Identification of glutathione (GSH)-independent glyoxalase III from *Schizosaccharomyces pombe*

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**Abstract**

**Background:** Reactive carbonyl species (RCS), such as methylglyoxal (MG) and glyoxal (GO), are synthesized as toxic metabolites in living systems. Mechanisms of RCS detoxification include the glutathione (GSH)-dependent system consisting of glyoxalase I (GLO1) and glyoxalase II (GLO2), and GSH-independent system involving glyoxalase III (GLO3). Hsp31 and DJ-1 proteins are weakly homologous to each other and belong to two different subfamilies of the DJ-1/Hsp31/PfpI superfamily. Recently, the *Escherichia coli* Hsp31 protein and the DJ-1 proteins from *Arabidopsis thaliana* and metazoans have been demonstrated to have GLO3 activity.

**Results:** We performed a systematic survey of homologs of DJ-1 and Hsp31 in fungi. We found that DJ-1 proteins have a very limited distribution in fungi, whereas Hsp31 proteins are widely distributed among different fungal groups. Phylogenetic analysis revealed that fungal and metazoan DJ-1 proteins and bacterial YajL proteins are most closely related and together form a sister clade to bacterial and fungal Hsp31 proteins. We showed that two *Schizosaccharomyces pombe* Hsp31 proteins (Hsp3101 and Hsp3102) and one *Saccharomyces cerevisiae* Hsp31 protein (ScHsp31) displayed significantly higher in vitro GLO3 activity than *S. pombe* DJ-1 (SpDJ-1). Overexpression of *hsp3101*, *hsp3102* and *ScHSP31* could confer MG and GO resistance on either wild-type *S. pombe* cells or GLO1 deletion of *S. pombe*. *S. pombe* DJ-1 and Hsp31 proteins exhibit different patterns of subcellular localization.

**Conclusions:** Our results suggest that fungal Hsp31 proteins are the major GLO3 that may have some role in protecting cells from RCS toxicity in fungi. Our results also support the view that the GLO3 activity of Hsp31 proteins may have evolved independently from that of DJ-1 proteins.

**Keywords:** Glyoxalase III, DJ-1, Hsp31, Methylglyoxal, Reactive carbonyl species

**Background**

Reactive carbonyl species (RCS) are usually produced as metabolites in living systems including prokaryotes, fungi, plants, and animals. Among RCS are the highly reactive α-carbonyl compounds methylglyoxal (MG) and glyoxal (GO). MG is formed primarily as a by-product of glycolysis via the β-elimination of phosphate from the glycolytic intermediates dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GA3P). GO is generated by oxidative degradation of lipid and DNA, and oxidation of glycolaldehyde. RCS can rapidly react with the amino groups in proteins, nucleic acids and lipids to form toxic or mutagenic advanced glycation endproducts (AGE) and cause carbonyl stress. RCS have been linked to diverse human diseases, including diabetes, neurodegenerative diseases (such as Parkinson and Alzheimer), and aging (for reviews, see [1-3]).

The major system for reactive α-carbonyl species detoxification in both prokaryotes and eukaryotes involves two enzymes, glyoxalase I (GLO1, EC 4.4.1.5) and glyoxalase II (GLO2, EC 3.1.2.6) (for reviews, see [4,5]). GLO1 converts MG into S-D-lactoylglutathione (SLG) with glutathione (GSH) as a catalytic cofactor, and then GLO2 hydrolyzes SLG to D-lactate and GSH. Most recently, a GSH-independent glyoxalase system was identified in *Escherichia coli*, *Caenorhabditis elegans*, mice and humans [6,7]. In this system, glyoxalase III (GLO3) converts MG directly into D-lactate and GSH. In animals, GLO3 activity appears to reside in DJ-1 proteins [6]. The GLO3 activity of DJ-1 proteins seems to play an important role in protecting cells against α-dicarbonyl-induced cell death [6]. Human DJ-1 (HsDJ-1)
is a cancer- and Parkinson’s disease (PD)-associated protein [8,9]. Besides playing a role in α-dicarbonyl detoxification, HsDJ-1 has multiple functions, including transcriptional regulation [10-19], regulation of mitochondrial function [20-25], molecular chaperone [26,27] and protease activities [28-30], and, most strikingly, protection against oxidative stress [31-36]. It appears that oxidation at the conserved cysteine residue (Cys106 in HsDJ-1) is critically required for DJ-1 function [22,37-39]. However, the mechanism of how HsDJ-1 executes these functions remains to be determined.

The closely related homologs of HsDJ-1 (hereafter referred to as DJ-1 proteins) are found in a wide variety of eukaryotes [40-43], but the species distribution of this gene appears to be complex. Unlike mammals, which contain a single DJ-1 gene, Drosophila melanogaster has two DJ-1 genes (DJ-1a and DJ-1b) [44], which differ in tissue distribution. DJ-1a is detected primarily in the tissues from the pupal to the adult stage, whereas DJ-1b is found in most tissues from embryo to adults [33,44,45].

Similar to Drosophila, C. elegans has two DJ-1 genes (cDJR-1.1 and cDJR-1.2), both of which encode GLO3 [6]. cDJR-1.1 is found in both the nucleus and cytoplasm of the intestine, while cDJR-1.2 is detected primarily in the tissues from the pupal to the adult stage, whereas DJ-1b is found in most tissues from embryo to adults [33,44,45].

The most closely related homologs of DJ-1 in prokaryotes are YajL proteins. The best characterized YajL protein is the E. coli YajL protein (EcYajL). Like DJ-1, EcYajL protects cells against oxidative stress [47]. The conserved cysteine 106 of EcYajL can form mixed disulfides with cytoplasmic proteins including chaperones, proteases, ribosomal proteins, catalases, peroxidases and FeS proteins. This conserved chaperone activity of EcYajL is required to protect against protein aggregation and sulfenylation during oxidative stress [48-50]. However, unlike DJ-1, EcYajL does not display any activity to MG [6].

The principal GLO3 enzyme responsible for converting MG to D-lactate without a cofactor in E. coli is EcHsp31 (formerly known as YedU) [7], which shares only 14% sequence identity to HsDJ-1. It has been suggested that EcHsp31 is involved in protecting stationary cells against dicarbonyl stress [7]. In addition, EcHsp31 is a heat-inducible molecular chaperone [51,52] that plays a role in protecting cells against multiple stresses including heat shock and starvation [53-55]. EcHsp31 is also involved in the management of protein misfolding under acid stress [56]. However, unlike DJ-1 proteins, EcHsp31 does not appear to be involved in oxidative stress response [56].

Close homologs of EcHsp31 (hereafter referred to as Hsp31 proteins) have been found in bacteria and fungi [41,55,57]. ScHsp31 (Accession number: YDR533C), a Saccharomyces cerevisiae homolog of EcHsp31, is involved in the protection of yeast cells against reactive oxygen species [58]. Based on sequence comparison, DJ-1 and Hsp31 proteins belong to different subfamilies of the DJ-1/Hsp31/PfpI superfamily, which encompasses a wide variety of functionally diverse proteins [40,41,43]. Both DJ-1 and Hsp31 proteins possess the Glu-Cys-His catalytic triad. The first two triad residues (e.g., Glu16 and Cys111 in SpDJ-1, and Glu30 and Cys138 in ScHsp31) are in the same locations in the two enzymes and are essential for GLO3 activity [6,7]. In contrast, the third triad residue His (e.g., His130 in SpDJ-1 and His139 in ScHsp31) resides at different sequence and structural positions, and is less important for enzyme activity [6,7]. In addition, Hsp31 proteins also have a potential cysteine protease-like catalytic triad (Cys138-His139-Glu170 in ScHsp31) of unknown functions [57,59-61].

