1 Rhodanese Rdl2 produces reactive sulfur species to scavenge hydroxyl radical and
2 protect mitochondria

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26 **Abstract**

27 During aerobic respiration, mitochondria generate superoxide anion ($O_2^{\cdot-}$), hydrogen
 28 peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$), and these reactive oxygen species (ROS) are
 29 detrimental to mitochondria. Mitochondrial damage is linked to a broad spectrum of
 30 pathologies such as Alzheimer's disease, hemochromatosis, and diabetes. Mitochondria
 31 contain several enzymes for rapidly removing superoxide anion and hydrogen peroxide, but
 32 how they antagonize $HO^{\cdot}$ is elusive, representing a loophole in the anti-ROS system. Herein,
 33 we discovered that Rhodanese 2 (Rdl2) is critical for maintaining the functionality and
 34 integrity of mitochondria under sub-lethal ROS stress in *Saccharomyces cerevisiae*. Rdl2
 35 converts stable sulfur species (thiosulfate and dialkyl polysulfide) to reactive sulfane sulfur
 36 including persulfide that protects mitochondrial DNA via scavenging $HO^{\cdot}$. Surprisingly,
 37 hydrogen sulfide (H_2S) promotes $HO^{\cdot}$ production through stimulating the Fenton reaction,
 38 leading to increased DNA damage. Our study may reveal an *ex-ante* mean for antagonizing
 39 $HO^{\cdot}$, patching the loophole of the anti-ROS system in mitochondria.

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41 *Keywords: Rhodanese, antioxidant, reactive sulfane sulfur, Fenton reaction, hydroxyl radical,*
 42 *mitochondria health*

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49 **Introduction**

50 Mitochondria are essential organelles in eukaryotic cells. They produce about 80% of
 51 cellular energy via oxidative phosphorylation under oxic condition [1]. Further, they have
 52 other key functions including heme production, iron-sulfur cluster biogenesis, and calcium
 53 homeostasis [2]. Maintaining the functionality and integrity of mitochondria is critical for
 54 human health, and failing to do so would lead to a broad spectrum of pathologies, such as
 55 Alzheimer's, Parkinson's disease, hemochromatosis, atherosclerosis, carcinogenesis, and
 56 diabetes mellitus [3-6].

57 As a hectic distribution station of metabolites, mitochondria incessantly experience many
 58 types of stresses. A widely documented one is mediated by reactive oxygen species (ROS)
 59 that includes the superoxide anion ($O_2^{\bullet-}$), the hydrogen peroxide (H_2O_2) and the hydroxyl
 60 radical ($HO^{\bullet}$) [7]. $O_2^{\bullet-}$ is directly produced by Complex I, III, and IV via transferring one
 61 electron to O_2 . The scavenging of $O_2^{\bullet-}$ is conducted by superoxide dismutases (SOD), which
 62 convert $O_2^{\bullet-}$ to H_2O_2 . GPX enzymes including peroxiredoxins, thioredoxins, and glutathione
 63 peroxidases are the major H_2O_2 scavengers [7,8]. SOD and GPX compose a major anti ROS
 64 system in mitochondria. Albeit the existence of these enzymes, a fraction of H_2O_2 still
 65 converts to $HO^{\bullet}$ via the Fenton reaction. Different from H_2O_2 , $HO^{\bullet}$ is a very strong and labile
 66 oxidant (2.33 V, at pH 7) that rapidly reacts with most organic molecules, such as DNA,
 67 proteins, lipids, and polysaccharides, causing structural damages to mitochondria if out of
 68 control. It is commonly believed that mitochondria only have *ex-post* means for antagonizing
 69 $HO^{\bullet}$ —resorting to the repair systems for mending the damages [9], and no a specific enzyme

70 or compound has been assigned for specifically eliminating it. Therefore, how mitochondria
71 maintain $\text{HO}^\bullet$ under lethal level is a loophole in their anti ROS system.

72 In recently years, a new type of cellular compounds defined as reactive sulfane sulfur (RSS)
73 has been discovered in both eukaryotic and prokaryotic cells [10-13]. RSS includes inorganic
74 polysulfide (HS_nH , $n \geq 2$), organic polysulfide (RS_nH , $n \geq 2$), and polysulfane (RS_nR , $n \geq 2$) [14].

