**Saccharomyces cerevisiae** DJ-1 paralogs maintain genome integrity through glycation repair of nucleic acids and proteins

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Abstract

Reactive carbonyl species (RCS) such as methylglyoxal and glyoxal are potent glycolytic intermediates that extensively damage cellular biomolecules leading to genetic aberration and protein misfolding. Hence, RCS levels are crucial indicators in the progression of various pathological diseases. Besides the glyoxalase system, emerging studies report highly conserved DJ-1 superfamily proteins as critical regulators of RCS. DJ-1 superfamily proteins, including the human DJ-1, a genetic determinant of Parkinson’s disease, possess diverse physiological functions paramount for combating multiple stressors. Although *S. cerevisiae* retains four DJ-1 orthologs (Hsp31, Hsp32, Hsp33, and Hsp34), their physiological relevance and collective requirement remain obscure. Here, we report for the first time that the yeast DJ-1 orthologs function as novel enzymes involved in the preferential scavenge of glyoxal and methylglyoxal, toxic metabolites, and genotoxic agents. Their collective loss stimulates chronic glycation of the proteome, and nucleic acids, inducing spectrum of genetic mutations and reduced mRNA translational efficiency. Furthermore, the Hsp31 paralogs efficiently repair severely glycated macromolecules derived from carbonyl modifications. Also, their absence elevates DNA damage response, making them vulnerable to various genotoxins. Interestingly, yeast DJ-1 orthologs preserve functional mitochondrial content, maintain ATP levels, and redistribute into mitochondria to alleviate the glycation damage of macromolecules. Together, our study uncovers a novel glycation repair pathway in *S. cerevisiae* and a possible neuroprotective mechanism of how hDJ-1 confers mitochondrial health during glycation toxicity.
**Introduction**

The structural and functional integrity of nucleic acids and proteins is critical for establishing a physiological balance to maintain normal cellular health. Reactive carbonyl species (RCS) are highly toxic compounds that covalently bind and damage vital cellular components such as protein, DNA, and fatty acids (Semchyshyn, 2014). RCS primarily constitutes methylglyoxal (MG), glyoxal (GO), and 3-deoxyglucosone (Semchyshyn, 2014). The MG and GO are metabolic by-products of reactive aldehyde and ketones, respectively (Allaman, Bélanger, & Magistretti, 2015; Lange, Wood, Knight, Assimos, & Holmes, 2012). At the basal physiological level, these dicarbonyls participate in numerous signaling processes essential for cellular development and stress response (Akhand et al., 2001; Rodrigues et al., 2020). On the contrary, MG and GO modifies the thiol and amine groups at elevated levels through the Maillard reaction, forming a myriad of irreversible intermediates followed by stable Advanced glycation end-products (AGEs) (Semchyshyn, 2014; Thornalley, 2008). They preferentially form adducts with side chains of arginine, lysine, and cysteine and indiscriminately impair the structure and function of proteins. Moreover, the glycation of essential proteins, including antioxidant machinery, can alter reactive oxygen species (ROS) homeostasis, mitochondrial performance, and proteostasis (Goudarzi, Kalantari, & Rezaei, 2018; Seo, Ki, & Shin, 2014). Likewise, chronic exposure of nucleic acids to RCS culminates into nucleotide AGEs that promote genetic aberrations and translational defects and induce a spectrum of mutations, including transition and G: C to T: A transversions (Murata-Kamiya, Kamiya, Kaji, & Kasai, 1997, 2000). Elevated glycation of protein and DNA has implications for several pathologies like diabetes, aging, and neurodegenerative diseases (Fournet, Bonté, & Desmoulière, 2018). Therefore, regulating endogenous RCS and
minimizing its detrimental effects on biomolecules is critically important. The
glyoxalase system that constitutes glyoxalase I and II efficiently detoxifies MG and
GO to D-lactate and glycolate by utilizing glutathione (GSH) as a cofactor (Inoue,
Maeta, & Nomura, 2011). Since excess levels of carbonyls concomitantly induce
ROS, the GSH pool is rapidly depleted during the anti-oxidation process (de Bari et
al., 2020). Hence, cells have evolved GSH independent pathway (glyoxalase III
system) to maintain a healthy redox balance, primarily comprising DJ-1/ThiJ/Pfp1
superfamily proteins (Bankapalli et al., 2015; J. Y. Lee et al., 2012; Melvin,
Bankapalli, D'Silva, & Shivaprasad, 2017).

DJ-1 superfamily members are highly conserved, multi-stress responding, and
ubiquitously present in most organisms (Bandyopadhyay & Cookson, 2004; Wei,
Ringe, Wilson, & Ondrechen, 2007). The superfamily includes human DJ-1(hDJ-
1/PARK7), a well-explored protein that induces a familial form of Parkinson's disease
(PD) through its genetic mutations (Bonifati et al., 2003). Besides having various
neuroprotective functions, hDJ-1 possesses methylglyoxalase and glyoxalase
activity that substantially modulates endogenous carbonyls (J. Y. Lee et al., 2012).
Moreover, the deletion of *E. Coli* DJ-1 members exhibited alterations in the glycation
*Saccharomyces cerevisiae* has four homologs of DJ-1, namely Hsp31, Hsp32,
Hsp33, and Hsp34, representing Hsp31 mini-family proteins (Wilson, St Amour,
Collins, Ringe, & Petsko, 2004). Interestingly, Hsp32, Hsp33, and Hsp34 share
~99.5% sequence identity within them and ~70% sequence identity with Hsp31. All
the paralogs possess a common catalytic triad constituting Cys138, His139, and
Glu170. A characteristic hallmark of DJ-1 superfamily members is the redox-sensing
catalytic cysteine, whose oxidation state determines the physiological function
(Wilson, 2011). The emerging evidence on bacteria, humans, and plant DJ-1 members reveals a unique deglycase machinery that relieves the MG and GO glycation adducts on DNA and proteins (Prasad et al., 2022; Richarme et al., 2017; Smith et al., 2022). This repair mechanism is further appreciated in preventing protein aggregation and altering the epigenetic landscape of the chromatin (Sharma, Rao, & Kalivendi, 2019; Zheng et al., 2019). Some S. cerevisiae Hsp31 members are reported to possess crucial functions in regulating ROS, mitochondrial dynamics, and chaperone activity (Aslam, Tsai, & Hazbun, 2016; Bankapalli et al., 2020; Miller-Fleming et al., 2014; Tsai et al., 2015). However, the biological relevance of having multiple DJ-1 orthologs in S. cerevisiae is poorly understood due to the lack of in vitro evidence and difficulties in targeting specific paralog as they are genetically almost identical.

In the current report, we have uncovered a primary role of the Hsp31 paralogs as glyoxalases and protectants of the genome and mitochondria against RCS. By, governing endogenous dicarbonyl levels, they prevent macromolecular glycation and abrogate genetic mutations. In response to glycation stress, mRNA translation activity was significantly reduced in the absence of yeast DJ-1 members. Furthermore, the novel deglycase machinery efficiently repairs glycated DNA and proteins, substantially reverting deleterious alterations on biomolecules. We also show that Hsp31 paralogs impart enhanced mitochondrial integrity by maintaining functional mitochondria content, sustained ATP levels, and attenuating glycation stress on mitochondria through redistribution. Together, the dual role is critical in providing multifaceted protection to cellular and organellar macromolecules during persistent carbonyl stress.
Results

Deletion of Hsp31 paralogs aggravates carbonyl toxicity and induces proteome glycation

The DJ-1 superfamily members strongly relate to regulating dicarbonyl stress primarily through scavenging excess intracellular MG and GO (J. Y. Lee et al., 2012; Subedi, Choi, Kim, Min, & Park, 2011). However, despite being conserved, the biological significance of *S. cerevisiae* Hsp31 paralogs is uncharacterized in the presence of carbonyl toxicity. Although the paralogs have very similar amino acid sequences, we utilized the ease of genetic manipulation in yeast to unravel their association with the homeostasis of RCS (*Figure 1-figure supplement 1A*). Hsp31 paralogs were sequentially deleted in the WT background in multiple combinations such as Δhsp31 (Δ31) to Δhsp34 (Δ34), Δ31Δ32 to Δ31Δ34, Δ31Δ32Δ33 (ΔT), and Δ31Δ32Δ33Δ34 (ΔQ) which simplifies the comprehensive understanding of the individual genes. As glyoxalase-1 (GLO1) extensively modulates endogenous RCS levels, Δglo1 strain was used for comparative analysis (Gomes et al., 2005).

Phenotypic analyses under GO stress revealed a mild growth sensitivity in single deletion strains (*Figure 1A*, compare panels glyoxal (GO) with control), and the viability was further affected in Δ31Δ32, Δ31Δ33, and Δ31Δ34. Interestingly, ΔT and ΔQ displayed synergistic worsening of growth compared to other strains, inferring their additive overlapping functions during GO toxicity. Subsequently, the effect of MG stress was tested in the deletion background of Hsp31 members. Among the paralogs, Δ31 alone displayed mild growth sensitivity, as reported earlier (*Figure 1A*, compare panels glyoxal (GO) with control).
compare panels methylglyoxal (MG) with control (Bankapalli et al., 2015). On the other hand, additional loss of Hsp32, Hsp33, and Hsp34 in Δ31 had no cumulative effect on viability.