Structural and bioinformatic studies reveal that Hsp31 proteins but not DJ-1, YajL and PfpI proteins have a P domain, which is likely involved in Hsp31 dimer formation and substrate binding [57,60]. Furthermore, Hsp31 proteins can clearly be divided into class I and class II proteins based on their structural and sequence similarity [57]. Class I proteins represented by EcHsp31 contain a complete P domain, whereas class II proteins represented by ScHsp31 contain a shorter P domain and have Glu instead of Asp as the third triad residue. Class II proteins but not DJ-1, YajL and PfpI proteins have a P domain, which is likely involved in Hsp31 dimer formation and substrate binding [57,60]. Furthermore, Hsp31 proteins can clearly be divided into class I and class II proteins based on their structural and sequence similarity [57]. Class I proteins represented by EcHsp31 contain a complete P domain, whereas class II proteins represented by ScHsp31 contain a shorter P domain and have Glu instead of Asp as the third member of a cysteine protease-like catalytic triad [57,60].

To better understand the origins and functions of GLO3, we investigated the distribution and diversity of DJ-1 and Hsp31 proteins in a broad diversity of fungal species since GLO3 activity is most likely to reside in these proteins. This is the first comprehensive survey of DJ-1 and Hsp31 proteins in fungi. We also provide evidence that fungal homologs of the DJ-1/Hsp31/PfpI superfamily may function as GLO3.

Results

Candidate DJ-1 proteins are present only in a very limited number of fungal species

To determine the distribution of DJ-1 proteins in fungi, we performed extensive BLAST searches against fungal databases using HsDJ-1 as a query sequence. In total, we have examined 191 sequenced fungal species, including 134 Ascomycota, 39 Basidiomycota, 3 Zygomycota, 3 Chytridiomycota and 12 Microsporidia. We identified 46 candidate DJ-1 proteins from a total of 43 fungal species (Table 1). Among them, only four (S. pombe, Alternaria
Table 1 Candidate fungal DJ-1 proteins identified in this study

| Species# | Taxonomy          | Accession number/name | No. aa | E value | Database |
|----------|-------------------|------------------------|--------|---------|----------|
| Alternaria arborescens | Ascomycota | - | 199 | 4E-24 | NCBI |
| Alternaria brassicicola | Ascomycota | Atlbr1_7229 | 197 | 2.32E-26 | JGI |
| Cochliobolus heterostrophus | Ascomycota | CocheC4_1_31923 | 197 | 4.97E-28 | JGI |
| Cochliobolus lunatus | Ascomycota | Coelu2_105387 | 196 | 1.04E-26 | JGI |
| Cochliobolus miyabeanus | Ascomycota | Coelm_87889 | 197 | 7.63E-27 | JGI |
| Cochliobolus sativus | Ascomycota | Coesal_1_181323 | 197 | 2.67E-26 | JGI |
| Cochliobolus victoriae | Ascomycota | Coevi1_89530 | 197 | 1.25E-27 | JGI |
| Pyrenophora teres | Ascomycota | PTT_13806 | 197 | 8.00E-34 | NCBI |
| Pyrenophora tritici | Ascomycota | PTRG_06163.1 | 197 | 2.67E-28 | JGI |
| Phaeosphaeria nodorum | Ascomycota | SNOG_07399 | 193 | 1.32E-27 | Broad |
| Schizosaccharomyces japonicus | Ascomycota | SJAG_06414.4 | 191 | 5.77E-15 | Broad |
| Schizosaccharomyces japonicus | Ascomycota | SJAG_02106.4 | 199 | 7.30E-10 | Broad |
| Schizosaccharomyces pombe | Ascomycota | SPAC22E12.03c/SpDJ-1 | 191 | 4.89E-14 | Broad |
| Schizosaccharomyces octosporus | Ascomycota | SOCG_00579S | 202 | 3.50E-12 | Broad |
| Schizosaccharomyces cryophilus | Ascomycota | SPOG_019263 | 202 | 7.80E-12 | Broad |
| Agaricus bisporus | Basidiomycota | Agabi_187606 | 200 | 1.06E-18 | JGI |
| Auricularia delicata | Basidiomycota | Aurlde1_110250 | 191 | 4.77E-21 | JGI |
| Ceriporiopsis subvermispora | Basidiomycota | Ceruis_140432 | 200 | 1.63E-20 | JGI |
| Coprinopsis cinerea | Basidiomycota | CCG1_10336.3 | 197 | 3.26E-26 | Broad |
| Dichomitus squalens | Basidiomycota | Dicsq1_140432 | 200 | 5.63E-20 | JGI |
| Ganoderma lucidum | Basidiomycota | Ganspl_117607 | 202 | 4.73E-22 | JGI |
| Heterobasidion annosum | Basidiomycota | Hetan2_436865 | 201 | 8.66E-21 | JGI |
| Heterobasidion irregulare | Basidiomycota | - | 201 | 2E-06 | NCBI |
| Malassezia globosa | Basidiomycota | MGL_3627 | 200 | 2.00E-23 | NCBI |
| Microbotryum violaceum | Basidiomycota | - | 188 | 7E-13 | NCBI |
| Omphalotus olearius | Basidiomycota | - | 196 | 2E-04 | NCBI |
| Phanerochaete camosus | Basidiomycota | Phaca1_160579 | 199 | 5.31E-18 | JGI |
| Phanerochaete chrysosporium | Basidiomycota | Pchcr1_3440 | 199 | 2.04E-6 | JGI |
| Postia placenta | Basidiomycota | POSPLDRRAFT_103847 | 199 | 3.00E-15 | NCBI |
| Postia placenta | Basidiomycota | POSPLDRRAFT_93097 | 135* | 1.00E-09 | NCBI |
| Punctularia striposozanata | Basidiomycota | Punst1_52328 | 197 | 3.75E-23 | JGI |
| Rhodotorula glutinis | Basidiomycota | RTG_01234 | 197 | 9.00E-29 | NCBI |
| Rhodotorula graminis | Basidiomycota | Rhobul1_52555 | 197 | 1.00E-122 | JGI |
| Schizophyllum commune | Basidiomycota | SCHCODRAFT_58862 | 196 | 1.00E-18 | NCBI |
| Serpula lacrymans | Basidiomycota | SERLA73DRAFT_191001 | 200 | 2.00E-29 | NCBI |
| Stereum hirsutum | Basidiomycota | Stehi1_124932 | 202 | 1.07E-25 | JGI |
| Trametes versicolor | Basidiomycota | Travell_171260 | 202 | 1.12E-22 | JGI |
| Ustilago maydis | Basidiomycota | Ummi10481 | 213 | 4.31E-25 | NCBI |
| Wallinia sebi | Basidiomycota | Walse1_59511 | 186 | 3.68E-27 | JGI |
| Spizellomyces punctatus | Chytridiomycota | SPPG_04052.2 | 191 | 1.04E-40 | Broad |
| Allomyces macrogynus | Chytridiomycota | AMAG_03424.1 | 205 | 2.94E-35 | Broad |
| Allomyces macrogynus | Chytridiomycota | AMAG_04742.1 | 205 | 1.62E-33 | Broad |
| Batrachochytrium dendrobatidis | Chytridiomycota | BDEG_07033 | 185 | 2.49E-34 | Broad |
brassicicola, Ustilago maydis and Coprinopsis cinerea) have been previously reported [41]. Identical results were obtained when EcYajL was used as a query sequence. However, no DJ-1 protein was found by the BLAST search by using EcHsp31 as a query.