75 Although several RSS generation enzymes including cystathionine gamma-lyase,
76 cystathionine beta-synthase, and 3-mercaptopyruvate sulfurtransferase (Mst) have been
77 identified in cytoplasm [15,16], mitochondria are thought to be the main RSS generation
78 organelles in mammals, where sulfide:quinone oxidoreductase (Sqr) oxidizes hydrogen
79 sulfide (H_2S) to RSS and excessive RSS is oxidized by persulfide dioxygenase (Pdo) to sulfite
80 [17]. CysteinyI-tRNA synthetase 2 (Crs2) also has unspecific RSS generation function via
81 converting cysteine to cysteine persulfide (CysSSH) in mitochondria [18]. Impairing RSS
82 biogenesis leads to mitochondrial dysfunction, intimating a close connection between RSS
83 biogenesis and mitochondrial health, but the underlying mechanism remains elusive [19,20].

84 *Saccharomyces cerevisiae* mitochondria have no Sqr or Pdo. In 2019, Nishimura et al.
85 discovered that *S. cerevisiae* cysteinyI-tRNA synthetase (Crs1) is expressed via an alternative
86 transcriptional initiation to locate into mitochondria, and hence it may pertain to
87 mitochondrial RSS generation [21]. Recently, we found that rhodanese 1 and 2 (Rdl1 and
88 Rdl2), members of the sulfurtransferase family, are responsible for thiosulfate assimilation in
89 *S. cerevisiae*, and during this process, glutathione persulfide (GSSH) is formed as an
90 intermediate [22]. Herein, we verified that rhodanese 2 (Rdl2) is the main RSS generation
91 enzyme in mitochondria and more importantly, it is crucial for maintaining mitochondrial

health under ROS stress. Knocking out Rdl2 results in the change of mitochondrial morphology, loss of mitochondrial DNA (mitDNA), reduction of oxidative phosphorylation efficiency, and change of iron metabolism. RSS generated by Rdl2 actively scavenges HO[•]. Thus, an active mechanism to remove HO[•] might be identified.

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97 **Materials and Methods**

98 *Strains and materials*

99 *S. cerevisiae* BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and CEN.PK2
100 (*MATα/MATα ura3-52/ura3-52; trp1-289/trp1-289; leu2-3,112/leu2-3,112; his3Δ 1/his3Δ 1;*
101 *MAL2-8C/MAL2-8C; SUC2/SUC2*) strains were cultured in yeast extract-peptone-dextrose
102 (YPD) medium or synthetic defined (SD) medium at 30°C. The constructed mutants are listed
103 in supplementary material (Table S1). *E. coli* DH5α and BL21(DE3) strains were cultured in
104 lysogeny broth (LB) medium at 37°C. Thiosulfate and sodium hydrosulfide (NaHS) were
105 purchased from Sigma-Aldrich (Saint Louis, MO). Dimethyl trisulfide (MeSSSMe) and S₈
106 were purchased from TCI (Shanghai, China) Company. HS_nH and GSSH were prepared
107 following the protocol of Luebke et al [23]. Other chemicals were purchased from local
108 companies if not specifically mentioned.

109 Key methods were described as below and others were provided in supplementary material
110 (Materials and methods).

111

112 *Subcellular Localization of Rdl1 and Rdl2.*

113 A GFP encoding sequence was fused to *RDL1* and *RDL2* C-termini in BY4742

114 chromosome by using the one-step PCR-mediated gene disruption method [24]. Yeast cells
115 were collected by centrifugation (10,000g, 5 min) and diluted to 1 OD₆₀₀ in sterile SD
116 medium containing 10 nM Mito Tracker Red CMSRos (Thermo Fisher, Waltham, MA). The
117 cell suspensions were incubated at 30°C for 20 min in dark place and then washed three times
118 to remove the extracellular CMSRos. Yeast cells were suspended in water and imaged by
119 using a fluorescence microscope (Olympus IX83). GFP and CMSRos co-localization was
120 analyzed.