To test whether the observed phenotypes are the consequence of impaired carbonyl homeostasis, protein modifications were examined in the deletion background of Hsp31 paralogs. To determine the GO and MG-induced alterations, anti-CML (carboxymethyl-lysine) and anti-MAGE (methylglyoxal AGE) antibodies were utilized to determine the intermediates of AGEs, respectively. The basal physiological glycation levels (absence of glycation stress) in ΔQ strain displayed an increment in GO-modified proteins compared to Δglo1 strain suggesting Hsp31 paralogs are essential for the RCS detoxification in vivo (Figure 1-figure supplement 1B, C). However, such basal glycation levels are tolerable to the cells without exhibiting growth sensitivity. Intriguingly, upon subjecting to additional external glycation stress, a significant elevation of CML levels was observed in the single mutants compared to WT (Figure 1B). At the same time, the deletion of additional members substantially further exacerbated the modifications. It cumulatively enhanced the protein glycation levels to a greater extent in ΔT and ΔQ than Δglo1, consistent with growth sensitivity (Figure 1C). In contrast, the effect of MG-induced glycation was restricted to Δ31 with a subtle increment of MAGE levels compared to WT, consistent with growth sensitivity in the presence of MG (Figure 1D and compare growth in the last panels, Fig 1A). Intriguingly, the collective loss of the members did not exhibit additional MAGE modification in agreement with the growth sensitivity in the presence of MG (Figure 1E and compare growth in the last panels, Fig 1A). This preliminary data demonstrate that yeast DJ-1 members act on specific RCS and collectively provide
superior tolerance towards GO toxicity than the GLO1. Strikingly, Hsp31 alone
imparts resistance for both GO and MG, preventing the persistent ongoing protein
glycation.

The absence of Hsp31 paralogs enhances nucleic acid glycation and abates
mRNA translation efficiency

The nucleic acids are highly susceptible to modifications and are constantly
challenged with several metabolic by-products that elicit mutations and chromosomal
aberrations (Chatterjee & Walker, 2017). Occasionally, the alterations are
irreversible and may not be repaired despite stringent repair systems, culminating to
disease conditions. Since the absence of Hsp31 paralogs augments protein
glycation, the glycation status of cellular nucleic acids was also assessed. Despite
the absence of GO stress, DNA glycation levels were elevated in both ΔT and ΔQ
(Figure 2-figure supplement 1A, B). However, in the presence of GO stress, the
single deletion strains exhibit further enhancement in the glycation compared to
WT (Figure 2A). At the same time, the additional loss of Hsp31 paralogs led to a
synergistic increment of DNA adducts, most evident in ΔT and ΔQ strains consistent
with the growth sensitivity (Figure 2B).

Besides the alterations in DNA, the glycation of RNA was also determined, as
its modifications lead to ribosome stalling, translational defects, and inefficient
binding with proteins (Mitchell et al., 2018; Zheng, Maksimovic, Upad, & David,
2020). Like DNA, the lack of Hsp31 paralogs led to the high frequency of RNA
modifications, which were quantitatively higher than Δglo1 (Figure 2C, D).
Furthermore, the effect of glycation toxicity on global mRNA translational activity was
evaluated between WT and ΔQ. In the absence of GO stress, WT and ΔQ showed
similar polysome profiles (Figure 2E). However, the mRNA translational activity was significantly reduced in ΔQ treated with GO, as indicated by low-intensity polysome peaks (Figure 2E). Moreover, the ratio of polysome to monosomes also suggested a reduced translation rate in ΔQ compared to WT in the presence of glycation stress (Figure 2F).

Next, the role of yeast DJ-1 orthologs in maintaining genome integrity was investigated during MG stress. Unlike Δ31, the DNA glycation in Δ32, Δ33, and Δ34 was minimally detected by α-MAGE (Figure 2G), inferring their lack of participation in MG detoxification. Further, the paralogs were cumulatively deleted to evaluate the possibility of additive effects on glycation levels. Interestingly, the MAGE-modified DNA was restricted to the loss of Hsp31 due to its role in MG homeostasis (Figure 2H). Also, deletion of Hsp31 alone led to substantial glycation of RNA when treated with MG (Figure 2I, J). In summary, our results highlight that the DJ-1 paralogs in yeast play an essential role in modulating the RCS-mediated glycation of DNA and RNA.

Yeast DJ-1 orthologs are robust glyoxalases that attenuate the glycation of macromolecules

DJ-1 superfamily members are reported to detoxify MG and GO independently by the same active site with varying kinetics (Bankapalli et al., 2015; J. Y. Lee et al., 2012). Hence, to establish a direct correlation of enhanced glycation with excess intracellular RCS, Hsp31 paralogs were purified using affinity chromatography (Figure 3-figure supplement 1A). Our in vitro enzyme activity assay suggested that Hsp31, Hsp32, Hsp33, and Hsp34 possess a robust glyoxalase activity compared to BSA, which served as negative control (Figure 3A). Furthermore, the kinetic parameters indicate that paralogs have relative catalytic efficiencies. Notably, the
paralogs exhibited 4-fold higher $k_{\text{cat}}$ for glyoxalase activity than hDJ-1 (J. Y. Lee et al., 2012) (Figure 3B and Figure 3-figure supplement 1B). To further appreciate their in vivo biological response during GO stress, Hsp31 members were overexpressed within ΔQ strain and observed a substantial rescue in the growth similar to WT (Figure 3C). The expression of the proteins was normalized to equal amounts (Figure 3-figure supplement 1C).

To further corroborate the above findings, ΔQ strain overexpressing Hsp31 paralogs was subjected to GO stress and probed for CML levels. In agreement with our data, the modifications on proteins and nucleic acids were substantially suppressed in the presence of yeast DJ-1 glyoxalase machinery (Figure 3D-F). Next, the role of nucleophile cysteine amino acid (Cys-138) in the glyoxalase activity was tested, as its mutations predominantly impair the functions of DJ-1 members (Bankapalli et al., 2015; J. Y. Lee et al., 2012; Zheng et al., 2019). Therefore, cysteine 138 was replaced with alanine (C138A), and the proteins were subjected to the glyoxalase activity assay. Upon in vitro analysis, all the mutant proteins had compromised activity suggesting the critical requirement of cysteine in mitigating intracellular carbonyl levels (Figure 3G). Furthermore, we observed that exposure of cells to excess GO concomitantly enhanced the steady-state levels of Hsp31 paralogs approximately three-fold (Figure 3-figure supplement 1D, E). These results indicate a direct role of yeast DJ-1 orthologs in modulating CML levels through regulating endogenous glyoxal.

**Hsp31 alone specifically prevents the accretion of methylglyoxal-derived AGEs**

MG is the most reactive dicarbonyl with the highest potency for glycating vital macromolecules, causing organellar damage due to membrane permeability (Allaman et al., 2015). To overcome MG toxicity, the glyoxalase system (GLO1 and
GLO2) inevitably exploits the GSH pool for detoxification and thus alters the cellular redox balance (de Bari et al., 2020). On the other hand, DJ-1 members are functionally independent and do not utilize GSH as a cofactor for detoxification. To determine the methylglyoxalase activity of Hsp31 paralogs in vitro, we purified proteins recombinantly and subjected them to activity analysis. Strikingly, Hsp31 alone exhibited robust methylglyoxalase activity compared to other paralogs (Figure 4A). This further confirms the specific enrichment of MAGE-modified macromolecules found earlier in Δ31 strain (Figure 1D, 2G, and 2I). On the contrary, Hsp32, Hsp33, and Hsp34 demonstrated significantly lower methylglyoxalase activity in agreement with the lack of MAGE modifications (Figure 1D, 2G, and 2I).

Remarkably, the overexpression of Hsp31 in ΔQ strain facilitated improved resistance towards MG treatment than its paralogs due to its potent methylglyoxalase activity (Figure 4B). Further, examining the proteome glycation by α-MAGE also indicated that Hsp31 markedly reduced MG glycated proteins compared to its paralogs (Figure 4C). To further substantiate the implications of methylglyoxalase activity of Hsp31 paralogs, modification of nucleic acids from the respective strains were investigated. Due to high intrinsic methylglyoxalase activity, Hsp31 alone could scavenge MG-associated glycation of nuclear DNA and global RNA (Figure 4D, and E). Besides, the role of a catalytic cysteine (C138) residue in methylglyoxalase activity was evaluated due to its pivotal role in abating GO toxicity. The mutation of C138 to alanine in Hsp31 completely abolished the enzymatic activity (Figure 4F), which further emphasizes the prominence of a conserved cysteine in the catalytic triad of DJ-1 superfamily members. Together, our data suggest that Hsp31 provides glycation defense against both MG and GO, unlike Hsp32, Hsp33, and Hsp34.
Hsp31 paralogs repair glyoxal glycated DNA and proteins, suppress genetic mutations, and combat genotoxic stress

Glycation of macromolecules in *S. cerevisiae* is a rapid and spontaneous event with specific targets allowing them to tune their physiological functions (Gomes et al., 2005). However, glycation is also an irreversible process that may erroneously alter biomolecule features without a dedicated restoration pathway. Emerging studies have reported hDJ-1 as deglycase (EC 3.5.1.124) that relieves advanced glycation adducts from MG and GO-modified nucleotides and proteins (Richarme et al., 2017; Richarme et al., 2015). This observation unraveled a gateway to regulating the glycation status of essential components within cells across species.

