Schizosaccharomyces pombe Homologs of Human DJ-1 Are Stationary Phase-Associated Proteins That Are Involved in Autophagy and Oxidative Stress Resistance

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Abstract

The Parkinson’s disease protein DJ-1 is involved in various cellular functions including detoxification of dicarbonyl compounds, autophagy and oxidative stress response. DJ-1 homologs are widely found in both prokaryotes and eukaryotes, constituting a superfamily of proteins that appear to be involved in stress response. Schizosaccharomyces pombe contains six DJ-1 homologs, designated Hsp3101-Hsp3105 and Sdj1 (previously named SpDJ-1). Here we show that deletion of any one of these six genes somehow affects autophagy during prolonged stationary phase. Furthermore, deletions of each of these DJ-1 homologs result in reduced stationary phase survival. Deletion of sdj1 also increases the sensitivity of stationary-phase cells to oxidative stress induced by hydrogen peroxide (H2O2) whereas overexpression of sdj1 has the opposite effect. Consistent with their role in stationary phase, expression of hsp3101, hsp3102, hsp3105 and sdj1, and to a lesser extent hsp3103 and hsp3104, is increased in stationary phase. The induction of hsp3101, hsp3102, hsp3105 and sdj1 involves the Sty1-regulated transcription factor Atf1 but not the transcription factor Pap1. Our results firmly establish that S. pombe homologs of DJ-1 are stationary-phase associated proteins and are likely involved in autophagy and antioxidant defense in stationary phase of S. pombe cells.

Introduction

DJ-1 and Hsp31 proteins belong to two different subfamilies of the DJ-1/Hsp31/PfpI superfamily, which is composed of a wide variety of structurally similar but functionally diverse proteins [1–3]. Nearly all members of the DJ-1/Hsp31/PfpI superfamily have a conserved cysteine residue.

Human DJ-1 is a multifunctional protein implicated in cancer- and Parkinson’s disease (PD) [4–6]. Human DJ-1 has been found to modulate autophagy through the activation of the...
stress-activated JNK signaling pathway [7, 8]. Autophagy is a highly conserved catabolic process that degrades and recycles intracellular proteins and organelles to maintain energy homeostasis under nutrient-limiting conditions and to eliminate unwanted cellular material [9]. Besides, human DJ-1 is also a glutathione-independent glyoxalase (glyoxalase III) that detoxifies reactive dicarbonyl compounds such as glyoxal and methylglyoxal, which are toxic metabolites in living organisms [10]. It is generally believed that DJ-1 is primarily involved in resistance to oxidative stress and in protection against mitochondrial damage [11, 12]. However, the detailed molecular mechanisms remain to be elucidated.

The budding yeast *Saccharomyces cerevisiae* has four DJ-1 homologs, Hsp31, Hsp32, Hsp33, and Hsp34 (collectively called *S. cerevisiae* Hsp31 proteins), the latter three of which have nearly identical amino acid sequences. *S. cerevisiae* Hsp31 proteins exhibit extremely weak sequence similarity to DJ-1, indicating extraordinary degree of divergence between Hsp31 proteins and DJ-1. In contrast, these proteins all have significant sequence similarity to the *Escherichia coli* chaperone Hsp31 [13]. However, unlike *E. coli* Hsp31 which is not involved in oxidative stress response [14] and like human DJ-1, *S. cerevisiae* Hsp31 has a role in the protection against oxidative stress [15].

It has recently been shown that all *S. cerevisiae* Hsp31 proteins associate with autophagy [16]. Deletions of each individual of *S. cerevisiae* HSP31 genes impair cell survival under nutrient-depleted environments and autophagy under carbon starvation. *S. cerevisiae* Hsp31 proteins appear to function upstream of the target of rapamycin complex 1 (TORC1) since deletion of HSP31 disrupts localization of TORC1 to processing bodies (P-bodies), and causes abnormal TORC1-mediated phosphorylation of Atg13. Under nutrient-rich condition, TORC1 phosphorylation of Atg13 prevents its binding to the autophagy-related (Atg) protein Atg1. The binding of Atg1 to Atg13 is required for the kinase activity of Atg1, which is required for the induction of autophagy. In addition, *S. cerevisiae* Hsp31 functions as a glyoxalase III [17–20], a stress-responsive chaperone [21] and a protein deglycase [22].