The distribution of DJ-1 proteins in the two phyla of higher fungi is different. The majority of fungal species within the largest and most phylogenetically diverse phylum of fungi, the Ascomycota, lack DJ-1. We identified 10 DJ-1 proteins from the Pezizomycotina, all of them belonging to the Dothideomycetes class. In addition, we identified DJ-1 proteins from all four sequenced Schizosaccharomyces species (S. pombe, Schizosaccharomyces octosporus, Schizosaccharomyces cryophilus and Schizosaccharomyces japonicus) belonging to the Taphrinomycotina, the basal subphylum of Ascomycota. These four fission yeast species are the only members of the Taphrinomycotina whose genomes have been sequenced so far. Unlike species in the Ascomycota, many sequenced species of the Basidiomycota phylum contain DJ-1 proteins. We identified 25 basidiomycete DJ-1 proteins in 39 species from all three subphyla of Basidiomycota.

Fungal species from the basal phyla Chytridiomycota, Zygomycota and Microsporidia are currently under-represented in the current genomic databases. Nevertheless, DJ-1 proteins were identified in all completely sequenced chytridiomycetes and zygomycetes. Unlike chytridiomycetes and zygomycetes, all sequenced microsporidian appear to lack DJ-1 proteins likely due to their extreme genome reduction and compaction [62].

Among some of fungal species that possess DJ-1 proteins, three species have two DJ-1 proteins. Fission yeast S. japonicus and chytrid species Allomyces macrognymus contain two candidate DJ-1 proteins with 70% and 94% sequence identities, respectively. In addition, the basidiomycete Postia placenta also appears to have two DJ-1 proteins (POSPLDRAFT_103847 and POSPLDRAFT_93097), although the full-length sequence of the latter candidate could not be accurately predicted due to the sequence gaps.

### Conservation of candidate fungal DJ-1 proteins
To evaluate the sequence conservation between fungal and higher eukaryotic DJ-1 proteins, we aligned DJ-1 sequences from fungi, human, fly, worm, and plant (Figure 1 and Additional file 1). We also included EcYajL and EcHsp31 for comparison. The fission yeast DJ-1 protein sequences were more divergent from human, fly, worm, and plant DJ-1 protein sequences than any other identified fungal DJ-1 sequences. Moreover, DJ-1 protein sequences from basal fungi are more similar to those from selected higher eukaryotes compared with their homologs from higher fungi. For example, DJ-1 proteins from basal and higher fungi share 40-43% and 23-39% overall sequence identity to HsDJ-1, respectively.

Fungal DJ-1 proteins exhibit a degree of amino acid sequence conservation with EcYajL, with identity ranging from 23-36% (Additional file 2). In contrast, fungal DJ-1 proteins exhibit extremely weak sequence similarity to EcHsp31, sharing as little as 9% amino acid identity with EcHsp31, illustrating extraordinary degree of divergence between DJ-1 and Hsp31 proteins (Additional file 2).

While the majority of fungal DJ-1 proteins have a conserved catalytic triad (Glu16-Cys111-His130 in SpDJ-1), the conserved His residue of the catalytic triad is replaced by either Phe or Tyr in three identified zygomycete DJ-1 proteins (RO3G_06344.3, Mucci2_157438 and Phybl2_131210) (Figure 1). Interestingly, this conserved His residue is also replaced by Tyr in the Bombbyx mori DJ-1 protein (BmDJ-1) [63].

### Candidate Hsp31 proteins are widely present in fungal species
To identify Hsp31 proteins from fungi, we conducted BLAST searches against fungal databases using the protein sequence of EcHsp31 as a query. Unlike DJ-1 proteins, candidate Hsp31 proteins were found in all fungal species we examined, except 7 Saccharomycotina species, 4 Basidiomycota species and 2 Chytridiomycota species. Overall, we identified a total of 142 fungal Hsp31 proteins from 83 fungal species, including 69 Ascomycota species, 10 Basidiomycota species, 1 Chytridiomycota species, and 3 Zygomycota species (Table 2 for representative list of fungal Hsp31 proteins. For a complete list see Additional file 3).

The number of Hsp31 proteins is highly variable among fungal species (Additional file 4). It is notable that while the majority of fungal species have one or two candidate Hsp31 proteins, some fungal species have as many as four Hsp31 proteins. The ascomycetous fungus Nectria haematococca (asexual name Fusarium solani), appears to possess the largest number of Hsp31 proteins (7) so far identified. Another ascomycete, Aspergillus niger, has the

### Table 1 Candidate fungal DJ-1 proteins identified in this study (Continued)

| Organism | Phylum          | Species                        | Accession   | AA | Identity | Location |
|----------|-----------------|--------------------------------|-------------|----|----------|----------|
| Rhizopus oryzae | Mucormycotina | RO3G_06344.3 | 191 | 3.49E-28 | Broad |
| Mucor circinelloides | Zygomycete | Mucci2_157438 | 191 | 6.70E-24 | JGI |
| Phycomyces blakesleeanus | Zygomycete | Phybl2_131210 | 192 | 1.11E-17 | JGI |

*The number of amino acids in the fungal DJ-1 proteins. ** Indicates the accession number is not available. *Indicates the sequence was incorrectly predicted by NCBI due to sequence gaps.
Alignment of DJ-1 proteins from representative fungal species.

| Species       | Accession | Sequence |
|---------------|-----------|----------|
| D. melanogaster | DmDJ-1    | EER      |
| A. thaliana   | AtDJ-1A   | EDR      |
| EcYajL        | EcYajL    | QELP     |
| ScHsp31       | ScHsp31   | DQP      |
| SpDJ-1        | SpDJ-1    | FAK-    |
| HsDJ-1        | HsDJ-1    | ENR      |
| BmDJ-1        | BmDJ-1    | GER      |
| SpDJ-1        | SpDJ-1    | DQP      |
| EcYajL        | EcYajL    | EDR      |
| ScHsp31       | ScHsp31   | QELP     |

