase and stationary phase (26). To assess whether Hsp31 expression increases under other stress conditions, we exposed cells to H2O2 and observed increased protein expression within 1 h of exposure (Fig. 4B). This increased expression in response to an ROS-inducing agent has been observed previously (22, 26).

We also examined the expression profile of Hsp31 to a proteotoxic stress in the form of αSyn overexpression, and the results indicated that increased expression of αSyn decreases cell viability and concomitantly increases the expression of Hsp31 during log phase compared with a strain not expressing αSyn (Fig. 4, C–E). The characteristic decrease in expression during log phase, 6–9 h postinoculation, is not observed when αSyn is expressed. Our data demonstrate that Hsp31 expression is rapidly elevated at the protein level in the event of
growth stress associated with diauxic shift, oxidative stress, and proteotoxicity.

Hsp31 Methylglyoxalase Activity Is Not Required for Rescue of αSyn-mediated Toxicity—Recent studies have shown that DJ-1 and Hsp31 are methylglyoxalases that convert MGO into D-lactate in a single step independent of GSH (53, 54). Hsp31, like other members of the DJ-1 superfamily, possesses the conserved Glu-Cys-His catalytic triad (18). The first two residues of this triad are critical for MGO activity of DJ-1 as well as Hsp31 in Schizosaccharomyces pombe (54). We determined the methylglyoxalase activity for recombinant Hsp31 (purified from E. coli with the GST tag removed to retain 3 amino acids, GPL, on the C terminus) and a catalytic triad mutant, Hsp31 C138D, using a commercially available D-lactate assay kit. As expected,
Hsp31 could convert MGO into D-lactate with a calculated specific activity of 28.4 μmol/min/mg enzyme (using a saturating substrate concentration of MGO at 6 mM). These enzymatic parameters indicate a more efficient enzyme activity than the previously reported Hsp31-specific activity of 10.5 D-lactate/μmol/min/mg (53). The Hsp31-MORF purified from yeast had similar enzymatic parameters as the recombinant Hsp31 (data not shown). Enzymatic parameters were calculated by measuring the time-dependent production of D-lactate with varying substrate concentrations and fitting to a Michaelis-Menten kinetics model, resulting in a $V_{\text{max}}$ of 0.0356 μmol of D-lactic acid/min (S.E. = 0.0016), and $K_m$ was 247.4 μM (S.E. = 31.5) (Fig. 5A). Specific activity for the mutant could not be determined because low levels of D-lactate were produced despite using 5 times the amount of protein (detection limit of 0.4 μmol/min/mg). The reaction products of these assays were examined by GC-MS, and a peak identical to the lactic acid standard (6.09-min elution time) was identified, confirming the production of lactic acid by Hsp31 (Fig. 5B). Lactic acid was undetectable by GC-MS analysis in the Hsp31 C138D sample (data not shown).

We next determined whether methylglyoxalase activity is required for rescue of αSyn toxicity. One copy of αSyn-YFP decreases fitness in the hsp31Δ strain, but GPD-driven expression of Hsp31 rescues that toxicity. The expression of GPD-driven Hsp31 C138D restored the αSyn-expressing strain to full viability comparable with expression of the wild-type Hsp31 (Fig. 5C). The lack of enzyme activity for this mutant and the
ability to rescue αSyn toxicity indicated that the in vivo mechanism of rescue is not dependent on methylglyoxalase activity. MGO is a toxic metabolite produced during glycolysis (53–55) that reacts with proteins to yield toxic advanced glycation end products (56, 57) and has been associated with increased αSyn cross-linking and formation of intracellular foci formation in neuroblastoma cells (58). We examined whether increased levels of MGO could increase the αSyn foci or toxicity by treating cells with exogenous MGO and found no increased foci in wild-type yeast, indicating that their formation is probably not influenced by MGO. In addition, the hsp31Δ strain also did not have increased foci, suggesting that lack of Hsp31 is not sufficient to increase foci formation due to MGO treatment (Fig. 6, A and B). We determined that MGO was toxic to wild-type yeast at 10 and 20 mM MGO, and viability in the hsp31Δ strains was similar to wild type. These results are consistent with the production of d-lactic acid by Hsp31 in the enzymatic reaction. C, overexpression of pGPD HSP31 or the C138D mutant rescues toxicity from αSyn-expressing strains.