121 To analyze the expression level of fused Rdl2-GFP in different growth phases, Microplate
122 Reader Synergy H1 was used with λ_{ex} of 488 nm and λ_{em} of 525 nm. To analyze the expression
123 level of fused Rdl2-GFP in the presence of H₂O₂, 1 OD₆₀₀ middle-log phase yeast cells (8 h
124 cultivation) were treated with 5 mM H₂O₂ at 30°C for 1 h, and then subjected to flow
125 cytometry analysis. For each sample, 10⁵ cells were analyzed in the FL1 channel and the
126 average fluorescence value was recorded.

127

128 *Transcriptomic analysis*

129 *S. cerevisiae* strains were cultured in YPG medium until OD₆₀₀ reached 0.8, and then
130 2 mM H₂O₂ was added and the cultivation was expanded for 12 h. Cells were harvested
131 for the omics analysis, which were performed at Shanghai Applied Protein Technology Co.,
132 Ltd (Shanghai, China). For transcriptomic analysis, total RNA was extracted. Magnetic beads
133 with Oligo (dT) were used to enrich mRNA and fragmentation buffer was added to randomly
134 interrupt the mRNA. The first strand of cDNA was obtained with six-base random primers
135 and the second strand of cDNA was synthesized by adding buffer, dNTPs and DNA

136 polymerase I. Double-stranded cDNA was purified with AMPure XP and then A-tailing and
 137 sequencing adapters were connected. The AMPure XP beads were used for fragment size
 138 selection and PCR enrichment was performed to obtain the final cDNA library. The library was
 139 sequenced on Illumina NovaSeq 6000 platform. Sequencing was performed at Shanghai
 140 Applied Protein Technology Co., Ltd. The clean data were obtained from raw data by
 141 removing reads containing adapter, poly-N and low quality reads. The clean reads were
 142 aligned with the genome of *S. cerevisiae* BY4742 by using HISAT2. The featureCounts
 143 software was used to calculate the FPKM value of each gene expression in each sample.
 144 Genes with a p-value < 0.05 and fold change > 2 were considered as significantly differentially
 145 expressed.

146

147 *Targeted metabolomics analysis*

148 For targeted metabolomics analysis, cells were harvested and quickly frozen in liquid
 149 nitrogen. After grinding with liquid nitrogen, 60 mg cells were mixed with 1 ml methanol
 150 acetonitrile aqueous solution (2:2:1, v/v) and vortexed for 60 s. Cells were then disrupted
 151 using the ultra-sound method. The broken cells were placed at -20 °C for 1 h and then
 152 centrifuged at 14,000g for 20 min at 4 °C. The obtained supernatant was subjected to
 153 freeze-dry and then LC-MS analysis. The Waters I-class ultra performance liquid
 154 chromatography was used and the mobile phase A was an aqueous solution with 25 mM
 155 ammonium acetate and 25 mM ammonia (pH, 9.75). Mobile phase B was acetonitrile. Protein
 156 sample was placed in a 4 °C autosampler, and the column temperature was set to 40 °C. The
 157 flow rate was 0.3 mL/min, and the injection volume was 2 µL. The liquid phase gradient was

158 set as: 0-1 min, phase B at 95%; 1-14 min, B linearly changing from 95% to 65%; 14-16 min,
159 B linearly changing from 65% to 40%; 16-18 min, B at 40%; 18-18.1 min, B linearly
160 changing from 40% to 95%; 18.1-23 min, B at 95%. In the sample cohort, one QC sample
161 was set every six experimental repeats to detect and evaluate the stability and repeatability of
162 the system.

163 The AB 5500 QqQ mass spectrometer (AB SCIEX, Framingham, MA) was used for mass
164 spectrometry analysis. The ESI source conditions were as: sheath gas temperature, 350°C; dry
165 gas temperature, 350°C; sheath gas flow, 11 L/min; dry gas flow, 10 L/min; capillary voltage
166 was 4000 V for positive mode and -3500 V for negative mode; nozzle voltage, 500 V; and
167 nebulizer pressure, 30 psi. Monitor was in MRM mode and the dwell time of each MRM
168 transition was 3 ms, and the total cycle time was 1.263 s. MRMANalyzer (R) was used to
169 extract the original MRM data of 200 metabolites to obtain the peak area of each metabolite.
170 Metabolites with p-value<0.05 and fold change>1.5 were considered as at significantly
171 different levels.