Candidate fungal DJ-1 proteins are from six ascomycetes, nine basidiomycetes and six basal fungal species. Accession numbers of fungal DJ-1 proteins are used; see Table 1 for details. DJ-1 proteins from human (Hdj-1), D. melanogaster (DmDJ-1), C. elegans (cdr-1), Bombyx mori (BmDJ-1) and A. thaliana (AtDJ-1A), EcYajL, EcHsp31 and ScHsp31 are included for comparison. For AtDJ-1A, only the C-terminal region is used for comparison. The alignment was constructed using Clustal W [91] and adjusted manually to improve the alignment. Identical residues are on a black ground and conserved residues shared in gray. The conserved catalytic triad residues of DJ-1 proteins and Hsp31 proteins are indicated below the alignment by stars and filled triangles, respectively. Hyphens represent gaps introduced into sequences for maximum alignment.
| Species                  | Taxonomy         | Accession number/name | Database | No. aa | Class Type | E-value |
|-------------------------|------------------|------------------------|----------|--------|------------|---------|
| *Ajellomyces dermatitidis* | Ascomycota       | BDGG_02344             | NCBI     | 231    | Class II   | 5.0E-19 |
| *Arthroderma benhamiae*  | Ascomycota       | ARB_05407              | NCBI     | 240    | Class II   | 1.0E-67 |
| *Aspergillus nidulans*   | Ascomycota       | AN6796.2               | NCBI     | 237*   | Class II   | 4.0E-15 |
| *Aspergillus nidulans*   | Ascomycota       | AN6810.2               | NCBI     | 256*   | Class III  | 1.0E-04 |
| *Candida albicans*       | Ascomycota       | CaO19.251/Glx3p        | NCBI     | 236    | Class II   | 5.0E-104|
| *Candida albicans*       | Ascomycota       | CaO19.7882             | NCBI     | 236    | Class II   | 2.0E-104|
| *Cordyceps militaris*    | Ascomycota       | CCM_05507              | NCBI     | 296    | Class I    | 5.0E-03 |
| *Cordyceps militaris*    | Ascomycota       | CCM_09248              | NCBI     | 225    | Class II   | 1.0E-23 |
| *Metarhizium acridum*    | Ascomycota       | MAC_07323              | NCBI     | 228    | Class II   | 9.0E-25 |
| *Metarhizium acridum*    | Ascomycota       | MAC_05717              | NCBI     | 294    | Class I    | 1.0E-03 |
| *Neurospora crassa*      | Ascomycota       | NCU06603               | NCBI     | 242    | Class II   | 8.0E-17 |
| *Phaeosphaeria nodorum*  | Ascomycota       | SNOG_00505             | NCBI     | 262    | Class III  | 1.0E-08 |
| *Phaeosphaeria nodorum*  | Ascomycota       | SNOG_04306             | NCBI     | 229    | Class II   | 6.0E-17 |
| *Pyrenophora teres f. teres* | Ascomycota       | PTT_13641             | NCBI     | 229    | Class II   | 2.0E-19 |
| *Pyrenophora teres f. teres* | Ascomycota       | PTT_19431             | NCBI     | 252    | Class III  | 1.0E-08 |
| *Saccharomyces cerevisiae* | Ascomycota       | YDR533C                | NCBI     | 237    | Class II   | 1.0E-171|
| *Saccharomyces cerevisiae* | Ascomycota       | YPL280W                | NCBI     | 237    | Class II   | 2.0E-117|
| *Saccharomyces cerevisiae* | Ascomycota       | YOR391C                | NCBI     | 237    | Class II   | 2.0E-04 |
| *Saccharomyces cerevisiae* | Ascomycota       | YMR322C                | NCBI     | 237    | Class II   | 2.0E-116|
| *Schizosaccharomyces pombe* | Ascomycota       | SPC757.03c/Hsp3101     | NCBI     | 244    | Class II   | 8.0E-63 |
| *Schizosaccharomyces pombe* | Ascomycota       | SPACSH10.02c/Hsp3102   | NCBI     | 240    | Class II   | 1.0E-56 |
| *Schizosaccharomyces pombe* | Ascomycota       | SPBC947.09/Hsp3103     | NCBI     | 262    | Class II   | 1.0E-55 |
| *Schizosaccharomyces pombe* | Ascomycota       | SPAC1D3.13/Hsp3104     | NCBI     | 222    | Class II   | 2.0E-42 |
| *Schizosaccharomyces pombe* | Ascomycota       | SPAC1F7.06/Hsp3105     | NCBI     | 251    | Class II   | 5.0E-33 |
| *Verticillium dahliae*   | Ascomycota       | VDAG_08958             | NCBI     | 293    | Class I    | 2.0E-03 |
| *Verticillium dahliae*   | Ascomycota       | VDAG_09321             | NCBI     | 235    | Class II   | 4.0E-16 |
| *Yarrowia lipolytica*    | Ascomycota       | YAL0C22000p            | NCBI     | 239    | Class II   | 7.0E-79 |
| *Yarrowia lipolytica*    | Ascomycota       | YAL10F00682p           | NCBI     | 250    | Class II   | 5.0E-70 |
| *Coprinopsis cinerea*    | Basidiomycota    | CC1G_10162             | NCBI     | 270    | Class III  | 1.0E-05 |
| *Coprinopsis cinerea*    | Basidiomycota    | CC1G_11702             | NCBI     | 231    | Class II   | 2.0E-09 |
| *Coprinopsis cinerea*    | Basidiomycota    | CC1G_00260             | NCBI     | 230    | Class II   | 4.0E-20 |
| *Malassezia globosa*     | Basidiomycota    | MGL_4192               | NCBI     | 240    | Class II   | 2.0E-73 |
| *Postia placenta*        | Basidiomycota    | Posp1_110200           | JGl      | 224    | Class II   | 1.4E-08 |
| *Postia placenta*        | Basidiomycota    | Posp1_115118           | JGl      | 249    | Class II   | 3.6E-19 |
| *Rhodotorula graminis*   | Basidiomycota    | Rhoba1_1_64335         | JGl      | 232    | Class II   | 7.0E-25 |
| *Schizophyllum commune*  | Basidiomycota    | SCHCODRAFT_46162      | NCBI     | 226    | Class II   | 6.0E-28 |
| *Schizophyllum commune*  | Basidiomycota    | SCHCODRAFT_49614      | NCBI     | 225    | Class II   | 2.0E-22 |
| *Serpula lacrymans*      | Basidiomycota    | SERL73DRAFT_120613    | NCBI     | 224    | Class II   | 6.0E-27 |
| *Ustilago maydis*        | Basidiomycota    | UM00094.1             | NCBI     | 235    | Class II   | 2.0E-16 |
| *Spizellomyces punctatus* | Chytridiomycota  | SPPG_02734.3           | BROAD    | 260    | Class II   | 5.5E-11 |
| *Spizellomyces punctatus* | Chytridiomycota  | SPPG_05672.3           | BROAD    | 231    | Class II   | 3.0E-18 |
second largest number of Hsp31 proteins (5). *S. cerevisiae* has four Hsp31 homologs, YDR533C, YPL280W, YOR391C and YMR322C, which have been named ScHsp31, ScHsp32, ScHsp33 and ScHsp34 (also called Sno4), respectively [60]. *S. pombe* has five Hsp31 proteins, which we named Hsp3101 (SPCC757.03c), Hsp3102 (SPAC5H10.02c), Hsp3103 (SPBC947.09), Hsp3104 (SPAC11D3.13) and Hsp3105 (SPAC1F7.06).

Conservation of candidate fungal Hsp31 proteins

To assess the extent of sequence conservation among fungal Hsp31 proteins, we performed multiple sequence alignment of Hsp31 protein sequences from taxonomically diverse fungal species (Figure 2; for a complete list see Additional file 5). For comparison, we also included HsDJ-1 and EcHsp31.