We utilized the modified protocols to test whether the yeast Hsp31 paralogs possess deglycase activity (Prasad et al., 2022; Richarme et al., 2017). Briefly, deoxyribonucleotide triphosphate (dNTPs) were pre-treated without or with GO to glycate the substrates completely. The glycation mixture was further diluted to 6-fold to suppress the effect of free GO on the activity of Hsp31 paralogs. Later, the glycated substrates were incubated for 3 h with purified Hsp31 paralogs and BSA as a control to test deglycase activity (*Experimental scheme; Figure 5A*). The deglycase activity was ascertained by amplifying the *S. cerevisiae sod1* gene generated by Phusion high fidelity DNA polymerase that incorporates repaired dNTPs during PCR reaction. Unlike GO and BSA controls, incubation with Hsp31 paralogs efficiently reverted the glycation adducts of dNTPs, noted by robust amplification of the PCR product (*Figure 5B*). As an additional substrate of DNA, the forward and reverse oligonucleotide primers of the *sod1* gene were glycated in the presence or absence of GO. Besides dNTPs, yeast DJ-1 orthologs prominently deglycated the primers,
allowing the reaction to yield an intense PCR product in contrast to GO and BSA controls (Figure 5C).

The chronic glycation of proteins induces multifactorial changes that potentially trigger aggregation, cross-linking, and cytotoxicity. Therefore, it is critical to study whether Hsp31 paralogs confer the glycation repair of proteins. To determine the deglycase property, human Sod1, and Lysozyme were pre-treated without or with GO separately for 2 h to form glycation adducts. The reaction mixture was diluted 6-fold and incubated for 3 h with purified Hsp31 members or BSA (negative control) (Experimental scheme; Figure 5A). The samples were separated on an SDS-PAGE and subjected to immunodetection by anti-CML antibody to confirm the degree of glycation. All the Hsp31 members exhibited robust deglycase activity, as the paralogs extensively reverted the glycation damage of proteins, unlike GO and BSA controls (Figure 5D and E). On the other hand, the active site mutation (C138A) in paralogs was tested to verify deglycase enzyme activity further. The active site mutants failed to relieve the glycation adducts on DNA and proteins, thus establishing the specificity of the deglycase action of Hsp31 paralogs (Figure 5-figure supplement 1A to D).

GO is known to induce random mutations that severely impair genome integrity. We scored the glycation-induced conversions at the DNA level to investigate the impact of glyoxalase and deglycase activity of Hsp31 paralogs on genome protection. Strains lacking Hsp31 paralogs were subjected to GO treatment, and mutation frequency was determined by amplifying specific genes such as mitochondrial-encoded (cox2) and nuclear-encoded (sod1 and rad14), which have vital in vivo functions. Due to the loss of Hsp31 paralogs, the ΔQ strain exhibited enhanced mutation frequency as determined by cox2, sod1, and rad14.
gene sequencing (Figure 5F). However, due to robust intrinsic glyoxalase and deglycase activity associated with the paralogs, the overexpression of Hsp31, Hsp32, Hsp33, and Hsp34 significantly attenuated the mutation frequency of all the genes tested, further highlighting their physiological role in the maintenance of genome integrity under the GO toxicity (Figure 5F and Figure 5-figure supplement 1E).

To further establish their role in offering genome integrity, WT and ΔQ strains were exposed to well-known genotoxic agents such as methyl methanesulfonate (MMS) and hydroxyurea (HU). The ΔQ strain showed severe growth sensitivity towards MMS and HU treatment compared to WT (Figure 5-figure supplement 1F). Furthermore, treatment with GO and genotoxins combined showed a synergistic increment in the growth sensitivity of ΔQ compared to their individual treatments (Figure 5G). These genotoxins induce DNA lesions and strand breaks, which could be monitored by the expression of DNA damage response genes such as RNR3 (subunit of Ribonucleotide Reductase) that specifically upregulates during genotoxic stress (Fu, Pastushok, & Xiao, 2008). Intriguingly, ΔQ exhibited a notable increase in the expression of RNR3 compared to WT cells treated with MMS and GO (Figure 5H). Besides RNR3, multiple DNA repair proteins, including RAD52, assemble at the strand break regions. Hence, the formation of RAD52-GFP foci indicates the extent of DNA damage in cells (Conde & San-Segundo, 2008). Intriguingly, ΔQ strain displayed a 3-fold increment in RAD52 foci formation compared to WT upon treatment with MMS and GO (Figure 5I and Figure 5-figure supplement 1G). In summary, our findings indicate that the Hsp31 members collectively attenuate the genotoxic damage and participate in an antiglycation pathway that globally detects and restores the GO-modified biomolecules.
Hsp31 alone explicitly reverts methylglyoxal glycated macromolecules and attenuates mutation frequency

Balancing the physiological amount of dicarbonyls is a major biological challenge as they have a short half-life and quickly react with nearby biomolecules. Besides glyoxalase-I and II systems, very few enzymes have been identified to detoxifying MG (Vander Jagt & Hunsaker, 2003). About 90-99% of cellular MG is bound to macromolecules, which may be reversible by the action of deglycases (Allaman et al., 2015). We utilized a modified protocol to test whether the paralogs attenuate MG-mediated modification of DNA and proteins (Richarme et al., 2017). As illustrated in schematic Figure 6A, the dNTPs or DNA oligonucleotide primers substrates were incubated in the absence or presence of MG, followed by 6-fold dilution of the reaction mixture to minimize the effects of free MG on the enzymatic activity of Hsp31 paralogs. Later, purified DJ-1 members were added, and deglycase activity was ascertained by PCR of the sod1 gene using Phusion high-fidelity DNA polymerase. In line with our findings, Hsp31 alone suppressed the MG-mediated glycation through its deglycase activity, yielding robust amplification of PCR product (Figure 6B and C).

To delineate the MG-associated protein deglycation, hSod1, and Lysozyme were treated without or with MG and probed with α-MAGE antibody. In the presence of Hsp31, the glycation adducts on the proteins was significantly reverted (Figure 6D, E). On the other hand, the Hsp32, Hsp33, and Hsp34 failed to revert the glycation of proteins in vitro, similar to DNA substrates (Figure 6D and E). The Hsp31 active site mutant (C138A) also failed to repress the MG adducts on DNA and proteins, underlining its significance in the catalytic reaction (Figure 6-figure supplement 1A-D). We also determined if the dual role of Hsp31 could provide genome integrity by
scoring the MG-induced DNA mutations. Strikingly, the ΔQ strain displayed an augmented mutation rate, which was notably abated by the expression of Hsp31 during MG toxicity, confirmed by amplification and sequencing of selected yeast-specific genes (Figure 6F and Figure 6-figure supplement 1E). Thus, our data establish a critical function of Hsp31 in suppressing the MG-mediated aberrant glycation of macromolecules in S. cerevisiae.

Dicarbonyl stress induces translocation of Hsp31 paralogs into mitochondria for organelle protection

DJ-1 superfamily members display dynamic subcellular localization into various organelles, such as the nucleus and mitochondria, during the stress response (Bankapalli et al., 2015; Conde & San-Segundo, 2008; Fu et al., 2008). However, the significance of the redistribution is still unclear. Therefore, we investigated whether Hsp31 paralogs localize to mitochondria during GO toxicity. To determine, the Hsp31 paralogs were genomically fused with GFP, and the mitochondria were decorated by targeting mCherry tagged with mitochondrial targeting sequence (MTS). Under normal physiological conditions, the paralogs were predominantly localized in the cytosol (Figure 7A-D, -GO untreated panels). Strikingly, upon treatment of cells with GO, all the Hsp31 paralogs translocate into mitochondria, as highlighted by the merged images of GFP and mCherry (Figure 7A-D, +GO stress panels).

To further support the microscopic findings, western analysis was performed to quantitatively measure the mitochondrial localization of Hsp31 paralogs from fractionated mitochondria of GO-treated and untreated cells. A ~2.5-fold level increase in the localization of Hsp31 paralogs into mitochondria was observed under GO toxicity (Figure 7-figure supplement 1A). As Hsp31 regulates MG-induced glycation of macromolecules, we determined its redistribution in response to MG
stress. The microscopic visualization suggests that Hsp31 up on MG toxicity localizes into mitochondria (Figure 7E), which was further quantified from probing isolated mitochondria treated with MG stress. The immunoblots indicate a 2-3-fold enrichment of Hsp31 in mitochondria upon exposure to MG (Figure 7-figure supplement 1B). In contrast, Hsp32, Hsp33, and Hsp34 do not display mitochondrial redistribution under MG toxicity (Figure 7-figure supplement 1C-E), further emphasizing that Hsp31 and not its paralogs respond and mitigate broader stress conditions. Tim23 and Ydj-1 were probed for mitochondrial and cytosol control proteins, respectively (Figure 7-figure supplement 1F).