172

173 *Mitochondria preparation and mitochondrial RSS analysis*

174 *S. cerevisiae* mitochondria were isolated using differential centrifugation as described
175 previously [25]. The fluorescence-based probe psGFP1.1 was expressed and localized into
176 mitochondrial matrix of BY4742 as reported previously [26]. Mitochondria containing
177 psGFP1.1 (mit-psGFP) were isolated and treated with sulfur containing chemicals in isolation
178 buffer (1 mM EDTA, 0.6 M sorbitol, 10 mM Tris-HCl, pH 7.4). The mixtures contained
179 mitochondria and 400 µM cysteine, thiosulfate, or MeSSSMe. After incubated at 30°C for 1 h,

180 treated mitochondria were centrifuged at 12,000g for 15 min and washed with isolation buffer
181 (1 mM EDTA, 0.6 M sorbitol, 10 mM Tris-HCl, pH 7.4). Fluorescence was detected using the
182 Synergy H1 microplate reader. The emission intensities at 515 nm excited by both 408 nm
183 and 488 nm were recorded, and the ratio of 408/488 was used to represent the reactive sulfane
184 sulfur level in mitochondria. Explanation of the calculation principle can be found in a
185 previous report [26].

186

187 *Radical-induced pDNA cleavage assay*

188 The pDNA cleavage assay was performed following the previously reported method [27].
189 The pBluescript II SK (+) plasmid was used as the model DNA. 25 µg/µL plasmid was mixed
190 with 50 µM Fe²⁺ and 50 µM H₂O₂ in distilled water. The mixture was incubated at room
191 temperature for 3 h and then analyzed by electrophoresis. The supercoiled (SC) and nicked
192 circular (NC) forms of plasmid was quantified using the software FluorChem Q (Protein
193 Simple, Inc.). SC ratio was calculated as SC/(SC+NC) and used as the DNA damage index.
194 For testing the protection effect of Rdl2 products, Rdl2 (2.5 µM) was incubated with 1 mM
195 thiosulfate or MeSSSMc at room temperature for 1 h, and then Rdl2 was removed using a
196 3-kDa filter. The filtrated products were diluted with HEPES buffer (100 mM, pH 7.4) to
197 prepare product-dilution solutions, which were added into the DNA-Fe²⁺-H₂O₂ mixture and
198 subjected to the same analysis. Thiosulfate, MeSSSMc, H₂S (in the form of NaHS), S₈,
199 laboratory prepared GSSH, GSH, and L-ascorbic acid were also tested in the same conditions.

200 For testing whether H₂S reduces Fe³⁺ to promote the Fenton reaction, 25 µg/µL plasmid
201 was incubated with 50 µM Fe³⁺, 50 µM H₂O₂ and NaHS or S₈ (20 µM-300 µM) at room

202 temperature for 3 h. As the control, 25 µg/µL plasmid was incubated with 50 µM H₂O₂ and
 203 NaHS (20 µM-300 µM) at room temperature for 3 h. The untreated-plasmid DNA and the
 204 Fenton solution-plasmid DNA mixture were also used as controls.

205

206 *In vitro analysis of produced H₂S and reactive sulfane sulfur*

207 A modified methylene blue method [28] was used to detect the produced H₂S. Briefly, 1%
 208 zinc acetate solution (900 µL) was added into the reaction solution (100 µL) to convert H₂S to
 209 ZnS. The mixture was centrifuged at 8,000g for 5 min, and washed with deionized and
 210 distilled water. The washing step was repeated three times. After removing the final
 211 supernatants, deionized and distilled water (100 µL) was added to the precipitates (ZnS) and
 212 adequately mixed. Then, the solution was incubated with 300 µL of 1% zinc acetate, 50 µL of
 213 20 mM DPDA (N,N-dimethyl-p-phenylenediamine) and 30 mM FeCl₃ in 7.2 N HCl for 30
 214 min. The samples were centrifuged at 8,000g for 5 min and transferred into 96-well plates to
 215 measure the OD₆₃₀. NaHS was used to make the standard curve.