We have identified six *Schizosaccharomyces pombe* DJ-1 homologs, designated Hsp3101-Hsp3105 (collectively called *S. pombe* Hsp31 proteins) and Sdj1 (previously named SpDJ-1) [19]. Unlike *S. cerevisiae* DJ-1 homologs, the sequences of *S. pombe* DJ-1 homologs are highly divergent; the percent identity varies from 10% to 58%. Among these genes, only Sdj1 exhibits a significant degree of sequence homology with human DJ-1 [19], whereas Hsp3101-Hsp3105 have significant sequence similarity to *E. coli* and *S. cerevisiae* Hsp31 proteins. We have recently shown that Hsp3101, Hsp3102 and Sdj1 are glyoxalase III enzymes and that the first two enzymes displayed significantly higher *in vitro* glyoxalase III activity than Sdj1 [19]. However, it is unclear whether *S. pombe* Hsp31 proteins and Sdj1 have a role in autophagy and oxidative stress.

In this study, we show that *S. pombe* DJ-1 homologs are somehow involved in autophagy and that Sdj1 is involved in oxidative stress. We also demonstrate that expression of *S. pombe* DJ-1 homologs are induced in stationary phase and that the induction is dependent on the mitogen-activated protein (MAP) kinase Sty1.

### Materials and Methods

#### Strains and media

*S. pombe* strains used in this study are listed in Table 1. Strain DY3510 expressing N-terminal CFP tagged Atg8 (CFP-Atg8) under the control of its endogenous promoter was obtained from L. L. Du. Strain ySY1 expressing N-terminal GFP tagged Atg8 (GFP-Atg8) under the control of its endogenous promoter was constructed as described [23]. The null mutants for DJ-1 homologs in *S. pombe* were constructed by one-step gene replacement [24]. For phenotypic analysis,
were constructed by cloning the 5' cassette for genes into which is a negative control in the CFP-Atg8 processing assay, or ySY1 to generate ySY15, template. The h+ his3-D1 leu1-32 ura4-D18 ade6-M210 Δhsp3102::kanMX6 GFP-atg8⋅::leu1

To assess the role of each DJ-1 homolog was deleted from the wild-type S. pombe strain yHL6381 as described [19]. To study the role of S. pombe DJ-1 homologs in autophagy, each DJ-1 homolog was deleted from DY3510 (for the CFP-Atg8 processing assay) or ySY1 (for fluorescence microscopy analysis). Briefly, the deletion cassettes for from DY3510 (for the CFP-Atg8 processing assay) or ySY1 (for fluorescence microscopy analysis). Briefly, the deletion cassettes for

| Strain | Genotype | Source |
|--------|----------|--------|
| yHL6381 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 | H. Levin |
| ySY1 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 GFP-atg8⋅::leu1 | This study |
| ySY2 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 Δhis3::GFP-atg8⋅::leu1 | This study |
| ySY3 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 Δhsp3101::his3 GFP-atg8⋅::leu1 | This study |
| ySY4 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 Δhsp3103::kanMX6 GFP-atg8⋅::leu1 | This study |
| ySY5 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 Δhsp3104::kanMX6 GFP-atg8⋅::leu1 | This study |
| ySY6 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 Δhsp3105::kanMX6 GFP-atg8⋅::leu1 | This study |
| ySY7 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 Δsdj1::kanMX6 GFP-atg8⋅::leu1 | This study |
| ySY8 | h+ leu1-32 Δhsp3101::kanMX6 CFP-atg8⋅::leu1 | This study |
| ySY9 | h+ leu1-32 Δhsp3102::kanMX6 CFP-atg8⋅::leu1 | This study |
| ySY10 | h+ leu1-32 Δhsp3103::kanMX6 CFP-atg8⋅::leu1 | This study |
| ySY11 | h+ leu1-32 Δhsp3104::kanMX6 CFP-atg8⋅::leu1 | This study |
| ySY12 | h+ leu1-32 Δhsp3105::kanMX6 CFP-atg8⋅::leu1 | This study |
| ySY13 | h+ leu1-32 Δsdj1::kanMX6 CFP-atg8⋅::leu1 | This study |
| ySY14 | h+ leu1-32 Δatg5::kanMX6 CFP-atg8⋅::leu1 | This study |
| ySY15 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 Δatg5::kanMX6 GFP-atg8⋅::leu1 | This study |
| yTW1 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 hsp3102-myc::kanMX6 | This study |
| yTW2 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 hsp3103-myc::kanMX6 | This study |
| yTW3 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 hsp3104-myc::kanMX6 | This study |
| yTW4 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 hsp3105-myc::kanMX6 | This study |
| ySY16 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 Δatf1::ura4 | This study |
| ySY17 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 Δsty1::ura4 | This study |
| ySY18 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 Δatf1::ura4 hsp3102-myc::kanMX6 | This study |
| ySY19 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 Δatf1::ura4 hsp3105-myc::kanMX6 | This study |
| ySY20 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 Δsty1::ura4 hsp3102-myc::kanMX6 | This study |
| ySY21 | h+ his3-D1 leu1-32 ura4-D18 ade6-M210 Δsty1::ura4 hsp3105-myc::kanMX6 | This study |