Hsp31 is a methylglyoxalase that produces d-lactic acid, and the enzyme activity is not necessary for rescuing αSyn toxicity. A, the plot of substrate concentration versus rate of d-lactate production by Hsp31 is depicted, and the Michaelis-Menten best fit model is represented by the solid line; V_max and K_m were determined based on this model. Mean values of triplicate experiments are plotted. Error bars, S.D. B, GC-MS trace demonstrates a peak (6.09 min) consistent with the production of d-lactic acid by Hsp31 in the enzymatic reaction. C, overexpression of pGPD HSP31 or the C138D mutant rescues toxicity from αSyn-expressing strains.
were present (Fig. 7B). The greatly reduced presence of soluble but SDS-resistant oligomers indicates that Hsp31 may interact with the monomer or an early oligomeric species to prevent formation of later stage oligomers. The same reactions were monitored using the ThioT assay, and only a baseline level of fluorescence was observed in the presence of Hsp31, further supporting the hypothesis that Hsp31 interacts with an species that precedes larger oligomers detected by ThioT (Fig. 7C). The ability of mutants to reduce fibrillization is consistent with our observation that the mutants suppress αSyn toxicity in yeast (Fig. 5C).

**Hsp31 Prevents Formation of Large Prion Aggregates**—The demonstration that Hsp31 possesses chaperone activity in a variety of anti-aggregation assays prompted us to investigate whether Hsp31 could also modulate the oligomeric state of yeast prion proteins, such as the Sup35 prion. The aggregation state of prions in living cells can be monitored by fusion of fluorescent protein tags to prion-forming domains (PrD), so we overexpressed Hsp31 to determine whether it modulated the aggregation of Sup35 PrD. Consistent with previous findings, cells overexpressing PrD in the presence of endogenous Sup35 showed characteristic ribbon-like or punctate aggregates, which were localized around the vacuoles and/or adjacent to plasma membranes (Fig. 8A). The number of cells co-expressing pGPD-HSP31-DsRed and pGAL-PrD-Sup35-EYFP had ~3-fold fewer Sup35 fluorescent aggregates compared with cells harboring pGAL-PrD-Sup35-EYFP plus vector control, indicating that Hsp31 can inhibit the formation of microscopically visible Sup35 prion aggregates (Fig. 8, A and B). The intrinsic fluorescence of Sup35-EYFP appears to increase upon aggregation, which allowed us to utilize flow cytometry as an alternative method to quantitatively measure and show decreased Sup35 aggregation (Fig. 8C). This phenomenon has not been exploited widely but was noted in at least one previous
study investigating the aggregation of a huntingtin-GFP fusion protein (61). Cell cultures co-expressing Sup35 and Hsp31 used in the microscopy experiments were prepared for flow cytometry and analyzed for EYFP fluorescence intensity. The flow cytometry results were similar to the microscopy results because Hsp31-expressing cells consistently exhibited lower median Sup35-EYFP fluorescence intensity when compared with cells containing Sup35-EYFP expression plasmid and empty vector (Fig. 8C). Individual traces depicting the number of counting events for each yeast strain also show the decrease in cellular fluorescence intensity when Hsp31 is expressed (data not shown).