216 Reactive sulfane sulfur was analyzed by using a previously reported method [29]. Briefly,
 217 the sample (50 µL) was mixed with 25 mM monobromobimane (mBBBr, 5 µL) in acetonitrile
 218 and incubated in the dark at room temperature for 30 min. An equal volume of acetic acid and
 219 acetonitrile mixture (v/v, 1:9) was added to precipitate proteins. The precipitates were
 220 removed via centrifugation at 12,000 g for 2 min. The obtained supernatant was subjected to
 221 LC-ESI-MS analysis (Ultimate 3000, Burkert impact HD).

222

223 *Statistical Analysis*

224 Transcriptomics and targeted metabolomics analysis were performed with six parallel
225 biological samples. The data have been deposited in <https://www.biosino.org/node/> with ID:
226 OEP001468. Other analysis were performed with at least 3 parallel biological samples. Data
227 are presented as mean $\pm$ S.D.

228

229 **Results**

230 *Rdl2 is the main enzyme for RSS biogenesis in S. cerevisiae mitochondria*

231 To check if Crs1 was mainly responsible for generating RSS in yeast mitochondria, we
232 expressed it in *Escherichia coli* BL21(DE3) and purified it. The enzymatic activity was
233 examined by mixing Crs1 with PLP (pyridoxal phosphate, the cofactor) and cysteine (the
234 substrate). The product was analyzed by using the sulfane sulfur prober 4 (SSP4) [30] and
235 LC-MS, and only trace amounts of cysteine persulfide (Cys-SSH) were produced (Fig. 1A
236 and supplementary material, Figure S1). Crs1 was overexpressed in *S. cerevisiae* BY4742
237 from two different promoters, the inducible promoter P_{gal} and the constitutive promoter P_{tefl} ;
238 neither one led to increased RSS production *in vivo* (Fig. 1B and 1C). Since knocking out
239 Crs1 in haploid strain BY4742 is lethal, we performed the knock-out experiment in the
240 diploid strain CEN.PK2. The Crs1 knock-out strain (*crs1*^{-/-}) showed no detectable difference
241 from its wild type (wt) in terms of intracellular RSS level (Fig. 1D). The above experiments
242 suggested that Crs1's contribution to the mitochondrial RSS is limited.

243 To check whether Rdl1 and Rdl2 were mainly responsible for RSS generation in yeast
244 mitochondria, we first investigated the locations of these two enzymes. A green fluorescent
245 protein (GFP) was fused with Rdl1 and Rdl2 by inserting the GFP ORF into the C-termini of

246 Rdl1 and 2 ORFs in *S. cerevisiae* BY4742 genome. The Mito Tracker Red CMSRos was used
247 to label BY4742 mitochondria. Rdl1/2-GFP and CMSRos co-location analysis showed that
248 Rdl2 particularly located in mitochondria; whereas, Rdl1 mainly located in cytoplasm (Fig.
249 1E). We isolated intact mitochondria from BY4742 (wt) and the *RDL2* deletion ($\Delta rdl2$)
250 mutant, in which the mitochondrial RSS detection probe mit-psGFP [26] was expressed. The
251 wt mitochondria contained more RSS than did the $\Delta rdl2$ counterparts, as represented by a
252 higher 408/488 fluorescence ratio (Fig. 2A). The isolated mitochondria were then treated with
253 cysteine, thiosulfate, or organic polysulfide (RS_nR , $n \geq 2$)—the three potential substrates of
254 sulfurtransferases. The addition of cysteine caused no RSS increase, indicating that again
255 Crs1 did not use cysteine to produce RSS. Thiosulfate and dimethyl trisulfide (MeSSSM_e)
256 caused 27% and 261% increases of RSS, respectively, in wt mitochondria (Fig. 2B). Whereas,
257 thiosulfate caused no increase, but MeSSSM_e caused 66% increase of RSS in $\Delta rdl2$
258 mitochondria (Fig. 2C). The *RDL2* gene was cloned and expressed in *E. coli* BL21 (DE3).
259 The addition of thiosulfate and MeSSSM_e increased RSS in *RDL2*-expressing *E. coli*, but did
260 not in the *E. coli* host (Fig. 2D and 2E).