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each DJ-1 homolog was deleted from the wild-type S. pombe strain yHL6381 as described [19]. To study the role of S. pombe DJ-1 homologs in autophagy, each DJ-1 homolog was deleted from DY3510 (for the CFP-Atg8 processing assay) or ySY1 (for fluorescence microscopy analysis). Briefly, the deletion cassettes for hsp3101 and hsp3103 were constructed by cloning the 5' and 3' flanks of the genes into the SpeI-PstI and the SalI-KpnI sites pAF1-KaMX6 (in which the his3' selectable marker in pAF1 [25] was replaced by the KanMX6 marker), respectively. The deletion cassettes for hsp3102 and sdj1 were constructed by cloning the 5' and 3' flanks of the genes into the SmaI-BglII and Pmel-EcoRI sites of pFA6a-kanMX6 [26], respectively. The deletion cassette for hsp3103 was constructed by cloning the 5' and 3' flanks of the gene into the SpeI-PstI and SacI-EcoRI sites of pFA6a-kanMX6, respectively. The deletion cassette for hsp3104 were constructed by cloning the 5' and 3' flanks of the gene into the SalI-BglII and SacI-EcoRI sites of pFA6a-kanMX6, respectively. To generate Δatg5 deletion mutants which serve as negative controls in assays of autophagy, the atg5 deletion cassette containing the KanMX6 selection marker flanked by the 5' and 3' flanks of atg5 was obtained by PCR using genomic DNA isolated from the Δatg5 deletion mutant Zd299 (obtained from obtained from L. L. Du) as a template. The atg5 deletion cassette was transformed into either DY3510 to generate ySY14, which is a negative control in the CFP-Atg8 processing assay, or ySY1 to generate ySY15,
which is a negative control for fluorescence microscopy analysis. To generate Δsty1 and Δatf1 deletion mutants, the sty1 and atf1 deletion cassettes containing the ura4 selection marker flanked by the 5' and 3' flanks of target genes were constructed by overlapping PCR. To generate strains expressing hsp3102, hsp3103, hsp3104 or hsp3105 tagged with a C-terminal c-Myc epitope tag at their genomic loci for Western blot analysis, the 5' upstream and 3' downstream of these genes were PCR-amplified, and cloned into the Sall-Smal and SacI-SacII sites of pFA6a-13myc-kanMX6 [26], respectively. PCR primers are available upon request. All constructs were verified by PCR.

S. pombe strains were grown in rich yeast extract supplements medium (YES) or synthetic Edinburgh minimal medium (EMM) [27]. Standard protocols for genetic manipulation of fission yeast were used [28].

Plasmid construction
For overexpression of genes in S. pombe, hsp3101-hsp3105 and Sdj1 were PCR-amplified from the genomic DNA, cloned into the BamH I and Sma I sites of the S. pombe expression plasmid pREP3X [29].

Quantitative real-time RT-PCR
RNA was isolated from wild type S. pombe cells (yHL6381) using an E.Z.N.A. Yeast RNA Kit (OMEGA). Contaminating genomic DNA in RNA was removed by treatment with RNase-free DNase (Fermentas). RNA samples were reversed transcribed with the oligo (dT)16 primer using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Comparative qPCR analysis was performed using the StepOne™ RT-PCR system (Applied Biosystems) with each primer sets (S1 Table). All reactions were performed in triplicate. Data analysis was performed by StepOne™ software. The C_T values were normalized against actin mRNA levels from the same preparations to give the ΔC_T values. Fold changes in gene expression at different growth phases compared to the early exponential phase (OD600 = 0.5) were calculated by using the 2^-ΔΔC_T method.

CFP-Atg8 processing assay
Cell lysates were prepared by alkaline extraction [30]. Total proteins were separated by 12% SDS/PAGE and immunobotted with anti-GFP antibody (Roche).

Western blot analysis
S. pombe whole cell extracts were prepared by bead beating using a FastPrep-24 machine (MP Biomedicals) [30, 31]. Proteins were separated by 15% SDS/PAGE and analyzed by immunoblotting using anti-Hsp3101 antibody (1:5000), anti-c-Myc antibody (1:5000) to detect Hsp3102-Myc, Hsp3103-Myc and Hsp3104-Myc, anti-Sdj1 antibody (1:5000) and anti-Sla1 antibody (1:5000) as a loading control. IRDye 800CW conjugated goat anti-rabbit IgG (LI-COR Biosciences) was used as secondary antibody. The fluorescent bands were detected using an Odyssey near-infrared fluorescence scanner (LI-COR Biosciences).