Based on sequence comparison, fungal Hsp31 proteins can be divided into different classes. The majority of fungal Hsp31 proteins belong to class II that contains a short P domain. In contrast, only five proteins belong to class I that contains a P domain. Interestingly, class I fungal Hsp31 proteins were only found in the Pezizomycotina species that belong to the order Hypocreales within the class Sordariomycetes. In addition to these previously noted classes, some fungal Hsp31 proteins appear to form a new class which we have termed class III. Class III Hsp31 proteins have a short P domain and are highly divergent in sequence from other classes of Hsp31 proteins. These proteins were found mainly in Pezizomycotina species, but were absent from all examined Saccharomycotina species.

The sequence similarity between different Hsp31 proteins of the same species varies considerably among different species. In *S. cerevisiae*, the four Hsp31 proteins, which all belong to class II, are highly similar to each other (84% average identity). Among these, ScHsp32, ScHsp33 and ScHsp34 are almost identical to each other (99.5% average identity). Similarly, two *Candida albicans* Hsp31 proteins, all belonging to class II, share 99.6% identity. In contrast, the two *Aspergillus nidulans* Hsp31 proteins belonging to class II and class III, respectively, share only 19% identity. Among the five *S. pombe* Hsp31 proteins, which all belong to class II, Hsp3101 and Hsp3102 are more closely related to each other than they are to other Hsp31 proteins (58% identity between Hsp3101 and Hsp3102).

The phylogenetic relationship between fungal DJ-1 and Hsp31 proteins

To examine the relationship between fungal DJ-1 and Hsp31 proteins, we generated a Bayesian phylogenetic tree from an alignment of representative fungal DJ-1 and Hsp31 proteins. In addition to fungal sequences, we also included Hsp31 and YajL proteins from representative bacterial species and DJ-1 proteins from representative metazoan species in our analyses. Our phylogenetic reconstruction revealed that bacterial YajL proteins and fungal and metazoan DJ-1 proteins form a monophyletic group to the exclusion of all Hsp31 proteins, indicating that DJ-1 and YajL proteins are very closely related to each other. By contrast, DJ-1 and Hsp31 proteins form two sister clades with Bayesian posterior probability of 1.00 (Figure 3). The phylogenetic reconstitution also revealed that fungal Hsp31 proteins can be further divided into different classes. This is congruent with the classification of fungal Hsp31 proteins based on sequence analysis. In addition, the phylogeny suggests that the presence of multiple *hsp31* genes identified in many fungal species is due to recent duplication events.

Prediction of subcellular localization of candidate fungal DJ-1 and Hsp31 proteins

We predicted the subcellular localization of DJ-1 and Hsp31 proteins using the programs Mitoprot, TargetP and iPSORT. Of all 46 fungal DJ-1 proteins analyzed, only DJ-1 from the basidiomycetous anamorphic yeast *Rhodotorula glutinis* (RgDJ-1, accession number: RTG_01234) has potential nuclear localization signals (NLSs). RgDJ-1 appears to have two NLS: 2RRR6 and 184RRKR187, which reside at the N- and C-terminus, respectively. However, despite a lack of NLSs in fungal DJ-1 proteins, it is possible that they are localized in nucleus given that a classical NLS could not be detected in HsDJ-1, but HsDJ-1 is localized in the nucleus [64-66], as well as the cytoplasm and mitochondria [22,67,68]. Indeed, we found that SpDJ-1 localizes to both the nucleus and cytoplasm (see below). Among 142 fungal Hsp31 proteins identified, 8 Hsp31 proteins are predicted to the nucleus, and 11 Hsp31 proteins are predicted to the nucleus, and 11 Hsp31 proteins are predicted to the nucleus.
Figure 2 Multiple sequence alignment of fungal Hsp31 proteins. Candidate fungal Hsp31 proteins are from thirteen ascomycetes, seven basidiomycetes and four basal fungal species. Ecthis31 and HsDJ-1 are included for comparison. See Table 2 for more information. The annotation of the alignment is as described in the legend to Figure 1.
Figure 3 Phylogenetic tree of candidate DJ-1 and Hsp31 proteins identified in fungi. The phylogenetic tree was constructed based on multiple sequence alignments of fungal DJ-1 and Hsp31 proteins whose accession numbers are shown in Table 1 and Additional file 3. Bacterial Hsp31 proteins from *E. coli* (EcHsp31), *Pseudomonas aeruginosa* (PaerPA_01001613), *Pseudomonas fluorescens* (Pfl01_2246), *Vibrio cholerae* (VCBJG01_3473), and *Staphylococcus aureus* (SAB0502), and bacterial YajL proteins from *E. coli* (EcYajL), *Citrobacter koseri* (CKO_02737), *Klebsiella oxytoca* (KOX_12625), and *Shigella sonnei* (SSON_0401) were also included in the analysis. Bayesian posterior probabilities generated by using Bayesian MCMC sampling are indicated at the nodes. The scale bar indicates 0.4 nucleotide substitutions per site.
proteins are predicted to be targeted to the mitochondria (Additional file 6).

Hsp3101, Hsp3102, ScHsp31 and SpDJ-1 possess GSH-independent glyoxalase activity
Candidate Hsp31 and DJ-1 proteins are present in fungi, but their roles in MG detoxification have not been studied in any fungal species. To determine whether fungal Hsp31 and DJ-1 proteins are indeed GLO3 enzymes, we chose to examine DJ-1 and Hsp31 proteins from \textit{S. pombe} and \textit{S. cerevisiae}, including SpDJ-1, Hsp3101-3104, ScHsp31 and ScHsp32. Hsp3105 was not examined because it lacks the conserved residues critical for GLO3 activity and is unlikely to possess GLO3 activity (Additional file 7). We also did not examine the GLO3 activity of ScHsp33 and ScHsp34 because their sequences are almost identical to the sequence of ScHsp32. We were able to obtain all but two of the soluble recombinant proteins by using either the pET expression system or the ESP® yeast protein expression. Two exceptions were Hsp3103 and Hsp3104, which were expressed as inclusion bodies in \textit{E. coli} and \textit{S. pombe}. As expected, SpDJ-1, Hsp3101, Hsp3102 and ScHsp31 could directly convert MG to lactate, which was detected by using the DNPH colorimetric method. The HPLC analysis of the reaction mixtures showed a peak with a retention time of 3.5 min identical to that expected for lactate, confirming the conversion of MG to lactate by these recombinant proteins (Figure 4). In contrast, recombinant ScHsp32 exhibited no detectable GLO3 activity.

We measured the $K_m$ and $k_{cat}$ values for the recombinant proteins that exhibited glyoxalase activity. Hsp3101, Hsp3102 and ScHsp31 have comparable apparent $k_{cat}/K_m$ values for MG (Table 3). These values are similar to those reported for HsDJ-1 [6] and EcHsp31 [7]. In contrast, the $k_{cat}/K_m$ value for SpDJ-1 is $7.9 \times 10^3$ min$^{-1}$ M$^{-1}$, which is about 15-fold lower than those for HsDJ-1 and EcHsp31 (Table 3). The reduction in catalytic efficiency for MG apparently is primarily caused by an increase in the $K_m$ value.