261 RSS production by Rdl2 was further tested with purified Rdl2. It produced RSS from
262 thiosulfate and MeSSSM_e as detected with SSP4; the amount of RSS was positively
263 correlated to the amount of added Rdl2 (Fig. 2F and 2G). No apparent production of H₂S was
264 detected by using a modified methylene blue method [28]. When the products of MeSSSM_e
265 reacting with Rdl2 were derivatized by monobromobimane (mBBr) and analyzed by using
266 LC-MS. A major peak corresponding to mB-SS-mB was detected (supplementary material,
267 Figure S2A), indicating the production of hydrogen persulfide (HS₂H, HS₂⁻, or S₂²⁻). A much

268 smaller peak of likely mB-SSS-mB was also observed (supplementary material, Figure S2B).
 269 MeSSMe, but neither MeSMe nor Me-SS-mB, was detected (supplementary material, Figure
 270 S2C), indicating that Rdl2 releases a sulfane sulfur atom from MeSSSMe to produce HS_nH
 271 and MeSSMe.

272

273 *Rdl2 is essential for maintaining mitochondrial health under sub-lethal levels of ROS*

274 Compared with wt strain, the $\Delta rdl2$ strain showed slightly growth retardation on both a
 275 fermentable carbon source (glucose) and a none-fermentable carbon source (glycerol). When
 276 a sub-lethal level of H₂O₂ was present, the $\Delta rdl2$ strain displayed more severe growth
 277 retardation than wt strain, especially on none-fermentable carbon source (Fig. 3A-3C),
 278 suggesting that mitochondria were impaired in the $\Delta rdl2$ strain.

279 A GFP ORF was fused with the mitochondrial citrate synthase 1 (Cit1) encoding gene in
 280 genomes of both wt and $\Delta rdl2$ strains, which were cultured in YPD medium without the
 281 addition of H₂O₂. The mitochondrial morphology was observed with a laser-scanning confocal
 282 microscope. Mitochondria in wt displayed normal morphology with mainly filamentous or
 283 granular shape; whereas, mitochondria in the $\Delta rdl2$ strain displayed irregular shape (Fig. 3D).
 284 The GFP fluorescence intensity was also obviously lower in $\Delta rdl2$ than in wt. These
 285 phenomena indicated that mitochondria in the $\Delta rdl2$ strain were abnormal.

286 Several mitochondria-related physiological characteristics were then examined. First, the
 287 mitochondrial membrane potential of $\Delta rdl2$ was lower than that of wt no matter with or
 288 without the addition of H₂O₂ (Fig. 3E). Second, the oxygen consumption rate of the $\Delta rdl2$
 289 strain was obviously lower than that of wt with or without the presence of glucose (Fig. 3F).

290 Third, the relative number of mitDNA (mitochondrial DNA normalized against nuclear DNA)
291 was lower in $\Delta rdl2$ than in wt, especially in the presence of H_2O_2 (Fig. 3G). These results
292 verified that the health of mitochondria was impaired by Rdl2 knock-out, and the impairment
293 made $\Delta rdl2$ more sensitive to ROS stress.

294

295 *Systematically investigation of the physiological changes caused by knocking out Rdl2*

296 Transcriptomics and metabolomics approaches were applied to systemically analyze the
297 consequences of Rdl2 knock-out. Both wt and $\Delta rdl2$ strains were treated with 2 mM H_2O_2
298 because their physiological differences were more obvious under ROS stress. In transcriptome
299 level, 215 genes were downregulated and 156 genes were upregulated (fold change >2,
300 $p < 0.05$) in the $\Delta rdl2$ strain compared to wt (Fig. 4A). For specification:

301 I) Among the 117 genes involved in glycolysis/gluconeogenesis/pentose phosphate
302 pathway, only 11 of them were changed at transcription level (Fig. 4B), indicating the
303 metabolism in cytoplasm was not severely affected by Rdl2 deletion. However, or 10
304 genes involved in tricarboxylic acid (TCA) cycle, 7 were downregulated and 3 were
305 upregulated (Fig. 4C); for 20 genes involved in fatty acid degradation, 8 of them were
306 downregulated (supplementary material, Figure S3); for 11 genes involved in
307 respiration chain, 9 of them were downregulated and 2 were upregulated (Fig. 4D);

308 II) For 13 genes involved in anti-ROS processes, 9 of them were downregulated and 4
309 were upregulated (Fig. 4E); for 38 genes involved in responding to DNA damage and
310 replication stress, 23 of them were downregulated and 15 were upregulated; for 4 genes
311 involved in maintaining iron homeostasis, 1 of them were downregulated and 3 were

312 upregulated (Fig. 4F).