Fluorescence microscopy
To detect the localization of GFP-Atg8 in wild type and mutant strains at different stages of growth, yeast strains expressing GFP-Atg8 from the promoter of Atg8 were grown in YES supplemented with 225 mg/l leucine, histidine, uracil and adenine at 30°C. Cells were harvested at different time points and cell pellets were suspended in PBS. The green fluorescence of EGFP
was detected using $\lambda_{ex}$ of 488 nm. All images were obtained on a Zeiss Axio Imager A1 microscope equipped with a PCO Sensicam CCD (charge-coupled-device) camera, and data were analyzed using MetaMorph image processing software (Universal Imaging).

Survival Assays

Cell viability was measured by counting colonies generated by viable individual cells. Cells were grown in YEPD media (0.5% yeast extract, 0.5% peptone and 1% glucose) overnight and diluted to an $OD_{600}$ of 0.2 in fresh medium and incubated at 30°C. An aliquot of each culture at the indicated time points were taken, serially diluted and plated on YES plates. Sensitivity to oxidative stress was evaluated by spotting assays. Fresh colonies were grown in liquid YES or leucine-selective media overnight, serially diluted, normalized to an $OD_{600}$ of 3.0, and 3 $\mu l$ of cells were spotted on rich YES or leucine-free minimal EMM plates without or with concentration of hydrogen peroxide. The plates were photographed after 5 days of incubation at 30°C.

Results

Deletion of $hsp3104$ and $sdj1$, and to a lesser extent, $hsp3102$ and $hsp3105$, leads to a delay in CFP-Atg8 processing in stationary phase

We investigated whether $S.\ pombe$ homologs of DJ-1 have a role in autophagy. To this end, we employed the CFP-Atg8 processing assay which is widely used to monitor autophagy over time from yeast to human [32–34]. This assay is based on the fact that upon induction of autophagy, CFP-Atg8 is attached on the inner membrane of the autophagosome, delivered to the vacuole and processed, generating the protease-resistant free CFP. Thus, the protein level of CFP detected by Western blot can be used to estimate the autophagic activity. We carried out systematic gene disruptions of individual genes encoding DJ-1 homologs in a $S.\ pombe$ strain expressing CFP-Atg8. All six genes could be disrupted individually without eliminating the growth of $S.\ pombe$ cells.

We first examined autophagy in single-gene deletion mutants of all six $S.\ pombe$ DJ-1-related genes during growth under rich-medium growth conditions. In the logarithmic growth phase (Log phase), only one protein band corresponding to CFP-Atg8 was detected in the wild-type and all deletion mutants (Fig 1A). Free CFP was detected in wild-type and all deletion mutants during prolonged stationary phase (Fig 1). However, the appearance of free CFP was considerably delayed in $\Delta hsp3104$ and $\Delta sdj1$ mutants and, to a lesser extent, in $\Delta hsp3102$ and $\Delta hsp3105$ mutants compared to the wild-type strain (Fig 1B and 1C). At 144 h time point, the level of free CFP in all deletion mutants was comparable to that of the wild-type strain. Deletion of autophagy-related gene $atg5$, which is necessary for autophagy, in the wild-type and deletion mutant background completely abolished CFP-Atg8 processing (Fig 1E), indicating that CFP-Atg8 processing in stationary-phase cells is the ATG-dependent autophagic process. Altogether, these results indicated that Hsp3104 and Sdj1 and, a lesser extent, Hsp3102, Hsp3105 are involved in, but not essential for the normal autophagy in prolonged stationary phase.

Since fission yeast autophagy is induced by nitrogen starvation but not carbon starvation and rapamycin treatment [35], we next examined whether $S.\ pombe$ DJ-1 homologs play a role in autophagy under nitrogen starvation conditions. Autophagy occurred at a basal level under normal growth conditions (data not shown). Under nitrogen starvation conditions, both wild-type strain and the deletion mutants showed a significant increased level of CFP-Atg8. In addition, the DJ-1-related gene deletion mutants had levels of autophagy similar to that of the wild-type strain (data not shown).
S. pombe DJ-1 homologs are involved in the localization of Atg8 at PAS