To understand the relevance of GO-dependent mitochondrial translocation of Hsp31 paralogs, mitochondria were isolated from cells supplemented with GO to analyze the altered glycation of mitochondrial macromolecules. Intriguingly, a significant portion of the mitochondrial proteome and DNA was glycated in ΔQ strain, while in the presence of Hsp31 paralogs, the glycation levels were substantially suppressed (Figure 7F and G). Since the Hsp31 paralogs govern the glycation of mitochondrial macromolecules, we studied mitochondrial health by determining the viability of deletion strains of yeast DJ-1 members on a non-fermentable carbon source (glycerol). Although we observe no growth difference in dextrose media, the collective absence of Hsp31 paralogs in ΔT and ΔQ induced sensitivity to glycerol media (Figure 8-figure supplement 1A). These results suggest that the disruption of yeast DJ-1 members compromises mitochondrial health.

Therefore, we further explored mitochondrial maintenance by yeast DJ-1 members by investigating various health parameters of the organelle. The mitochondrial morphology indicates the dynamic balance of the fusion-fission network, which could be affected by altered mitochondrial health. Under
physiological conditions, ΔQ displayed a highly reticular mitochondrial network than WT, possibly due to elevated expression of Mgm1 and Fzo1 as reported by Bankapalli et al., 2020 in Δ31Δ34 (Figure 8A -GO panel). However, under glycation stress, WT showed fragmented morphology of mitochondria as compared to no stress (Figure 8A +GO panel). Strikingly, despite the increase in the reticular network, we observed higher fragmentation of mitochondria in ΔQ than WT upon glycation toxicity (Figure 8A +GO panel). As the glycation affected the mitochondrial morphology, we examined the impact on total and functional mitochondrial content through flow cytometric analysis using NAO and TMRE dyes, respectively. In the absence of GO stress, ΔQ showed an increase in overall mitochondrial content than WT, possibly due to hyperfusion of mitochondria (Figure 8B). Although the mass of mitochondria remained almost similar in WT, an increase in total mitochondrial content was found in ΔQ upon GO stress (Figure 8B). Next, functional mitochondrial mass was determined through TMRE dye, we observed comparable functional mitochondria in WT treated and untreated, while a significant loss of functional mitochondria was noted in ΔQ under glycation toxicity (Figure 8C). To score the glycation-induced damage on mitochondrial health, ATP levels were estimated from isolated mitochondria. Intriguingly, the glycation stress led to a substantial loss of mitochondrial ATP levels by ~40% in ΔQ compared to WT (Figure 8D). These results infer that glycation stress induces extensive mitochondrial damage and impaired clearances of dysfunctional mitochondria in the absence of Hsp31 paralogs.

As mtDNA is prone to glycation in the absence of yeast DJ-1 members (Figure 7G), we examined the integrity of mtDNA by staining with mtDNA-specific SYTO18 stain (Ingavale et al., 2008; Matta, Pareek, Bankapalli, Oblesha, & D'Silva, 2017). Incubation of WT with GO led to a reduction in SYTO18 staining with
coalescent nucleoid morphology compared to untreated cells (Figure 8E, left panel). On the other hand, the lack of Hsp31 paralogs led to a significant reduction in SYTO18 staining with punctate nucleoid under GO stress compared to untreated cells (Figure 8E, right panel), suggesting their importance in maintaining mtDNA quantity and quality. In conclusion, the detoxification of carbonyls and macromolecular repair by yeast DJ-1 members is critical for preserving the global integrity of healthy mitochondria.

Discussion

Glyoxalase defence mechanism of the Hsp31 class of proteins is more robust than GLO-1 system.

The present study highlights robust glyoxalase activities of yeast Hsp31 mini-family proteins essential to combat aberrant intracellular levels of RCS. Our in vitro experiments firmly establish the specificity of Hsp31 paralogs towards GO substrates, while Hsp31 alone detoxifies both MG and GO. Since Hsp32 and Hsp34 are structurally uncharacterized, we considered overlapping structural features with Hsp33 for comparative analysis due to its solved crystal structure and close sequence similarity. Strikingly, Hsp32, Hsp33, and Hsp34 possess a similar active site offering different substrate specificity than Hsp31. Such preferential scavenging of carbonyls was also observed in bacteria and plant DJ-1 members where YajL, ElbB, AtDJ-1A, and AtDJ-1B are GO specific, and some orthologs detoxify both substrates (Kwon et al., 2013; C. Lee et al., 2016). Although MG and GO are detoxified through distinct pathways, certain NADPH-dependent enzymes like...
aldehyde dehydrogenase and aldose reductase scavenge both the carbonyls, with their activities stringently governed by cellular redox status (Vander Jagt & Hunsaker, 2003).

In contrast to MG catabolism, an antioxidant such as GSH inefficiently reacts with GO making it a poor substrate for the GLO1 glyoxalase system (Yang et al., 2011). Hence, S. cerevisiae has bonafide multiple DJ-1 orthologs, and their steady-state levels are rapidly upregulated in response to GO allowing efficient carbonyl homeostasis. Our results highlight that the deletion of individual paralogs shows moderate sensitivity for GO. However, the collective loss of yeast DJ-1 orthologs (ΔQ) renders severe growth deficiency more than Δglo1. At the same time, their absence promotes severe accumulation of toxic carbonyls leading to a higher fold macromolecular glycation than Δglo1. Besides scavenging RCS, Hsp31 is also reported to provide partial tolerance toward acetic acid stress, also a deleterious glycolytic intermediate (Ansari, Chaudhary, & Dash, 2018; Natkańska, Skoneczna, Sieńko, & Skoneczny, 2017). These findings suggest that Hsp31 members are paramount in regulating a broad group of toxic metabolites compared to the GLO system (1 and 2), which is restricted to specific carbonyls. Moreover, under oxidative damage, the activity of the GLO system (1 and 2) is impaired due to extensive utilization of the limiting pool of GSH. In contrast, the GSH-independent Hsp31 paralogs aid in restoring redox balance and simultaneously provide robust RCS homeostasis, hence, indispensable for cellular viability under carbonyl stress.

Global surveillance of nucleic acid glycation by yeast DJ-1 orthologs regulates the genome integrity
Despite several harmful effects, the physiological amounts of glycation are critical for post-translation modification, required for signaling in numerous pathways (Akhand et al., 2001; Rodrigues et al., 2020). Both MG and GO are known to glycate histone proteins. Hitherto, 28 site-specific modifications on histones have been identified as targets of MG (Ansari et al., 2018; Galligan et al., 2018; Ray et al., 2022). Such non-enzymatic covalent epigenetic modifications regulate stress adaptations by altering chromatin architecture and gene expression. We highlight that Hsp31 paralogs are essential repair enzymes that regulate the degree of nucleic acid glycation by indirectly detoxifying the carbonyls or directly removing adducts by deglycation. This corroborates well with the idea that Hsp31 paralogs preserve genome integrity by lowering deleterious mutations as a counter-response (Figure 9). Also, the absence of paralogs elicits enhanced sensitivity to DNA damage stress when exposed to multiple genotoxins, as indicated by enriched expression of RNR3 and RAD52 foci formation, suggesting their strong association with genome maintenance. At the molecular level, the antiglycation property of the paralogs could also prevent the replication stress by replenishing repaired dNTP pool. Hsp31 members are widely recognized for metabolic and transcriptional reprogramming during the diauxic shift as they extensively tune the expression of necessary proteins for rapid acclimatization (Miller-Fleming et al., 2014). These observations may point to a unique regulatory mechanism by Hsp31 class of proteins similar to hDJ-1 in governing the chromatin landscape’s carbonyl biology (Zheng et al., 2019).

Besides DNA, we found enriched glycation of RNA in ΔQ due to detrimental levels of MG and GO, accompanied by the disrupted mRNA translation at the global scale, possibly through compromised binding or stalling of ribosomes. Notably, yeast DJ-1 members extensively reverted the AGE modifications on RNA, suggesting their
comprehensive role in translation regulation. The enhanced spectrum of mutations observed in various essential genes of ΔQ is a crucial indicator of a dedicated glycation repair pathway in *S. cerevisiae* (*Figure 9*). Interestingly, a recent report on *Arabidopsis thaliana* identified a novel glycation repair pathway similar to *S. cerevisiae*, involving AtDJ-1D in the repair of DNA and proteins (Prasad et al., 2022).

Supporting the previous studies (Galligan et al., 2018; Nair et al., 2018; Richarme et al., 2017), we report yeast DJ-1 orthologs as a novel class of deglycases that repair RCS-mediated glycation of nucleic acids.