Fungal DJ-1 and Hsp31 proteins have similar predicted catalytic triads (Glu16-Cys111-His130 in SpDJ-1 and Glu30-Cys138-His139 in EcHsp31). To test whether the proposed catalytic triad is important for the GLO3 activity of SpDJ-1, we individually mutated these residues to Ala and purified the mutant enzymes (SpDJ-1E16A, SpDJ-1C111A and SpDJ-1H130A). Mutations of Glu16 and Cys111 to Ala nearly completely abolished the enzymatic activity, whereas the H130A substitution led to a 5- to 6-fold reduction in the catalytic efficiency.

\textbf{Overexpression of hsp3101, hsp3102, ScHSP31 and EcHsp31 can confer resistance to MG and GO}
Since Hsp3101, Hsp3102 and SpDJ-1 possess GLO3 activity \textit{in vitro}, we wanted to assess whether Hsp3101-3103 and SpDJ-1 can function as GLO3 \textit{in vivo}. We first deleted their corresponding genes individually or in combination and examined the sensitivity of the mutant strains to MG and GO. These mutant strains showed sensitivity similar
to that of the isogenic wild-type strain, suggesting that Hsp3101-3103 and SpDJ-1 may be functionally redundant for MG and GO detoxification (Additional file 8 and data not shown).

Next, we wanted to determine whether overexpression of hsp3101-3103 and SpDJ-1 under the control of the nmt1 promoter [69] could modulate the sensitivity of S. pombe cells to MG and GO. Overexpression of hsp3102 and, to a lesser extent, hsp3102 increased the survival of wild-type S. pombe cells during growth in the presence of either MG or GO. In fact, overexpression of hsp3102 displayed increased MG and GO resistance to a degree similar to overexpression of Spglo1 (S. pombe GLO1). Interestingly, overexpression of ScHSP31 and, to a lesser extent, EcHsp31 in wild-type pombe cells increased resistance to MG or GO, suggesting functional conservation of Hsp31 proteins across species. Unlike overexpression of hsp3101-3103, overexpression of SpDJ-1 led to enhanced resistance to GO, but not to MG (Figure 5A and B). In contrast, overexpression of hsp3103, ScHSP32 and HspDJ-1 did not affect the sensitivity of wild-type S. pombe cells to MG or GO (Figure 5A and B and Data not shown).

We also tested the effect of overexpression of hsp3101-3103 and SpDJ-1 in a ΔSpglo1 strain on sensitivity to MG. The ΔSpglo1 strain is much more sensitive to MG than the wild-type strain, which is consistent with its role as the major enzyme responsible for MG detoxification (Figure 5A and [42]). Similar to overexpression of Spglo1, overexpression of ScHSP31, EcHsp31, hsp3101 and hsp3102 markedly suppressed the MG sensitivity of ΔSpglo1 cells, indicating that overexpression of these hsp31 genes were able to compensate for the absence of Spglo1 in S. pombe (Figure 5C). In contrast, overexpression of hsp3103, ScHSP32, SpDJ-1 and HsdDJ-1 did not affect the sensitivity of the ΔSpglo1 mutant to MG (Figure 5C and Data not shown).

### Table 3 The enzyme kinetic parameters of SpDJ-1, the predicted catalytically inactive mutants of SpDJ-1, Hsp3101, Hsp3102, ScHsp31 and ScHsp32

| Enzyme     | $K_m$ (mM) | $k_{cat}$ (min⁻¹) | $k_{cat}/K_m$ (min⁻¹ M⁻¹) |
|------------|------------|-------------------|--------------------------|
| SpDJ-1     | 1.08       | 85.7              | 7.9 × 10⁴                 |
| SpDJ-1E16A | ND         | ND                | ND                       |
| SpDJ-1C111A| ND         | ND                | ND                       |
| SpDJ-1H130A| 37.3       | 65.5              | 1.8 × 10⁴                 |
| Hsp3101    | 1.4        | 31.1              | 2.3 × 10⁴                 |
| Hsp3102    | 2.7        | 58.0              | 2.1 × 10⁴                 |
| ScHsp31    | 1.5        | 75.0              | 5.1 × 10⁴                 |
| ScHsp32    | ND         | ND                | ND                       |

All enzyme assays were done in triplicate, and the mean values are presented. ND, not determined. The kinetic parameters of SpDJ-1E16A, SpDJ-1C111A and ScHsp32 cannot be accurately measured because of the extremely low activity of these proteins.

Hsp3101 and SpDJ-1 are both nuclear and cytoplasmic whereas Hsp3102 is exclusively cytoplasmic

A previous genome-wide subcellular localization study using enhanced green fluorescent protein (GFP) fusions under the control of the intermediate-strength nmt1 promoter [69] showed that SpDJ-1, Hsp3101, and Hsp3102 are present in both the nucleus and cytoplasm, Hsp3104 is cytoplasmic, and Hsp3105 is predominantly nuclear [70]. However, the subcellular localization of Hsp3103 is unclear. Since the GFP fusions used in the genome-wide study may be mislocalized due to overexpression of these fusions [69]. To determine the subcellular localization of Hsp3103 and to verify the results from the genome-wide study, we examined the localization of the C-terminal GFP fusions of SpDJ-1 and Hsp3101-3105 expressed from their endogenous loci. Consistent with results obtained by the genome-wide study, SpDJ-1-GFP and Hsp3101-GFP are localized in the nucleus and cytoplasm (Figure 6A and B). However, in our analysis, Hsp3102-GFP is only localized in the cytoplasm, not in nucleus (Figure 6C). The localization of other GFP-tagged Hsp31 proteins could not be unequivocally determined, most likely due to very low levels of expression of these proteins.

### Discussion

To better understand the origin and evolution of GLO3, we performed the first comprehensive survey of DJ-1 and Hsp31 proteins in a wide range of taxonomically diverse fungal species. Our studies revealed that most fungal species examined lack DJ-1 proteins. The fact that DJ-1 proteins are present in many basidiomycetes and all sequenced basal fungi, but are absent from most species in the Ascomycota, suggests that DJ-1 proteins were lost in recent fungal evolution. Unlike DJ-1 proteins, Hsp31 proteins are widely distributed among phylogenetically distant fungal species, indicating that Hsp31 proteins were retained during fungal evolution. The distribution of DJ-1 and Hsp31 proteins in fungi appears to be in sharp contrast to the situation in metazoans, where DJ-1 proteins are ubiquitous, whereas Hsp31 proteins are largely absent (Z. Wang and Y. Huang, unpublished results). These results imply that DJ-1 proteins appeared before the divergence of metazoans from fungi, and that Hsp31 proteins were lost after the divergence of metazoan and fungal lineages.
Although direct evidence demonstrating the in vivo role of fungal Hsp31 proteins in MG and GO detoxification is still lacking, we showed that overexpression of the two most closely related S. pombe hsp31 genes (hsp3101 and hsp3102) could confer MG and GO resistance on S. pombe wild-type and ΔSpglo1 cells, suggesting that these two S. pombe Hsp31 proteins may play some role in protecting cells from MG and GO toxicity. We also found that overexpression of ScHSP31 and EcHsp31 could also increase the resistance of S. pombe cells to MG and GO, suggesting functional conservation of Hsp31 proteins from E. coli, S. cerevisiae and S. pombe. It will be important in future studies to elucidate the physiological function of GLO3 enzymes.