Next, we examined whether S. pombe DJ-1 homologs play a role in the localization of Atg8 at the phagophore assembly site/pre-autophagosomal structure (PAS), which is a unique site where most autophagy-related proteins act together to form the double-membraned vesicles known as autophagosomes. Since GFP gives a brighter signal than CFP in our hands, we integrated a GFP tag at the N-terminus of endogenous Atg8 in a wild-type strain and all deletion mutants of S. pombe DJ-1 homologs. Fluorescence microscopy revealed that GFP-Atg8 was
dispersed in the cytoplasm during log phase. After entering stationary phase, a few GFP-Atg8 punctate signals corresponding to the PAS were readily detected in wild type cells and all deletion mutants but not in the atg5 deletion mutant (Fig 2A). However, the detection of the GFP-Atg8 puncta in Δsdj1 cells were 12 h later than wild-type and other deletion mutants (Fig 2A). In addition, all deletion mutants of S. pombe DJ-1 homologs accumulated more GFP-Atg8 puncta than the wild type cells (Fig 2A). Similar results were observed in deletion mutants of S. pombe autophagy-related genes atg2, atg18b and atg18c [23]. Quantitative analysis of GFP-Atg8 puncta in the wild-type and deletion mutants of S. pombe DJ-1 homologs is summarized in Fig 2B.

sdj1 deletion resulted in increased sensitivity to H2O2, while sdj1 overexpression led to increased resistance to H2O2.

Since DJ-1 is involved in oxidative stress, we were next interested in determining whether S. pombe Hsp3101-Hsp3105 and Sdj1 are also involved in resistance to hydrogen peroxide-induced oxidative stress. To this end, wild-type cells and deletion mutants of S. pombe DJ-1 homologs were grown to either log or stationary phase, and sensitivity to oxidant H2O2 were determined by spotting assays using H2O2 concentrations in a range of 0–4.5 mM. As shown in Fig 3, sdj1 deletion did not alter log phase cell sensitivity to H2O2. However, the sdj1 deletion mutant in stationary phase showed increased sensitivity to H2O2 compared to the wild-type strain. In contrast, other deletion mutants showed similar sensitivity to H2O2 compared to the isogenic wild-type strain (data not shown).

We next determined whether overexpression of sdj1 in the wild-type cells could modulate the sensitivity of S. pombe cells to H2O2. The wild-type cells that overexpressed sdj1 from the pREP3X nmt1 promoter survived better than cells transformed with the empty pREP3X vector (Fig 3B). These results showed that overexpression of sdj1 increased the survival of wild-type stationary-phase cells in the presence of exogenous H2O2.

Deletion of S. pombe DJ-1 homologs reduces the survival of stationary phase cells

In our prior study, we have previously shown that deletion of hsp3101-hsp3104 and sdj1 has no effect on cell growth under normal conditions [19]. Because the S. pombe homologs of DJ-1 appeared to function in stationary phase, we determined whether deletion of S. pombe DJ-1 homologs affected cell survival in stationary phase. When cells were grown in rich YES medium, there was no difference in survival between the wild-type and deletion mutants of S. pombe DJ-1 homologs (data not shown). Deletion of S. pombe homologs of DJ-1 resulted in reduced viability in stationary phase when cells were grown in low-glucose rich YEPD medium (1% glucose) (Fig 4).

Genes encoding S. pombe DJ-1 homologs are up-regulated during stationary phase

Because S. pombe homologs of DJ-1 seem to function in stationary phase, we asked whether their expression was induced in stationary phase. To this end, we first determined the relative mRNA levels of the genes encoding S. pombe DJ-1 homologs using quantitative RT-PCR (qRT-PCR) and primers specific for each S. pombe DJ-1 homolog. As shown in Fig 5A, expression of all S. pombe DJ-1 homologs was up-regulated at stationary phase. hsp3101 and hsp3102 were induced ~170-fold and ~58-fold, respectively, at the 36 h time point and were the most highly induced genes in the stationary phase. In contrast, hsp3103 and hsp3104 were induced
The Role of S. pombe DJ-1 Homologs during Stationary Phase

A

| 12 h | 24 h | 36 h | 48 h | 60 h | 72 h |
|------|------|------|------|------|------|
| WT   | Δhsp3101 | Δhsp3102 | Δhsp3103 | Δhsp3104 | Δhsp3105 |
|      | Δsdj1   | Δatg5   |          |          |        |

B

![Graph showing percentage of cells exhibiting more than one punctum over time](image)

- WT
- Δhsp3101
- Δhsp3102
- Δhsp3103
- Δhsp3104
- Δhsp3105
- Δsdj1
- Δatg5

Time (hours)

0 10 20 30 40 50 60 70 80 90 100

Percentage of cells exhibiting more than one punctum
only ~3-fold and ~9-fold, respectively (Fig 5A). hsp3105 and sdj1 were induced ~37-fold and ~17-fold, respectively.