*Hsp31 paralogs regulate the proteome glycation homeostasis.*

Proteins are the most abundant biomolecules with considerably longer half-lives, making them highly vulnerable to multiple non-enzymatic covalent modifications. Under physiological conditions, glycation on arginine, lysine, and cysteine modulates the functional diversity of various proteins (Sun, Suttapitugsakul, Xiao, & Wu, 2019). We show that the absence of Hsp31 paralogs triggers severe glycation of the proteome, which may contribute to protein aggregation, misfolding, and cross-linking. Furthermore, under these circumstances, the conventional proteasomal degradation pathway fails to provide quality turnover resulting in AGE accumulation (Raupbach, Ott, Koenig, & Grune, 2020). As a compensatory response mechanism, amino acid biosynthesis and protein translation were upregulated in the single deletions of Hsp31 mini-family proteins (Miller-Fleming et al., 2014). This primarily indicates the biogenesis of functional proteins to promote cell survival during glycation stress.
Therefore, scavenging excess endogenous RCS by the Hsp31 paralogs is imperative to evade persistent glycation and maintain balanced proteostasis. Besides, their robust deglycase activity repairs severely glycated proteins from different cellular compartments, including mitochondria. This enables cells to efficiently reutilize the cellular proteome pool (Figure 9). In contrast, human DJ-1 exhibited weaker methylglyoxalase and deglycase activity than yeast and plant counterparts (Mazza et al., 2022).

Due to its chemical properties and redox-sensing ability, the catalytic cysteine in DJ-1 superfamily proteins holds critical importance during multi-stress responses (Wilson, 2011). In agreement with hDJ-1, C138A mutants of Hsp31 paralogs failed to repair macromolecules and detoxify RCS, suggesting a common multipurpose catalytic site.

Yeast DJ-1 orthologs provide mitochondrial protection during carbonyl stress. MG and GO toxicity is one of the leading factors contributing to mitochondrial dysfunction in most neurodegenerative diseases (Wang, Xu, Musich, & Lin, 2019). Mechanistically, they disrupt the mitochondrial morphology and electron transport chain and elevate ROS levels (Videira & Castro-Caldas, 2018). Although DJ-1 superfamily proteins display dynamic subcellular localization during the stress response (Junn, Jang, Zhao, Jeong, & Mouradian, 2009; Miller et al., 2003; Nair et al., 2018), their physiological relevance in mitochondrial protection was not well-studied. In a previous report, we showed the maintenance of mitochondrial morphology by regulating the fusion-fission network by Hsp31 and Hsp34 (Bankapalli et al., 2020). Here, we provide evidence of mitochondrial maintenance by yeast DJ-1 orthologs, effectively through global regulation of glycation stress and attenuation of glycation damage on mitochondrial macromolecules. Interestingly,
Hsp31 members redistribute into mitochondria in response to carbonyl toxicity that may substantially lower the glycation of mitochondrial proteome and DNA, contributing to enhanced mitochondrial survival. Moreover, the mitochondrial translocation of the paralogs could elevate the glycolate synthesis from glyoxal, a critical antioxidant that maintains mitochondrial membrane potential during stress conditions and re-establishes redox balance by producing GSH (Diez, Traikov, Schmeisser, Adhikari, & Kurzchalia, 2021; Toyoda et al., 2014). Hence, under carbonyl stress, their absence leads to the accumulation of fragmented and dysfunctional mitochondria, significantly attenuating the availability of the ATP pool and contributing to detrimental mitochondrial health. The dysfunctional mitochondria are accompanied by poor maintenance of mitochondrial DNA quality, which may further suppress the activity of mitochondrial DNA-encoded respiratory complexes leading to compromised ATP generation. These pathogenic alterations of mitochondria are central to neurodegeneration and the rapid progression of PD.

Besides impaired carbonyl homeostasis, a major contributor to PD progression is α-synuclein toxicity (Spillantini et al., 1997). Its physiological role involves interaction with mitochondrial complex I and ATP synthase. However, MG glycated α-synuclein forms cytotoxic oligomers, which may induce mitophagy and mitochondrial fragmentation (Faustini et al., 2017; Vicente Miranda et al., 2017). Therefore, cells rely on DJ-1 member proteins that possess glyoxalase and deglycases to abrogate the toxic accumulation of glycated products. Our findings highlight a possible neuroprotective function of PD-associated hDJ-1 involving the repair of damaged macromolecules and mitochondrial quality control, promoting healthy maintenance of neuronal cells with intrinsically higher levels of RCS (Angeloni, Zambonin, & Hrelia, 2014).
In summary, our findings significantly contribute to understanding the diversified response by Hsp31 paralogs in the field of carbonyl biology. Through the novel repair pathway identified in *S. cerevisiae*, the AGEs formation is efficiently suppressed, and mitochondrial performance is enhanced during chronic exposure to RCS. Although Hsp31 paralogs display stress-induced mitochondrial redistribution, their translocation pathway is poorly studied. Therefore, mechanistic insights into the translocation process and their sub-compartment distribution would reveal critical mitochondrial-associated functions of Hsp31 paralogs. Also, further exploration of genotoxic damage induced in the absence of hsp31 paralogs may suggest how hDJ-1 encounters PD-linked chromatin aberrations.

**Materials And Methods**

**Strains and plasmid constructions**

The yeast strains used in this study are mentioned in *Supplementary file 1*. The details of the primers and plasmids utilized in this study are described in *Supplementary file 1*. Knockouts of Hsp31 paralogs were generated through homologous recombination in haploid yeast strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). Deletion of hsp32 (Δ32), hsp33(Δ33), and glo1(Δglo1), was performed by transforming suitable cassettes generated by primers P1-P4, constituting the *hphNT1* marker. Subsequently, the deletions were confirmed through PCR using primers P5-P8. The strains lacking hsp31 (Δ31) and hsp34 (Δ34) were previously generated in the lab. The double, triple (ΔT), and quadruple (ΔQ) deletion strains were generated by transforming DNA cassettes in Δ31Δ34. All the deletion strains were confirmed through PCR using primers P5-P8.
RNR3 and Hsp31 paralogs expression was determined by genomically tagging at the C-terminus with heme agglutinin (HA) tag using primer P9-P11 and P12-P14, respectively, with *hphNT1* cassette as described above. Primers P15-P18 were used to tag RAD52 with GFP at the carboxy terminus in WT and ΔQ.

For overexpression studies, Hsp31 paralogs containing C-terminus heme agglutinin (HA) tag were cloned into pRS415 vector with GPD (glyceraldehyde-6-phosphate dehydrogenase) promoter and transformed into ΔQ. For protein expression and purification, Hsp31 family proteins were cloned into pRSF-Duet and purified with a previously published protocol (Bankapalli et al., 2015).

**Western blot analysis**

Indicated respective strains grown till the mid-log phase (*A*<sub>600</sub>~0.6) were treated with 10 mM MG or 15 mM GO for 12 h in the culture tubes. Later, the cell lysates were prepared by incubating with 10% Trichloro acetic acid solution at 4°C. Following acetone washes, 1X SDS dye (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 100 mM b-mercaptoethanol) and acid-washed glass bead was added and vortexed thoroughly. Subsequently, the samples were heated at 92°C, and 30 µg of the sample was loaded. The lysates were resolved on 12% SDS-PAGE and transferred to the PVDF membrane. Subsequently, the membrane was blocked with 5% BSA in phosphate-buffered saline (PBST-Tween 0.05%). Later, the membrane was incubated overnight with anti-CML or anti-MAGE antibody 1:1000 at 4°C. Following 3 washes with PBST, the membrane was incubated with the secondary antibody (1:10,000) at room temperature. Subsequently, the membrane was washed with PBST and exposed to luminol solutions (BIO-RAD). Equal loading of samples was confirmed through ponceau S staining of the membrane.
To determine the expression levels of Hsp31 paralogs in response to GO treatment, the WT strain containing genomic HA tag on the C-terminus of Hsp31 members was incubated in the absence or presence of 15 mM GO for 12 h. Subsequently, the cell lysates were resolved on 15% SDS-PAGE and probed with an anti-HA antibody. Similarly, WT and ΔQ were incubated without or with 0.01% MMS, and 15 mM GO for 3 h to estimate the expression of RNR3. The fold change in all experiments was calculated by measuring the band intensities using Multi Gauge V3.0 software.

**Nucleic acid glycation and DNA dot blot analysis**

Yeast strains grown till the mid-exponential phase were treated with 10 mM MG or 15 mM GO in the culture tubes for 12 h (Brown, 2001). Subsequently, genomic DNA was isolated using Wizard genomic DNA extraction kit (Promega, Cat No: A1120). For RNA isolation, cells were treated with 10 mM Tris pH 7.5, 1 mM EDTA, and 0.5% SDS (TES) buffer with hot acidic phenol for 1 h at 65°C. The resulting organic layer obtained after centrifugation was mixed with chloroform and centrifuged to acquire pure RNA. DNA and RNA were sheared through sonication and dotted (3 µg) on the nitrocellulose membrane (BIO-RAD, Cat No: 1620112). The membrane was baked at 80°C for 2 h and blocked-in phosphate-buffered saline (PBS-Tween 0.05%) with 5% BSA for 2 h at room temperature. Consequently, the membrane was probed overnight with an anti-CML or anti-MAGE antibody. Following the washes, the membrane was incubated with a secondary anti-mouse antibody. The membrane was exposed to luminol solutions. The membrane was stained with methylene blue to confirm equal loading of the samples.