It is not clear at present why many fungal species bear more than one Hsp31 gene. Some of them do not appear to encode GLO3. Among five S. pombe Hsp31 proteins, Hsp3105 lacks the conserved residues critical for GLO3 activity, and is thus unlikely to function as a GLO3. Our biochemical studies reveal that S. cerevisiae ScHsp32 did not exhibit any GLO3 activity. Since the sequence of ScHsp32 is nearly identical to those of ScHsp33 and ScHsp34, it is likely that only one of the four S. cerevisiae Hsp31 proteins (ScHsp31) has GLO3 activity. Other Hsp31 proteins (Hsp32, 33, and 34) may have other in vivo substrates. In support of this idea, it has recently found that the size and orientation of the active site pockets of the Hsp31 and Hsp33 are markedly different [71].

Unlike Hsp3102, Hsp3101 and SpDJ-1 are both nuclear and cytoplasmic. Although the physiological relevance of the differences in subcellular distribution of these proteins is unclear at present, it seems likely that nuclear SpDJ-1 and Hsp3101 pools may play a role in the regulation of gene expression. This hypothesis is consistent with previous studies reporting that a nuclear pool of HsDJ-1 regulates the expression of some genes through stabilization of the antioxidant transcriptional master regulator Nrf2.

**Figure 5** Overexpression of hsp3101, 3102, ScHSP31 and EcHsp31 increases resistance of wild-type and ΔSpglo1 cells to α-dicarbonyls. (A-B) Wild-type cells harboring pREP3X (empty plasmid) or indicated constructs overexpressing Spglo1 (positive control), EcHsp31, ScHSP31, hsp3101-3103 and SpDJ-1. (C) Wild-type cells harboring pREP3X and ΔSpglo1 cells harboring indicated constructs. Cells were grown to stationary phase in leucine-selective medium. Equivalent numbers of cells, in 10-fold serial dilutions (from left to right), were spotted on leucine-selective media without or with the indicated concentration of either MG (A and C) or GO (B) and were incubated at 30°C.
which is comparable to our $k_{cat}/K_m$ values for Hsp3101, Hsp3102 and ScHsp31. While in this paper, Hasim et al. mainly focused on the C. albicans Glx3p, they also reported the specific activities of SpDJ-1 and ScHsp31. However, the kinetic parameters for these two enzymes were not reported. Consistent with our results, they found that Glx3p and ScHsp31 are considerably more active than SpDJ-1 (Table 3 in [79]). However, in contrast to our findings that single and combined deletions of S. pombe DJ-1 and hsp31 genes did not result in an increase in sensitivity to MG, they found that deleting glx3 caused increased sensitivity to MG. We believe that the difference may be due to functional redundancy of S. pombe DJ-1 and Hsp31 proteins. Furthermore, our results extend their findings to show that some of fungal Hsp31 proteins may not be GLO3.

Conclusions
This study represents the first large-scale identification and analysis of fungal DJ-1 and Hsp31 proteins. Our survey shows that Hsp31 proteins are widespread throughout the fungal kingdom, whereas DJ-1 proteins are restricted to certain fungal phyla. The apparent lack of DJ-1 genes in the majority of fungal species suggests that they may have lost during fungal evolution. Sequence alignment and phylogenetic analyses reveal that fungal Hsp31 proteins can be further divided into different classes. Our analysis reveals that fungal Hsp31 proteins may serve as the major GLO3 in fungi, although some of them may not be GLO3. The GLO3 activity of Hsp31 proteins may have occurred independently from that of DJ-1 proteins. Our results further suggest that Hsp3101 and Hsp3102 are the major GLO3 that may have some role in protecting cells from RCS toxicity in S. pombe, and that SpDJ-1 and Hsp3101 may also participate in nuclear-related functions.

Methods
Strains, media and genetic manipulation
All S. pombe strains used in this study are listed in Additional file 9. Deletion mutants were created by the one-step gene replacement method [80]. The deletion cassettes to generate the Spglo1 and SpDJ-1 null alleles were generated by cloning the 5’ and 3’ flanks of Spglo1 and SpDJ-1 into pAF1 [81] and pFA6a-kanMX6 [82], respectively. The deletion cassettes were transformed into yHL6381. Primers used for deletion cassette construction and other plasmid constructions are available upon request. All deletions were verified by PCR.

Fungal genome database search and protein sequence analysis
Fungal DJ-1 and Hsp31 proteins were identified by BLAST searches using known DJ-1 and Hsp31 proteins as queries, respectively, and the cut-off E-value of 0.01.
Fungal genome databases used include the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE= BlastSearch& SPEC=MicrobialGenomes), the Broad Institute (http://www.broadinstitute.org/science/data), the Joint Genome Institute (http://genome.jgi-psf.org/pages/fungi/home.jsf), the Genome Center at Washington University (http://genome.wustl.edu/), FungiDB (http://fungidb.org/fungidb), and the Universal Protein Resource (http://www.uniprot.org). All sequences were subjected to manual curation and further analysis as described [83-85].

Phylogenetic analysis
The phylogenetic tree of fungal DJ-1 and Hsp31 proteins was constructed using methods as described [83-85]. Briefly, fungal DJ-1 and Hsp31 protein sequences were aligned using the Clustal W program implemented in MEGA5.0 [86] and adjusted manually to conform to the optimized alignment of amino acid sequences. Model selection was performed using ProtTest 2.4 [87]. The Bayesian phylogenetic tree was inferred by using MrBayes using I + G distribution. Statistical confidence was assessed by using Markov Chain Monte Carlo (MCMC) sampling approaches. Two separate runs including a total of four independent tree searches were conducted. All searches consisted of one ‘cold’ and three ‘heated’ Markov chains estimated for 10⁷ generations, and every 100 generations were sampled. The burn-in parameter was estimated by plotting -lnL against the generation number using Tracer (v1.4.1, http://beast.bio.ed.ac.uk/software/Tracer), and the retained trees were used to estimate the consensus tree and the Bayesian posterior probabilities.

Plasmid construction
To make recombinant proteins with an addition of a 6-histidine tag at their carboxyl-terminal end, ScHSP31-32, hsp3101-3104 and SpDJ-1 were PCR-amplified from genomic DNA, cloned into the NcoI and XhoI sites (for ScHSP31, hsp3101-3104 and SpDJ-1) of pET28a (Novagen) or the Ndel and Xhol (for ScHSP32) of pET23a (Novagen), hsp3102-3104, which formed inclusion bodies when expressed in E. coli, were also cloned into the Ndel and BamHI sites of the yeast expression vector pESP3 (Stratagene), and were overexpressed in S. pombe as C-terminally His-tagged fusion proteins. The point mutations of SpDJ-1 (SpDJ-1E16A, SpDJ-1C111A and SpDJ-1H130A) were generated by overlapping PCR with two sets of PCR primers including overlapping primer pairs containing the desired mutations. For expression in S. pombe, the coding regions of Spglo1, DJ-1 and Hsp31 genes were PCR-amplified from genomic DNA (for Spglo1, hchA, ScHSP31-32, hsp3101-3103 and SpDJ-1) or plasmid p3xFlag-myc-CMV-24-DJ-1 [89] (for HsDJ-1). The PCR fragments were digested with BamHI and Smal for DJ-1 and hsp31 genes or with XhoI and Smal for Spglo1, and cloned into the BamHI and Smal or the XhoI and Smal sites of pREP3X [69].