To validate the results of qRT-PCR, we examined whether the protein levels for S. pombe Hsp3101-Hsp3105 and Sdj1 were increased in stationary phase. Because only antibodies against Hsp3101 and Sdj1 are available, we generated four strains that endogenously expressed C-terminally thirteen-Myc-tagged Hsp3102-Hsp3105 in the wild type strain yHL6381. In agreement with the qRT-PCR data, Western blot analysis revealed that the proteins levels for Hsp3101, Hsp3102, Hsp3105 and Sdj1 were significantly up-regulated in stationary phase (Fig 5B). However, despite our efforts, we were unable to detect Hsp3103-Myc and Hsp3104-Myc by Western blot using anti-Myc antibody, most likely due to very low levels of expression of these two proteins. These results suggest that the S. pombe homologs of DJ-1 are functionally associated with the stationary phase.

We next tested whether the stress-activated MAP kinase Sty1 (also known as Spc1), which plays an essential role in the cellular response to nutrient deprivation and environmental stresses, was required for the induction of S. pombe DJ-1 homologs. To this end, we disrupted sty1 in the wild-type yHL6381 and measured the mRNA expression levels of S. pombe hsp31 genes and sdj1 in Δstry1. Consistent with the previous findings that deletion of any component of the Spc1-Atf1 pathway causes rapid loss of cell viability after cells enter stationary phase [36], Δstry1 cells lost viability dramatically in stationary phase (data not shown). It was very difficult to isolate high quality RNA after 48 h incubation due to RNA degradation. Deleting sty1 almost completely abolished the stationary phase induction of S. pombe hsp3101-hsp3105 and sdj1 (Fig 6A). Since Sty1 regulates stress-dependent transcription, at least in part, through the basic-region leucine-zipper (bZIP) transcription factor Atf1 [37, 38], we constructed an Δatf1 strain and found that induction of hsp3102 and sdj1 expression was essentially abolished in Δatf1 cells, whereas induction of hsp3101 and hsp3105 was attenuated (Fig 6B). hsp3101 and hsp3105 expression was induced ~19-fold and ~8-fold, respectively, at 48 h time point (Fig 6B). By contrast, induction of hsp3103 and hsp3104 expression appeared to be unaffected in Δatf1 cells (Fig 6B).

Besides the Sty1 pathway, the Pap1-mediated regulatory pathway is required for the expression of genes involved in the adaptation to oxidative stress in fission yeast [39]. To determine if Pap1 is involved in the induction of S. pombe DJ-1 homologs, we deleted pap1 in the wild-type strain. Deletion of pap1 did not affect the induction of S. pombe DJ-1 homologs (Fig 6C).

To verify these results, we performed Western blotting with antibodies against Hsp3101 and Sdj1, and anti-Myc antibody to detect Hsp3102 and Hsp3105. Consistent with the qRT-PCR results, deletion of either sty1 or atf1 resulted in the reduction of Hsp3101, Hsp3102, Hsp3105 and Sdj1 protein levels (Fig 7).

We analyzed the 5’ upstream regions of S. pombe hsp31 genes and sdj1 to understand how these genes are activated. We found that the promoter regions of hsp3101-hsp3103 and sdj1 possess a core environmental stress response (CESR) motif, which is homologous to the ATF/CRE motif and is found in S. pombe genes in response to stress condition [40] (S2 Table). In addition, the promoter region of sdj1 contains an ATF/CRE motif, which is the binding site for the ATF/CREB family of transcription factors, and is required for stress-dependent activation of transcription [36, 38]. These motifs are candidate Aft1 binding sites [40].
### A

|   | LP | SP | \(H_2O_2\) |
|---|----|----|------------|
| WT | ![WT LP](image1) | ![WT SP](image2) | 0 mM |
| \(\Delta sdj1\) | ![\(\Delta sdj1\) LP](image3) | ![\(\Delta sdj1\) SP](image4) | 1.5 mM |
| WT | ![WT LP](image5) | ![WT SP](image6) | 2.5 mM |
| \(\Delta sdj1\) | ![\(\Delta sdj1\) LP](image7) | ![\(\Delta sdj1\) SP](image8) | 3.5 mM |
| WT | ![WT LP](image9) | ![WT SP](image10) | 4.5 mM |
| \(\Delta sdj1\) | ![\(\Delta sdj1\) LP](image11) | ![\(\Delta sdj1\) SP](image12) | |