Expression of Hsp31 with the GPD promoter was diffuse and cytoplasmic and varied in intensity in individual cells across the total cell population. The cytoplasmic localization profile of Hsp31 was not altered when PrD-Sup35 was expressed (Fig. 8A). Interestingly, we observed overlapping localization in cells with Sup35 aggregates and relatively high levels of Hsp31, but the proteins do not appear to mutually co-localize (Fig. 9A). In some cells, it was evident that the DsRed signal was decreased in the area where there was a Sup35 aggregate, suggesting that Hsp31 is not incorporated in aggregates (Fig. 9A). The aggregate appears to occlude Hsp31, and the diffuse overlapping signal observed in some cells is consistent with the presence of Hsp31 throughout the cytoplasm surrounding the aggregate. We also confirmed that Hsp31 overexpression reduces the formation of in vivo aggregates based on assessing cell lysates by SDD-AGE. We found that SDS-resistant Sup35 aggregates were greatly reduced when Hsp31 was overexpressed. The positive control, Hsp104, completely eliminated detectable Sup35 aggregates (Fig. 9B). These results are consistent with the ability of Hsp31 to inhibit the formation of large protein aggregates.

We also ascertained whether other chaperones are induced upon overexpression of Hsp31. We demonstrated that Hsp70 and Hsp104 expression are not altered, and hence elevated expression of these chaperones is not responsible for the observed suppression of prion aggregates (Fig. 9C). This is in agreement with our in vitro aggregation studies establishing that purified and recombinant Hsp31 protein is solely sufficient.

FIGURE 7. Hsp31 and catalytic mutants prevent the formation of αSyn oligomers. A, flow chart depicting the fractionation of the αSyn fibrillization assay and detection of monomeric and oligomeric species. SDS-PAGE of the pellet and supernatant fractions was performed, and antibodies were used to detect αSyn followed by stripping of the membrane and reprobing with anti-HA tag antibody to detect Hsp31 (note that Hsp31 was purified using the MORF tag, but Protein A was removed proteolytically, and the HA tag is retained). B, Hsp31 suppresses the formation of SDS-resistant higher order oligomers detected with Western blotting of the pellet and supernatant fractions. C, Hsp31 catalytic triad mutants suppress αSyn fibrillization. All proteins were added at the same level of 2 μM. A.U., arbitrary units.
to prevent protein aggregation, although we cannot exclude the possibility that Hsp31 could collaborate with other chaperones to prevent prion aggregation in the cell.

Hsp31 Rescue of αSyn-mediated Toxicity Is Independent of the Autophagy Pathway—Recently, in several studies, autophagy has been identified as one of the main mechanisms to clear and recycle misfolded proteins and aggregates in the cytosol (62, 63). In addition to the ubiquitin proteasome system, autophagy is a major cellular function involved in clearance of misfolded and aggregated proteins, including αSyn aggregates (62). Deletion of autophagy genes, such as ATG5 or ATG7, leads to neurodegenerative disease in mice (64, 65). In yeast, deletion of the ATG1 gene reduced the clearance of αSyn aggregates (66). Deletion of HSP31 was also demonstrated to impair autophagy under carbon starvation conditions (22). These studies raise the possibility that Hsp31 could be promoting autophagy of aggregated protein. In order to exclude the possibility that the Hsp31 rescue effect on αSyn toxicity depends on autophagy, we deleted the ATG8 gene in the αSyn strains and assessed the synthetic lethal effects of αSyn expression in these strains. As expected, expression of αSyn in the atg8Δ strain resulted in decreased viability compared with the wild-type strain. The atg8Δ hsp31Δ strain had viability similar to that of the atg8Δ strain when αSyn was expressed (Fig. 10A). These results confirm that autophagy is a protective mechanism against αSyn toxicity. The lack of synthetic lethal defect between ATG8 and HSP31 with and without αSyn expression is consistent with genes that are in the same pathway and hence do not buffer each other. Expression of Hsp31 and the C138D mutant in the autophagy strains, atg8Δ αSyn or atg8Δ hsp31Δ αSyn, increased viability in rich YEP media compared with vector controls (Fig. 10B). We found that the rescue effect in synthetic media was not easily observed because expression of αSyn was not toxic in the atg8Δ strain background, but it was toxic in the hsp31Δ, indicating that media conditions are an important factor in this complex relationship (Fig. 10B, mid-
These results demonstrate that Hsp31 can rescue αSyn toxicity in autophagy-deficient cells.