**Polysome profiling**
The experiment was performed using an established protocol (Shaffer & Rollins, 2020). Briefly, WT and ΔQ strains were grown till the mid-log phase and treated with 15 mM GO for 4 h at 30°C, and post-treatment cycloheximide was added to immobilize the ribosomes on mRNA. The cell lysates were prepared by adding lysis buffer (10 mM Tris pH 7.4, 100 mM NaCl, 30 mM MgCl₂, 100 µg/ml cycloheximide, 10 U RiboLock, and 1 mM PMSF) to the pellet and vortexed with glass beads. The lysates were loaded on SW41 tubes containing a 10% to 50% sucrose density gradient, followed by ultra-centrifugation. Later, the fractions were analyzed using a polysome profiler (Piston gradient fractionator; BIOCOMP).

The area under the curve of polysome and monosome was calculated using Origin 8.0, and the ratio was plotted in Prism GraphPad 5.0.

**Phenotypic analysis and growth conditions**

Individual yeast strains were grown till mid-log phase ($A_{600}$~0.6) in YPD (yeast extract-1%, peptone-2%, and dextrose-2%) or synthetic dropout (SD) lacking leucine amino acid (0.67% yeast nitrogen base without amino acids, 0.072% Leu dropout supplement, and 2% dextrose). Consequently, cells were pelleted and serially diluted 10-fold until $10^{-5}$ dilution factor. Each dilution was spotted on the media plate without or with 15 mM GO. Parallelly, the cell pellet was treated with water or 10 mM MG for 6 h, serially diluted, and spotted on media plates. The plates were incubated at 30°C for 36 h and imaged. Phenotype under DNA damaging agents was determined by spotting cells from the mid-exponential phase onto YPD media plates containing 0.01% MMS (methyl methanesulfonate) or 150 mM HU (hydroxyurea). Also, the cells were spotted on YPD media plates containing 15 mM GO or 0.01% MMS and plates with both genotoxins. Images of spot assay were taken at 36 h. For growth assay on
non-fermentable carbon source, mid-log phase cells were spotted on S.D. Glycerol (2%) media.

**In vitro glyoxalase and methylglyoxalase activity**

A previously established protocol was used to determine the activity (Bankapalli et al., 2015; C. Lee et al., 2016). To determine the enzyme activity, 5 µg of purified Hsp31 paralogs WT and C138A mutants were incubated with 0.5 mM GO or MG in HEPES KOH pH 7 buffer for 30 min at 30°C. Later, the reaction was terminated using 0.1% DNPH (2,4-dinitrophenylhydrazone), followed by adding 10% NaOH and incubating at 42°C for 10 min. The results were calorimetrically analyzed using Biospectrometer (Eppendorf), 570 nm for GO, and 530 nm for MG. The kinetic parameters were determined by incubating Hsp31 members with varying GO concentrations, and the readings were noted at different time points. The experiments were performed in three biological replicates and triplicates. Subsequently, the values were plotted on Prism GraphPad 5.0 to estimate the $K_m$ and $V_{max}$.

**In vitro deglycation assay of DNA and Proteins**

The deglycation of DNA and protein was performed using previously published protocols with minor modifications (Richarme et al., 2017). For DNA deglycation experiments, 500 µM dNTPs or 150 µM forward and reverse primers of yeast sod1 (P19-P20) were incubated without or with GO/MG (2 mM) in HEPES KOH buffer pH 7 at 30°C for 2 h. Before supplementing the reactions with Hsp31 paralogs, the glycation mixtures were first diluted (6-fold) to minimize the free GO or MG levels. Post glycation reaction, 5 µg of purified Hsp31 paralogs and BSA were supplemented and incubated for 3 h at 30°C. The deglycase activity of Hsp31
paralogs was determined by subjecting the samples to a polymerase chain reaction (PCR) to amplify the \textit{sod1} gene in the presence of Phusion enzyme high fidelity DNA polymerase.

To measure the protein deglycation activity, 2 µg of purified human Sod1 and Lysozyme were treated without or in the presence of 2 mM of GO/MG in HEPES KOH buffer pH 7 at 30°C for 2 h (Prasad et al., 2022). Subsequently, the samples were diluted 6-fold to minimize GO or MG residual levels. Post glycation of protein samples, the reaction mixture was supplemented with 5 µg of purified Hsp31 paralogs and incubated for 3 h for deglycation. The deglycation status of proteins was determined by resolving the samples on 15% SDS-PAGE, followed by western analysis and immunodetected with anti-CML or anti-MAGE antibodies.

**Genetic mutation and sequencing**

Respective strains were grown till the mid-log phase and treated with 15 mM GO or 10 mM MG in the culture flasks for 24 h at 30°C. Total DNA was isolated as described above, and PCR amplified for \textit{sod1}, \textit{rad14}, and \textit{cox2} ORFs using primers P19-P24. Subsequently, the amplicons were sequenced by the Sangar method, and the raw reads were aligned with the WT sequences. The number of mutations was determined using the multi-sequence alignment tool Clustal Omega and the data was plotted on GraphPad Prism 5.0.

**Microscopic analysis**

To analyse the formation of RAD52 GFP foci, WT and ΔQ cells were grown until the exponential phase and treated with 0.03% MMS and 15 mM GO for 1 h. Subsequently, the \(A_{600} = 0.5\) OD cells were harvested and washed with 1X PBS before spreading over 2% agarose pads. The number of foci were quantified and plotted.
The localization of yeast DJ-1 members was performed by genomically
tagging with GFP at the carboxy terminus using primers P12-P14. Consequently, the
cells were transformed with a pRS415_{TEF} vector expressing MTS-mCherry to
decorate mitochondria (Bankapalli et al., 2015). The transformed strains grown until
the mid-exponential phase were harvested (A_{600} = 0.5) and incubated with water or
15 mM GO for 2 h. Parallelly, the cells were treated with water or 10 mM MG for 2 h
and harvested. The cell pellet was washed with 1X PBS and processed for imaging.
All the images were acquired in confocal microscopy (Olympus FV3000) with a 10
µm scale bar.

For mitochondrial DNA visualization, selected strains were grown till the mid-
log phase and treated with 15 mM GO for 3h. Later, cells were harvested and
stained with 10 µM SYTO 18 for 15 min at 30°C, followed by visualization under
confocal microscopy (Olympus FV3000). Images contain 10 µm scale bar.

**Analysis of mitochondrial mass**

WT and ∆Q were grown to the early log phase and treated without or with 15 mM
GO for 3h. Later, the cells were stained with 10 µm Nonyl Acridine Orange (NAO) for
total mitochondrial mass or 10 µm TetraMethylRhodamine ethyl ester (TMRE) for
measuring functional mass. Later, the cells were washed and examined under a
cytoFLEX flow cytometer.

**Measurement of mitochondrial ATP levels**

30 µg of fractionated mitochondria from WT and ∆Q strains treated with GO were
lysed and incubated with ATP detection solution from a Mitochondrial ToxGlo Assay
kit (Promega, Madison, USA). The luminescence was recorded using TECAN
pro800.

**Chemicals and antibodies**
Chemicals used in the study are Glyoxal (Sigma, 128465), Methylglyoxal (Sigma, M0252), Methyl Methanesulfonate (TCI, M0369), Hydroxyurea (Sigma, H8627), Lysozyme (Sigma, 4403), and nitrocellulose membrane (BIO-RAD, 1620112).

Antibodies used in the study are anti-CML (Carboxymethyl lysine; MAB3247 R&D Systems, Minneapolis, MN, USA), anti-MAGE (Argpyrimidine specific; ab243074 Abcam), anti-Tim23 (BD Bioscience), anti-HA antibody (GT4810, Sigma), secondary mouse (GE-bioscience).

**Miscellaneous**

Mitochondria were isolated from cells treated with 15 mM GO or 10 mM MG for 16 h using a previously published protocol (Kumar, Matta, & D'Silva, 2020). For mitochondrial DNA, fractionated mitochondria were lysed and subjected to alkaline lysis DNA purification (Sambrook & Russell, 2006).

**Statistical analysis**

Glycation intensities of DNA and proteins were measured through quantitation of complete dots or lanes, respectively, in Multi Gauge V3.0 software. The values were normalized with respect to WT in all experiments and plotted in GraphPad Prism 5.0 software. Error bars represent the standard deviation derived from three biological replicates. One-way ANOVA with Dunnett's multiple comparisons test was used to determine significance analysis, comparing multiple columns against the WT. Asterisks used in the figures represent the following significance values: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$; NS, not significant.

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**Additional information**

**Competing interests**

The authors declare that no competing interests exist.

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Additional files

- Source data files contain both the original image data and Image data before trimming used in Figures and Figure supplements.
- Supplementary file 1. List of strains, primers, and plasmids used in this study.
- MDAR checklist

Data Availability

All data generated or analysed during this study are included in the manuscript and supporting files; Source data files have been provided.