### B

|   | LP | SP | \(H_2O_2\) |
|---|----|----|------------|
| pREP3X sdj1 | ![pREP3X SDj1 LP](image13) | ![pREP3X SDj1 SP](image14) | 0 mM |
| pREP3X sdj1 | ![pREP3X SDj1 LP](image15) | ![pREP3X SDj1 SP](image16) | 1.5 mM |
| pREP3X sdj1 | ![pREP3X SDj1 LP](image17) | ![pREP3X SDj1 SP](image18) | 2.5 mM |
| pREP3X sdj1 | ![pREP3X SDj1 LP](image19) | ![pREP3X SDj1 SP](image20) | 3.5 mM |
| pREP3X sdj1 | ![pREP3X SDj1 LP](image21) | ![pREP3X SDj1 SP](image22) | 4.5 mM |
Discussion

Stationary phase cells undergo major metabolic and transcriptional reprogramming to adapt to nutrient starvation and various stress conditions due to accumulation of toxic metabolites, characteristic of stationary-phase cultures. We have demonstrated that in *S. pombe* under normal growth conditions, autophagy is induced in prolonged stationary phase. Induced
A

![Graph showing relative fold increase of various genes (
sp3101, hsp3102, hsp3103, hsp3104, hsp3105, and sdj1) at different time points (7 h, 12 h, 17 h, 36 h, and 48 h).]

B

| Time (hrs) | 12 | 24 | 36 | 48 | 60 | 72 |
|------------|----|----|----|----|----|----|
| Anti-Hsp3101 | ![Image of Hsp3101 antibody at different time points](image) | ![Image of Hsp3102 antibody at different time points](image) | ![Image of Sdj1 antibody at different time points](image) | ![Image of Sla1 antibody at different time points](image) | ![Image of Hsp3105 antibody at different time points](image) | ![Image of Sla1 antibody at different time points](image) |
The Role of S. pombe DJ-1 Homologs during Stationary Phase

Fig 5. Induction of S. pombe homologs of DJ-1. (A) qRT-PCR analysis of expression of S. pombe hsp3101-hsp3105 and sdj1 genes in the wild-type cells. Total RNA was isolated from the wild-type cells grown in YES medium at the indicated time points. All mRNA levels were normalized to the control act1+ mRNA level and were expressed as fold change relative to the mRNA levels at the 7 h time point, which was set at a value of 1. Data are presented as mean ± SD (p ≤ 0.01; t test). (B) Immunoblot analyses of Hsp3101, Hsp3102, Hsp3105 and Sdj1 expression in wild-type cells. Crude extracts were prepared from the wild-type cells at indicated time points (h). Total proteins were separated on SDS/PAGE gels and immunoblotted using anti-Hsp3101 Ab, anti-Sdj1 Ab, anti-Myc Ab, which detects Hsp3102-Myc and Hsp3105-Myc, and anti-Sla1 Ab (serves as a loading control).

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Autophagy may provide a way for cells in prolonged stationary phase to overcome nutrient-depletion conditions. Deletion of hsp3102-hsp3105 and sdj1 delays vacuolar degradation of CFP-Atg8, as indicated by delayed proteolytic cleavage of CFP-Atg8. Further, the localization of Atg8 at PAS appears to be influenced by S. pombe Hsp3101-Hsp3105 and Sdj1 proteins. The number of GFP-Atg8 puncta is increased in all deletion mutants of S. pombe DJ-1 homologs. Generation of Atg8 puncta is somewhat delayed in the Δsdj1 deletion mutant. Thus, in S. pombe, individual DJ-1 homologs appear to be involved in, but are not absolutely required for the execution of autophagy in prolonged stationary phase. However, we cannot rule out the possibility that the deletion of all S. pombe homologs of DJ-1 may have a synergistic effect on autophagy. Addressing this possibility would require the generation of a mutant strain in which all S. pombe DJ-1 homologs are deleted. However, we failed to obtain such a mutant strain due to technical difficulties. In addition, S. pombe DJ-1 homologs are not involved in autophagy under nitrogen starvation conditions (data not shown). These results are in sharp contrast to the situation in S. cerevisiae, where deletion of each of HSP31 genes severely impairs autophagy during stationary phase and under carbon starvation conditions, indicating that each S. cerevisiae HSP31 gene is required for autophagy under these conditions.

Autophagy protein Atg13 plays a central role in the initial step of PAS formation and Atg13 phosphorylation is crucial for autophagy in S. cerevisiae cells [41]. As Atg13 phosphorylation has been shown to be altered in S. cerevisiae ΔHSP31 cells [16], we tried to examine whether there was an alteration in phosphorylated Atg13 in deletion mutants of S. pombe DJ-1 homologs. However, despite our efforts, we could not detect any endogenous Atg13 protein in S. pombe cells grown in the late stationary phase by Western blotting likely due to an extremely low level of endogenous Atg13 expression in S. pombe (data not shown). This is consistent with a previous report, which showed that endogenous Atg13 was barely detected in S. pombe cells grown under nitrogen starvation conditions [35].