**Discussion**

The roles of chaperones and proteases in maintaining integrity and activity of folded, partially folded, and misfolded proteins are well established. Members of the DJ-1/ThiJ/PfpI superfamily exist in prokaryotes and eukaryotes, and structural and biochemical analyses have established that they have multiple activities, including chaperone, protease, and enzymatic functions. Because of the multiple activities associated with this superfamily, we sought to characterize the chaperone activity of Hsp31 and delineate its contribution in protecting against cellular stress.

The crystal structure of yeast Hsp31 has been solved, and structural evidence shows that Hsp31 and hchA share high similarity, in terms of the position of a hydrophobic patch and a catalytic triad. Yeast Hsp31, *E. coli* hchA, and human DJ-1 are in the same DJ-1/PfpI/ThiJ superfamily, and the literature suggests that both DJ-1 and hchA have chaperone-like effects against protein aggregation. Despite these similarities, the structures differ in important details, and it could have been argued that Hsp31 might not possess chaperone activity based on the absence of 45 amino acids present in hchA and the distinct dimerization. We investigated whether the chaperone-like activities are conserved in Hsp31 and found that the protein showed broad chaperone activity on three different substrate proteins, with aggregation induced by different mechanisms.
CS aggregation is triggered by elevated temperature, and we demonstrated that Hsp31 suppresses CS aggregation 6-fold more efficiently than hchA and DJ-1. Hsp31 also suppresses insulin aggregation, demonstrating that Hsp31 can maintain chaperone activity in a reducing environment in contrast to the redox-dependent DJ-1, which does not have activity in this assay.

Hsp31 also exhibited considerable activity in an in vitro fibrillization assay and completely suppressed fibrillization at a molar ratio of 8.75:1 (αSyn/Hsp31) and maintained considerable activity at a ratio of 35:1. DJ-1 also had activity at substoichiometric levels but required higher molar ratios compared with Hsp31. We consistently observed that the Hsp31-MORF purified from yeast was more active than recombinant Hsp31, DJ-1, or hchA (Fig. 1, B and D). This could be due to the fusion tag type or fusion orientation affecting protein activity, which has been noted previously for hchA (17, 18). Further studies are needed to delineate the source of these activity differences, including the possibility that it is related to the purification procedure, oxidation state of the protein, oligomeric state, or post-translational modifications. The broad range of substrates and substoichiometric activity all indicate that Hsp31 is a chaperone with robust activity compared with other members of this superfamily.

Despite the fact that its cellular function is incompletely understood, αSyn is known to aggregate and form inclusions. These protein inclusions are precursors of Lewy bodies, the pathological hallmark of PD. Elucidating mechanisms of αSyn aggregation is important to understand PD pathogenesis, and the heterologous expression of αSyn in yeast established a useful platform to investigate αSyn biology (9). We extended the αSyn yeast model by demonstrating the modulation of αSyn toxicity and subcellular localization by Hsp31. Expression of 2xGAL-αSyn is toxic to yeast cells, and constitutive expression of Hsp31 from the GPD promoter rescued cells from αSyn-mediated toxicity (Fig. 2). In addition, expression of one copy of GAL-αSyn is not toxic to wild-type cells but is toxic in the hsp31Δ strain. Taken together, it appears that the chaperone effect of Hsp31 is necessary to maintain cellular fitness upon αSyn expression.

Hsp31 also altered the subcellular localization profile of αSyn. Expression of 2xGAL-αSyn results in cytosolic accumulations (foci), whereas the expression of one copy of GAL-αSyn results in primarily plasma membrane localization. The in vivo...
cytoplasmic foci are associated with vesicular membranes (39), and analysis of cellular lysates indicates the presence of higher order oligomeric forms of αSyn, although the nature of this aggregation is unclear (23, 67). Expression of GAL-HSP31 results in more than 2-fold suppression of foci and increases the localization of αSyn to the plasma membrane (Fig. 3A). This effect was transiently observed at 8 h postinduction, but a high rate of puncta formation returned after 24 h, possibly because Hsp31 expression was induced at the same time as αSyn. This is consistent with the result where GAL-HSP31 does not rescue the synthetic lethal effect of αSyn expression in the hsp31Δ strain, whereas the constitutive GPD-HSP31 rescues hsp31Δ. These results indicate that a higher threshold level of Hsp31 is needed in the cell prior to αSyn induction to prevent toxicity. The exact temporal relationship between Hsp31 expression and αSyn foci suppression could provide insight into the aggregation state of αSyn species targeted by Hsp31.