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**Figure legends**

**Figure 1. Deletion of Hsp31 paralogs induces protein glycation.** (A) Yeast phenotypic analysis. Cells were grown until the mid-log phase and harvested, subsequently treated with 10 mM MG before being spotted or spotted on YPD medium plates containing 15 mM GO. The plates were incubated at 30°C and imaged at 36 h. (B, C) Proteome glycation profile. Yeast strains were treated with 15 mM GO in the YPD culture medium and allowed to grow for 12 h, followed by western analysis with anti-CML antibody. (D, E) MAGE detection by western blotting. Cells from the mid-log phase were incubated with 10 mM MG for 12 h, and MAGE levels were estimated using an anti-MAGE antibody. Protein glycation levels were determined by measuring the whole lane intensities by densitometry and plotted with respect to WT. The blots stained with Ponceau S were used as the loading control. One-way ANOVA with Dunnet's multiple comparisons test was used to determine
significance from 3 independent biological replicates, *, \( p \leq 0.05 \); **, \( p \leq 0.01 \); ***, \( p \leq 0.001 \); NS, not significant.

Figure 1-figure supplement 1. Amino acid sequence alignment of Hsp31 paralogs and protein glycation profile in the absence of GO stress. (A) Amino acid sequences of Hsp31 paralogs were aligned using the online tool Clustal Omega. Black shade indicates identical amino acids. Red boxes indicate amino acids of the catalytic triad. (B, C) Respective strains were grown till the stationary phase and subsequently lysed. The protein glycation levels were estimated by immunodetection using anti-CML antibody.

Figure 2. Loss of Hsp31 paralogs aggravates the glycation of DNA and RNA, affecting translational activity. (A, B) Immunodetection of DNA using dot-blot assay. 3 µg of total genomic DNA from respective strains treated overnight with 15 mM GO was dotted on nitrocellulose membrane and probed with anti-CML antibody. (C, D) RNA glycation profile. Global RNA extracted from cells incubated with 15 mM GO were dotted and analyzed using anti-CML antibody. (E) Polysome profiling. Untreated (-GO) and treated (+GO) WT and ΔQ cells with GO were subjected to polysome profiling. (F) Ratio of polysomes to monosomes (P/M). The area occupied by the polysome and the monosome peak was determined using Origin 8.0 for the respective samples, and the ratios were plotted. WT (+GO) was compared with WT (-GO), and ΔQ (-GO) was compared with ΔQ (+GO). (G-J) Estimation of MAGE-modified nucleic acids. Strains lacking Hsp31 paralogs were treated with 10 mM MG for 12 h, and the genomic DNA and RNA were dotted on the membrane and probed with anti-MAGE antibody. The relative intensity of dots representing the glycation levels was calculated and compared to WT. One-way
ANOVA with Dunnet's multiple comparisons test was used to determine significance from 3 independent biological replicates, *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; NS, not significant.

Figure 2-figure supplement 1. Glycation profile of DNA in the absence of GO stress. (A, B) DNA extracted from stationary phase cells was dotted (3 µg) on nitrocellulose membrane and probed with anti-CML to determine the glycation status.

Figure 3. Hsp31 paralogs prevent glyoxal toxicity. (A) Measurement of the glyoxalase activity in vitro. 5 µg of purified Hsp31 paralogs were incubated with 0.5 mM GO, and the absorbance was monitored at 570 nm. (B) Estimating the kinetic parameters of the enzyme. The kinetic efficiency was determined by calculating their reaction velocities against multiple GO concentrations. (C) Growth phenotypic analysis. WT and ΔQ overexpressing Hsp31 paralogs were grown until the mid-log phase, spotted on SD Leu- media plates containing 15 mM GO, and incubated at 30°C. (D) Yeast DJ-1 homologs alleviate proteome glycation. Respective strains were grown in SD Leu- culture tubes and treated with 15 mM GO for 12 h, and the whole cell lysates were processed and probed for CML modifications. (E, F) Analysis of nucleic acid modifications by dot-blot assay. Genomic DNA and global RNA extracted from cells treated with 15 mM GO were dotted on the membrane and immunoreacted with anti-CML-antibody. (G) C138 amino acid residue is essential for glyoxalase activity. All the proteins were purified and incubated (5 µg) with 0.5 mM GO; subsequently, the absorbance was monitored at 570 nm. Significance was determined by Dunnett's multiple comparison test with WT or BSA (enzyme activity). Glycation levels represent the relative intensities of the dots and lanes compared.
with WT. Data from 3 independent biological replicates was used to determine
significance through one-way ANOVA with Dunnet's multiple comparisons test *, \( p \leq 0.05 \); **, \( p \leq 0.01 \); ***, \( p \leq 0.001 \); NS, not significant.

**Figure 3-figure supplement 1. Enzyme kinetics of yeast DJ-1 orthologs and their expression under GO stress.** (A) SDS-PAGE of purified Hsp31 paralogs. 7 µg of Hsp31 paralogs were loaded on SDS-PAGE gel along with the protein marker (M). (B) Raw values of enzyme kinetics. GO was incubated at various concentrations with 5 µg Hsp31 paralogs, and the reaction was terminated at different time intervals. The corresponding readings were plotted on Prism GraphPad 5.0 and calculated for \( K_m \), \( k_{cat} \), and \( V_{max} \). (C) Hsp31 members expressed under the plasmid pRS415GPD in \( \Delta Q \), confirmed with western analysis. (D, E) GO-induced expression of Hsp31 paralogs. Cells expressing Hsp31 members with genomic tagged HA were incubated without or with 15 mM GO for 12 h, and the expression was determined by immunoblotting with an anti-HA antibody. Gel stained with Coomassie brilliant blue (CBB) was used as a loading control. The fold change in the expression was calculated using Multi-Gauge V3.0 and plotted in Prism GraphPad 5.0. The experiments were performed in 3 independent biological replicates and statistically analysed by paired t-test, *, \( p \leq 0.05 \); **, \( p \leq 0.01 \); ***, \( p \leq 0.001 \); NS, not significant.

**Figure 4. Hsp31 is a potent scavenger of methylglyoxal.** (A) In vitro methylglyoxalase activity. Purified Hsp31 members 5 µg each were incubated with 0.5 mM MG, and the absorbance at 530 nm was monitored. (B) Spot assay. Respective strains grown till the mid-log phase were harvested and treated with 10 mM MG for 5 h before spotting on SD Leu- plates. Images were captured at 36 h. (C-E) Hsp31 reduces MG-derived AGE modifications. Individual strains were treated
with 10 mM MG in the culture tubes for 12 h, and the macromolecule glycation was analyzed using an anti-MAGE antibody. (F) C138 amino acid residue is critical for methylglyoxalase activity. 5 µg of Hsp31-WT and Hsp31\textsuperscript{C138A} mutant were incubated with MG, and the activity was examined at 530 nm. Significance was determined by Dunnett's multiple comparison test with WT or BSA (enzyme activity). Glycation levels represent the relative intensities of the dots and lanes compared with WT. One-way ANOVA with Dunnett's multiple comparisons test was used to determine significance from 3 independent biological replicates, *, p≤ 0.05; **, p≤0.01; ***, p≤ 0.001; NS, not significant.

Figure 5. \textit{In vitro} deglycation of DNA and proteins, and genotoxic sensitivity in the absence of yeast DJ-1 orthologs. (A) Schematic representation of the experimental procedure followed for DNA and protein deglycation reactions. (B, C) Hsp31 paralogs deglycate DNA. 500 µM dNTPs (B) or 150 µM forward and reverse primers (C) were incubated without (UN) or with 2 mM GO for 2 h, followed by 3 h incubation with 5 µg Hsp31 paralogs. The samples were examined for deglycation through PCR. (D, E) Yeast DJ-1 members repair glycated proteins. 2 µg of purified protein hSod1 (D) or Lysozyme (E) were treated without (UN) or with 2 mM GO for 2 h, and the reactions were further incubated for 3 h with Hsp31 paralogs. Anti-CML antibody was used to determine glycation levels. BSA was used as a negative control in all experiments. 10 kb DNA ladder was used as a marker (M) for DNA gels, and the Ponceau S stain indicates the equal loading of protein samples. (F) Hsp31 paralogs attenuate genetic mutations. Individual genes were PCR amplified, and Sanger sequenced from the isolated genome of strains treated with 15 mM GO. The number of genetic mutations was calculated and plotted on GraphPad prism 5.0 (n=2). (G) Growth phenotypic analysis. Cells grown until the
mid-log phase were spotted on plates containing 0.01% MMS or 15 mM GO or
0.01% MMS and 15 mM GO. The plates were incubated at 30°C and imaged at 36
h. (H) Western analysis of RNR3 levels. WT and ΔQ grown till the mid-log phase
were supplemented with 0.01% MMS, and 15 mM GO in the culture media and
further incubated for 3 h. Subsequently, RNR3 levels were probed using an anti-HA
antibody. (I) RAD52 foci formation. Cells were grown until the mid-exponential
phase and were treated without (-) or with 0.03% MMS and 15 mM GO for 1 h.
Subsequently, cells were imaged using a confocal microscope (Olympus FV3000);
representative images have a 10 µm scale. All the experiments were performed in 3
independent biological replicates.