Green fluorescent protein (GFP) tagging of S. pombe DJ-1 and Hsp31 proteins
S. pombe DJ-1 and Hsp31 proteins were C-terminally tagged with GFP (S65T) at their respective chromosomal loci by homologous recombination. To place a GFP tag at the C terminus of spDJ-1, Hsp3101 and Hsp3102, the GFP tagging cassette containing the coding region before the stop codon, coding regions for GFP and the kanamycin resistance marker (KanMX6), and the 3′ untranslated region after the stop codon was generated by fusion PCR using pFA6a-GFP (S65T)-kanMX6 as template. To place a GFP tag at the C terminus of Hsp3103-3105, the 5′ coding region before the stop codon and the 3′ untranslated region after the stop codon were subcloned into the Sall and BamHI sites, and the Spel and SacII sites of pFA6a-GFP (S65T)-kanMX6, respectively. The GFP tagging cassette was then generated by PCR. The GFP tagging cassettes were gel-purified and used to transform yHL6381 to G418 resistance by lithium acetate-mediated transformation. Correct integration of the GFP tag into the desired locus was verified by PCR. GFP fusion proteins were visualized by fluorescence microscopy as described [90].

Expression and purification of recombinant proteins
Recombinant plasmids carrying wild-type SpDJ-1, hsp3101 or ScHSP31, or the predicted catalytically inactive mutants of SpDJ-1 (pET28a-SpDJ-1E16A, pET28a-SpDJ-1C111A and pET28a-SpDJ-1H130A) were transformed into E. coli BL21 (DE3) cells. The recombinant His-tagged proteins were induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 hrs. Cells were harvested and lysed by sonification in buffer containing 20 mM sodium phosphate, pH7.5, 14.3 mM β-mercaptoethanol and 0.5 mM Phenylmethylsulfonyl fluoride (PMSF), and further clarified with centrifugation. The supernatant was incubated with Ni-NTA resin (Qiagen) with rocking at 4°C. The resin was washed using buffer containing 20 mM sodium phosphate, pH7.5, and 25 mM imidazole. The proteins were eluted with 20 mM sodium phosphate buffer (pH7.5) containing 250 mM imidazole. Purified proteins were dialyzed against buffer containing 100 mM sodium phosphate, pH6.0, and 1 mM DTT, and quantitated using the Bradford assay. Recombinant Hsp3102, which formed inclusion bodies when expressed in E. coli, was produced with the ESP® yeast protein expression system in S. pombe (Stratagene). The plasmid pES3 expressing hsp3102 (pESP3-Hsp3102) was transformed into S. pombe strain yAS56, and the
recombinant protein was produced according to manufacturer’s instructions, except that the recombinant protein was affinity purified with Ni-NTA resin. The purified recombinant proteins were used in the in vitro assays and for antiserum production.

GLO3 activity assay
For the determination of GLO3 activity in vitro, a colorimetric assay was used as described in references [6,7] with modifications. The reaction mixtures (200 μl) contain 100 mM Na3PO4, pH 6.8, different concentrations of MG (0.5, 1, 1.5, 2, 2.5, and 3 mM) and purified recombinant wild-type or mutant SpDJ-1 proteins, or ScHsp31 were incubated for predetermined time periods at 45°C. The reactions were stopped by the addition of 700 μl 100 mM Na3PO4, pH 6.8 and 300 μl of 2,4-dinitrophenylhydrazine (DNPH). After incubation for 15 min at room temperature, 10 ml of 10% NaOH was added. The mixtures were further incubated for 15 min, and the formation of phenylhydrazone was determined by measuring absorbance at 540 nm. Steady-state kinetic parameters were determined from initial rates, which were calculated from absorbance at 540 nm. Steady-state kinetic parameters were determined using OriginPro 8 software (OriginLab Corporation).

HPLC analysis
The reactions catalyzed by SpDJ-1, Hsp3101, Hsp3102 and ScHsp31 were monitored by HPLC according to a previously published protocol with modifications [6]. Briefly, HPLC analysis was carried out using an Agilent 1260 Infinity HPLC system with the Ultimate AQ-C18 column (4.6 x 150 mm, 5 μm column, Welch Materials, Inc.) at a flow rate of 1.0 ml/min. The mobile phase was a mixture of 10 mM H3PO4 (pH 2.5). Production of lactate was detected by UV absorbance at 210 nm. The retention of D-lactate (Sigma) was around 3.5 min.

Methylglyoxal and glyoxal sensitivity assays
S. pombe cells were grown at 30°C overnight, diluted and grown to stationary phase. Cells were suspended in water and normalized to an OD600 = 3.0. 3 μl of cells were spotted in 10-fold serial dilution onto medium containing different concentrations of MG or GO (Sigma). The plates were photographed after 7 to 10 days of incubation at 30°C.

Additional files

Additional file 1: Multiple sequence alignment of candidate fungal DJ-1 proteins. The accession numbers for the candidates are listed in Additional file 3. The annotation of the alignment is described in the legend to Figure 1.

Additional file 2: Percentage amino acid identities of fungal DJ-1 proteins compared with those from C. elegans (DJKR-1.1), D. melanogaster (DmDJ-1α), A. thaliana (AtDJ-1A), and human (HsDJ-1), E. coli YajL (EcYajL), and E. coli Hsp31 (EcHsp31). The first column is accession numbers (For proteins lacking a accession number, species names are used). See Table 1 for details. The pair-wise percent identity scores were generated with Clustal W [91]. For AtDJ-1A, only the C-terminal region is used for comparison.

Additional file 3: Distribution of candidate Hsp31 proteins identified in fungi. *The number of amino acids in fungal Hsp31. "Indicates that mispredicted sequences obtained from the databases have been corrected. Indicates the sequence could not be correctly predicted due to sequence gaps. , the protein sequence could not be identified by BLAST searches. "#, the accession number is not available.

Additional file 4: The number of Hsp31 proteins in fungal species.

Additional file 5: Multiple sequence alignment of candidate DJ-1 and Hsp31 proteins from representative fungal species. The accession numbers for the candidates are listed in Table 1 and Additional file 3. The annotation of the alignment is described in the legend to Figure 1.

Additional file 6: Candidate fungal Hsp31 proteins predicted to localize to nucleus or mitochondria. Predicted NLSs are shown. *+ indicates the predicted mitochondrial localization.

Additional file 7: Multiple sequence alignment of candidate S. pombe Hsp31 proteins.

Additional file 8: Deletion of individual SpDJ-1 and hsp3101-3103 or in combination does not affect the MG sensitivity of cells.

Additional file 9: List of S. pombe strains used in this study.

Abbreviations
MG: Methylglyoxal; GO: Glyoxal; GSH: Glutathione; SLG: S-D- lactoylglutathione; GLO1: Glyoxalase I; GLO2: Glyoxalase II; GLO3: Glyoxalase III; GATase1: Glutamine amidotransferase.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
QZ performed biochemical assays. ZW did online database searches and sequence analysis.  YS performed all spotting assays. CC and TW carried out localization analysis. YH conceived this study, analyzed the data and drafted the manuscript. All authors have read and approved the final version of the manuscript.

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