Several orthologous experiments suggest that Hsp31 interacts early in the misfolding process and prevents the formation of larger oligomeric species. The addition of Hsp31 to αSyn monomers in a fibrillization assay resulted in a baseline level of ThioT fluorescence signal, indicating that the formation of larger oligomeric species was prevented (Fig. 1D). Analysis of the SDS-resistant oligomeric species by SDS-PAGE demonstrated that the presence of Hsp31 prevented the formation of higher order oligomeric αSyn species (Fig. 7A). Our prion aggregation studies indicate that Hsp31 inhibits prion assembly before the formation of subcellular visible GFP-tagged PrD aggregates and those detected by SDD-AGE (Fig. 9B). Furthermore, the Sup35 aggregates occlude Hsp31, indicating that the anti-aggregation activity of Hsp31 commences prior to the formation of the visible aggregate and probably does not act to remove or disassemble any preformed aggregate (Fig. 9A). This is in contrast to large chaperones, Hsp104, Ssa1/2, Sis1, and Sse1, which mutually colocalize with prion aggregates (68). A previous study has demonstrated that small HSPs can inhibit the formation of Sup35 aggregates and found that Hsp26 and Hsp42 inhibit rare transient oligomers at distinctly different steps in the prion formation process (69). Further studies will be required to determine whether Hsp31 inhibits protein misfolding with a similar or distinct mechanism as Hsp26 or Hsp42. Overall, we demonstrate that Hsp31 can inhibit oligomerization or aggregation of many different proteins by intervening early in the process.

The role of Hsp31 in protecting cells from αSyn-mediated toxicity led us to investigate the expression of Hsp31 in response to proteotoxic stress and other types of stress. The expression pattern of Hsp31 in a genomically tagged strain decreased during log phase growth and increased at the diauxic shift and at saturation (Fig. 4). The increased expression of Hsp31 at the diauxic shift and in stationary phase could possibly be due to the stress of decreased nutrients or accumulation of metabolites associated with these growth phases and is part of a global transcriptional response program (70). An increase in Hsp31 expression in response to ROS has been observed previously (71), and we confirmed that our tagged strain behaved in a similar manner by demonstrating an increase in expression upon exposure to H₂O₂ (Fig. 4B). This ROS response has been implicated to be dependent on the Yap1 stress response transcription factor, and a shortened Hsp31 promoter (−313 bp relative to the ATG), which removes the Yap1 binding site (−363 and −353 bp relative to the ATG), has decreased Hsp31 expression in response to oxidative stress (72). However, we note that the shortened promoter also removes an additional stress response element (a CCCCT site at −379 to −383 from the ATG) in proximity to the Yap1 binding site. Stress response elements have been demonstrated to be the binding site for Msn2/4, and numerous stress-related yeast genes have been documented to be transcriptionally activated by these transcription factors (26), including genes up-regulated after the diauxic shift in stationary phase cells (26). Hsp31 appears to be part of the global transcriptional stress response by Yap1 and Msn2/4 or other stress-related transcription factors, such as Hsf1 or Gis1.

Finally, we also examined the response of Hsp31 expression during αSyn-initiated proteotoxic stress and observed an increased expression in cells in log phase. We measured an increase in superoxide levels upon αSyn expression consistent with a previous report that indicated that αSyn expression is associated with caspase-mediated ROS generation (73) (Fig. 3D). Deletion of HSP31 also leads to increased superoxide levels with or without αSyn expression. These results establish that Hsp31 has a role in response to ROS, and its expression is induced in response to several types of stress.