In fission yeast, the Sty1 stress-activated protein kinase pathway, which is similar to the S. cerevisiae Hog1 pathway and the mammalian p38 MAP kinase pathway, plays a critical role in regulating transcriptional responses to nutrient starvation and other stresses [42, 43]. In this study we show that all S. pombe DJ-1 homologs are induced in stationary phase, albeit hsp3103 and hsp3104 are induced to a much lesser extent. Furthermore, the induction of these genes are dependent on the mitogen-activated protein kinase (MAPK) Sty1. Interestingly, deletion of atf1, which is the main substrate of Sty1 and regulates a large set of stress response genes, essentially abolishes hsp3102 and sdj1 induction, but does not affect the induction of hsp3103 and hsp3104 and only partially abolishes induction of hsp3101 and hsp3105 expression. Thus, the induction of hsp3102 and sdj1 is solely dependent on Aft1; hsp3103 and hsp3104 are induced by transcription factor (s) other than Aft1; the full induction of hsp3101 and hsp3105 expression requires Aft1 and additional transcription factor (s). Consistent with our results, the Pap1-mediated regulatory pathway is not involved in the induction of S. pombe DJ-1 homologs.

It is important to note that expression of S. pombe DJ-1 homologs is also induced by other stresses. A global analysis of transcriptional response of fission yeast to environment stresses shows that the expression of hsp3101, hsp3102, hsp3104 and sdj1 are increased during cellular
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A

![Graph A]

B

![Graph B]

C

![Graph C]
response to oxidative stress, osmotic stress, heat stress, and heavy metal stress (i.e., treatment with cadmium sulfate) [40]. In addition, a genome-wide analysis of gene expression under nitrogen-limiting reveals that hsp3105 expression is increased during nitrogen depletion [44].

Our previous and current data suggest that S. pombe DJ-1 homologs are involved in the stationary-phase stress responses. This conclusion is based on the following observations. First, S. pombe Hsp3101 and Hsp3102 are the major GSH-independent glyoxalase III that may have some role in protecting cells from reactive carbonyl species toxicity accumulated during the

![Image of Western blot analysis](doi:10.1371/journal.pone.0143888.g006)

**Fig 6. Expression levels of S. pombe DJ-1 homologs in wild-type, Δsty1, Δatf1 and Δpap1 cells.** S. pombe hsp3101-hsp3105 and sdj1 in Δsty1 (A), Δatf1 (B) and Δpap1 (C) were grown in YES medium, and total RNAs were extracted at indicated times and analyzed by qRT-PCR. Columns indicate mean values of at least three independent experiments; error bars represent standard error of the mean (SEM) (n = 3, separate experiments). Statistical analyses were performed using Student t test (*p < 0.05, **p < 0.01). Reliable data could not be obtained beyond the 48 h time point due to the loss of viability caused by sty1 or atf1 depletion.

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![Image of Western blot analysis](doi:10.1371/journal.pone.0143888.g007)

**Fig 7. Western blot analysis of Hsp3101, Hsp3102, Hsp3105 and Sdj1 in wild-type, Δsty1 and Δatf1 cells.** Wild-type, Δsty1 and Δatf1 cells were grown in YES medium. Cell lysates were prepared, and proteins were separated by SDS/PAGE and analyzed by immunoblotting with indicated antibodies. Sla1 serves as the loading control.

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stationary phase of yeast growth [19]. Second, *S. pombe* DJ-1 homologs appear to be involved but not essential for autophagy in stationary phase. Third, Sdj1 is likely to play a role in defense against oxidative stress during stationary phase. Fourth, expression of *S. pombe* DJ-1 homologs are induced in stationary phase in a Sty1-dependent manner. Interestingly, *S. pombe* DJ-1 homologs exhibit different patterns of subcellular localization [19]. Moreover, induction of *S. pombe* DJ-1 homologs may involve different transcription factors. Based on these observations, we speculate that *S. pombe* DJ-1 homologs may play overlapping yet distinct roles in stress responses.

**Supporting Information**

**S1 Table.** Nucleotide sequences of primers for qRT-PCR analysis.

(DOCX)

**S2 Table.** Identification of the CESR and ATF/CRE motifs in *S. pombe* homologs of DJ-1.

The CESR motif: TKACGT, where K is T or G; the ATF/CRE motif: KWCGTCA, where K is T or G and W is T or A.

(DOCX)

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**Author Contributions**

Conceived and designed the experiments: YH YS CC LH. Performed the experiments: YS CC LH. Analyzed the data: YH YS CC LH JY. Contributed reagents/materials/analysis tools: YH YS. Wrote the paper: YH YS.

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