Figure 5-figure Supplement 1. GO-associated macromolecular deglycation,
mutation frequency profile, and genotoxic damage. (A, B) Deglycation of DNA by
C138A mutants of Hsp31 paralogs. dNTPs (500 µM) and DNA oligonucleotides (150
µM) were incubated for 2 h in the absence and presence of 2 mM GO, followed by
deglycation with Hsp31 paralogs and C138A mutants for 3 h. (C, D) C138 amino
acid residue is crucial for protein deglycase activity. 2 µg glycated hSod1 and
Lysozyme were individually supplemented with 5 µg of Hsp31 paralogs (WT), or
mutant paralogs (C138A) and subjected to western analysis to determine the
glycation status (E) Respective genes were PCR amplified from cells treated with 15
mM GO for 16 h and Sanger sequenced. The number of genetic mutations from
various genes is represented here. (F) Phenotypic analysis under DNA damaging
agents. The cells harvested from the mid-log phase were spotted on plates
containing 0.01% MMS or 150 mM HU. Plates were incubated at 30°C and imaged
at 36 h. (G) Quantitation of RAD52 foci formation. Foci of individual strains were
quantitated from 3 independent biological replicates, statically analysed through paired t-test and plotted on Prism GraphPad 5.0.

**Figure 6. Hsp31 repairs MG-derived AGE modifications on DNA and proteins.**

(A) Schematic representation of the experimental procedure used for DNA and protein deglycation reactions. (B, C) DNA deglycation by Hsp31. 500 µM dNTPs (B) or 150 µM forward and reverse primers (C) were incubated without (UN) or with 2 mM MG for 2 h. Subsequently, 5 µg Hsp31 paralogs were added and incubated for 3 h. The samples were subjected for PCR analysis. (D, E) Hsp31 reverts MG modification on proteins. hSod1 (D) and Lysozyme (E) were glycate for 2 h with 2 mM MG and incubated with Hsp31 paralogs. The glycation status was determined through western analysis against anti-MAGE antibody. 10 kb DNA ladder was used as a marker (M) for DNA gels, and the Ponceau S stain indicates the equal loading of protein samples. BSA was used as a negative control in all experiments. The experiments were performed in 3 independent biological replicates. (F) The dual role of Hsp31 reduces MG-induced DNA mutations. Respective genes were PCR amplified, and Sanger sequenced from strains treated with 10 mM MG. The number of genetic mutations was plotted on GraphPad prism 5.0 (n=2).

**Figure 6-figure supplement 1. Hsp31^C138A fails to repair glycated DNA and proteins.** (A-D) Hsp31^C138A mutant lacks deglycase activity. Macromolecules were treated with 2 mM MG for 2 h and incubated with Hsp31-WT and Hsp31^C138A mutant (2 µg each). The glycation status was determined through PCR and western analysis. (E) Individual genes, as indicated, were PCR amplified from various strains incubated with 10 mM MG for 16 h and Sanger sequenced. The number of genetic mutations was determined and represented here.
Figure 7. Glyoxal induced translocation of yeast DJ-1 orthologs into mitochondria. (A-E) Mitochondrial translocation of Hsp31 paralogs. WT strain expressing genomic GFP tagged Hsp31 paralogs, and MTS-mCherry (decorates mitochondria) plasmid were treated with either buffer (-GO/-MG) or 15 mM GO (+GO) or 10mM MG (+MG) for 3 h. Consequently, images were captured in a confocal microscope (Olympus FV3000) and represented with 10 µm scale. (F-G) Mitochondrial protein and DNA glycation levels. WT and ΔQ overexpressing Hsp31 class of proteins were treated with 15 mM GO stress, followed by isolation of mitochondria and western analysis to determine glyoxal modifications using an anti-CML antibody. The intensity of each lane and dot was quantitated densitometrically and plotted in the graph. One-way ANOVA with Dunnett’s multiple comparisons test was used to determine significance from 3 independent biological replicates, *, p≤ 0.05; **, p≤ 0.01; ***, p≤ 0.001; NS, not significant.

Figure 7-figure supplement 1. Western and microscopic analysis of mitochondrial translocated Hsp31 paralogs. (A) Hsp31 paralogs genomically tagged with HA were treated with GO at log phase, and their levels were probed from fractionated mitochondria. For fold change, the band intensities were quantified and normalized with no treatment (-GO). The purity is indicated by the mitochondrial and cytosol control proteins, Tim23 and Ydj-1, respectively. (B) Probing mitochondrial translocated Hsp31. Mitochondria were isolated from MG-treated cells expressing Hsp31-HA. and subjected to immunoblotting to determine the levels. The band intensities from respective lanes were quantified and normalized with –MG. (C-E) MG does not affect the localization of Hsp32, Hsp33, and Hsp34. Cells containing genomic GFP-tagged Hsp31 paralogs and MTS-mCherry were incubated without (-MG) or with MG (+MG) and visualized under the microscope. (F) Purity of isolated
mitochondria. Mitochondria used in the study (Figure 7F, G) were analyzed for quality by probing with mitochondrial control protein, Tim23, and cytosol control Ydj-1. Paired t-test was used to determine significance of 3 independent biological replicates, *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; NS, not significant.

**Figure 8. Loss of yeast DJ-1 members induces mitochondrial dysfunction.** (A) Visualization of mitochondrial morphology. WT and △Q strains expressing MTS-mCherry (decorates mitochondria) were either treated with buffer (-GO) or 15 mM GO (+GO), followed by imaging. (B, C) FACS analysis to estimate total and functional mitochondrial mass. Respective strains were grown till the early log phase, followed by incubation with GO. Later, the cells were stained with Nonyl Acridine Orange (NAO) for total mass and TetraMethylRhodamine ethyl ester (TMRE) for determining functional mass. (D) Measurement of ATP levels. Selected strains were exposed to GO treatment at the mid-log phase. Consequently, the mitochondria were isolated, and the ATP levels were estimated through a fluorescence assay. (E) mtDNA staining by SYTO18. Following the treatment with GO, WT, and △Q were stained with SYTO18 dye. The microscopic analysis was performed in a confocal microscope (Olympus FV3000) and with 10 µm scale in images. All experiments were performed in 3 independent biological replicates and analysed through paired t-test to determine significance, *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; NS, not significant.

**Figure 8-figure supplement 1. Phenotypic analysis of Hsp31 deletion strains in non-fermentable carbon source.** (A) Selected strains were grown till the early log phase and spotted on media plates containing YP dextrose (2%) or YP glycerol (2%). Plates were incubated at 30°C, and images were acquired at 36 h.
Figure 9. Model depicting the role of yeast DJ-1 orthologs in the maintenance and protection against carbonyls. Various metabolic pathways generate MG and GO that spontaneously glycate cytosol and organellar macromolecules (1). Hsp31 paralogs scavenge excess endogenous RCS and regulate their levels (2). The dual role of paralogs provides nucleic acid protection by efficient detoxification of carbonyls and reversal of damaged molecules (3). Deglycation of proteins preserves the functional integrity of proteins (4). Dicarbonyl stress-induced translocation of Hsp31 paralogs into mitochondria confers enhanced organellar protection by attenuating damage to mitochondrial biomolecules (5).
**Table B**

<table>
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<th>Protein</th>
<th>$K_m$ (M)</th>
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<th>$V_{max}$ (min$^{-1}$ M)</th>
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<td>809.5</td>
<td>2.5x10$^{-2}$</td>
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<td>795.7</td>
<td>2.4x10$^{-2}$</td>
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</table>
**Graph A**: DNA gel with bands labeled UN, GO, 31, 32, 33, 34, 31C138A, 32C138A, 33C138A, 34C138A, and BSA. The gel is marked with 500 bp markers.

**Graph B**: RNA gel with bands labeled UN, GO, 31, 32, 33, 34, 31C138A, 32C138A, 33C138A, 34C138A, and BSA. The gel is marked with 500 bp markers.

**Graph C**: hSod1 + GO gel with bands labeled UN, GO, 31, 32, 33, 34, 31C138A, 32C138A, 33C138A, and 34C138A. The gel is marked with 20 kDa markers.

**Graph D**: Lysozyme + GO gel with bands labeled UN, GO, 31, 32, 33, 34, 31C138A, 32C138A, 33C138A, and 34C138A. The gel is marked with 15 kDa markers.

**Graph E**: Table showing gene expression.

<table>
<thead>
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<th>Gene</th>
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<th>ΔQ</th>
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<th>ΔQ/32↑</th>
<th>ΔQ/33↑</th>
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<tr>
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<td>0</td>
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<td>10</td>
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</tbody>
</table>

**Graph F**: Comparison of WT and ΔQ under control, 0.01% MMS, and 150 mM HU conditions.

**Graph G**: Bar chart showing the number of foci with WT and ΔQ, where ΔQ has significantly more foci compared to WT (**p < 0.001**).