We demonstrated the robust chaperone activity of Hsp31, but the in vivo rescue effects could have been mediated by the Hsp31 methylglyoxalase activity or interaction with other biological pathways, such as the metabolic or autophagy pathway. We confirmed that Hsp31 does have methylglyoxalase activity and demonstrated that mutation of the catalytic triad residue, C138, results in greatly reduced methylglyoxalase activity (Fig. 5A and B). However, this mutation did not inactivate the chaperone activity in the αSyn fibrillization assay (Fig. 7C), and expression of GPD-HSP31 C138D rescued cells from αSyn-mediated toxicity (Fig. 5C). Furthermore, we showed that that exogenous MGO did not differentially affect viability, suggesting that Hsp31 does not rescue by reducing accumulation of this toxic metabolite in αSyn-expressing cells (Fig. 6).

We also demonstrated that the autophagy pathway is an important mediator in the αSyn toxicity because deletion of ATG8 resulted in synthetic lethal interaction with αSyn expression (Fig. 10A). This is consistent with atg1Δ or atg7Δ strains having synthetic lethal interactions with αSyn expression and having defects in clearing αSyn foci (74). However, we show that the hsp31Δ atg8Δ strain does not have increased αSyn-mediated toxicity when compared with atg8Δ, consistent with these genes occurring within the same synthetic lethal pathway. A recent result indicates that hsp31Δ strains are defective in autophagy (51), so the addition deletion of an autophagy gene should not increase toxicity. Interestingly, overexpression of Hsp31 in these autophagy-deficient strains can rescue cells from αSyn toxicity, indicating that the autophagy pathway is not essential for chaperone rescue (Fig. 10B). Autophagy may be beneficial for controlling αSyn toxicity, but it appears that chaperone activity of Hsp31 is also important. These experiments also are the first to delineate that enzymatic methylgly-
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Lysosomal activity of Hsp31 is not essential to rescue αSyn toxicity.

Our results suggest that Hsp31 functions as a cytoplasmic chaperone against a variety of misfolded proteins. Recent studies have demonstrated the facility of yeast to investigate pathogenic mechanisms underlying αSyn toxicity, including the identification of novel biological pathways that impinge on αSyn biology and small molecule modulators of αSyn toxicity (66, 69). In addition, the action of Hsp31 on αSyn may provide insight into the mode of toxicity of αSyn because recent evidence suggests that the αSyn toxic species is smaller than the visible aggregate (66). It should be noted that the prion expression studies done in this study were not completed with [PSI+] strains, but further studies using such strains would be of interest to determine the effect of Hsp31 on prion initiation and propagation.

We have established a framework that extends the yeast model for investigating mechanisms of αSyn toxicity in the context of the DJ-1/ThiJ/PfpI superfamily in yeast. Extension of this work will assist in elucidation of the chaperone-like mechanisms of Hsp31, and a comparison with DJ-1 may provide evolutionary insights into the activities of the DJ-1/Ppl/ThiJ superfamily. Our model may also be used to further delineate the nature of oligomeric species in the pathogenesis of PD and possibly what species of αSyn should be targeted therapeutically.

Author Contributions—C. T., K. A., J.-C. R., and T. H. wrote the paper. J.-C. R. and T. H. conceived and coordinated the study. C. T. constructed plasmids, strains, and purified proteins for this study. C. T. performed experiments and analyzed experiments in Figs. 1, 2, 3, 4, and 7. K. A. performed and analyzed experiments in Figs. 5, 6, 8, and 9. T. H. M. D. performed and analyzed the citrate synthase studies, K. M. G. ascertained the Hsp31 expression with oxidative stress, and J. M. A. provided technical assistance and analysis of αSyn protein purification and assessment. L. N. P. provided technical assistance and contributed to sedimentation ultracentrifugation studies. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank Dr. Susan Lindquist (MIT) and Dr. François Baneys (University of Washington) for strains and plasmids. Confocal imaging was assisted by Dr. Li Li (Purdue Live Cell Imaging Facility).

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