313 Together, these results indicated that the energy generation processes that mainly happen in
314 mitochondria were impaired, and some anti-DNA damage processes were activated by Rdl2
315 knock-out. It is noteworthy that *OAC1*, the gene involved in TCA cycle but also responsible
316 for thiosulfate importing into mitochondria was downregulated, implying a less thiosulfate
317 import to mitochondria. The well-known anti-ROS genes including mitochondrial thioredoxin
318 reductase (*TRR1*), thiol-specific peroxiredoxin (*AHP1*), and cytosolic catalase (*CTT1*) were
319 all downregulated in $\Delta rdl2$ strain, implying that their anti-ROS functions are not over-lapped
320 with Rdl2 (otherwise, they should be upregulated to compensate for the loss of Rdl2 function).
321 The upregulation of plasma membrane ferric importing/reduction enzymes (*FRE7* and *FRE1*)
322 and the mitochondrial iron transporter (*GGC1*), together with the downregulation of
323 mitochondrial iron-sulfur assembly enzyme (*ISU1*, which consumes reduced iron) implied
324 that there was a lack of reduced iron (Fe^{2+}) in mitochondria of $\Delta rdl2$ strain.

325 For metabolomics analysis, we targeted 200 metabolites of central and energetic
326 metabolism (supplementary material, sheet 1). Among them, 131 compounds were identified
327 with 36 showing significant abundance change between wt and $\Delta rdl2$ strains (fold
328 change >1.5, $p < 0.05$) (Fig. 4G). For specification:

329 I) The abundance of 23 metabolites was increased in $\Delta rdl2$ strain compared to that of wt
330 strain (Fig. 4H). The increase of reduced nicotinamide adenine dinucleotide (NADH)
331 and lactic acid indicated that the consumption of NADH through the respiration chain
332 was reduced.

333 II) The abundance of 13 metabolites was decreased in the $\Delta rdl2$ strain compared to that of

334 wt strain, including flavin mononucleotide (FMN), the main cofactor of enzymes
335 composing the respiration chain (Fig. 4H).

336 The metabolome included no iron, we performed iron analysis additionally. Without ROS
337 stress, the *Δrdl2* strain contained less Fe^{2+} and more Fe^{3+} compared to wt strain. The addition
338 of H_2O_2 increased the content of Fe^{3+} and decreased the content of Fe^{2+} in wt strain (Fig. 4I).
339 In *Δrdl2* strain, the content of Fe^{3+} was increased but content of Fe^{2+} was not by H_2O_2 ,
340 indicating that more Fe^{3+} was imported into the cell (Fig. 4I). These phenomena were in
341 consistent with the results of transcriptomic analysis.

342

343 *Rdl2-generated RSS protects DNA from hydroxyl radical-induced damage*

344 We suspected that the Rdl2-generated RSS may protect mitDNA via interfering with the
345 Fenton reaction. To test this hypothesis, the classical radical-induced plasmid DNA (pDNA)
346 cleavage assay [27] was performed. The principle of this assay is when Fe^{2+} , H_2O_2 , and pDNA
347 are mixed in deionized water, $\text{HO}^\bullet$ generated from the Fenton reaction cleaves the supercoiled
348 plasmids (SC) and converts most of them to nicked-circular form (NC), but when a $\text{HO}^\bullet$ or
349 H_2O_2 scavenger is present, the damage to pDNA is inhibited. Fe^{2+} (50 μM), H_2O_2 (50 μM),
350 and pDNA (25 $\mu\text{g}/\mu\text{l}$) were used. We first tested thiosulfate and MeSSSMe (20 μM ~ 300 μM),
351 and observed no protection of SC by either one. Second, we tested the products of Rdl2 +
352 thiosulfate and Rdl2 + MeSSSMe reactions, SC percentage was apparently increased by each
353 one, indicating a pDNA protection effect (Fig. 5A). As two controls, laboratory prepared
354 glutathione persulfide (GSSH, made via mixing GSH with S_8) and S_8 also showed SC
355 protection effect (Fig. 5B and 5C). These results indicated that RSS indeed interfere with the

356 Fenton reaction.

357 RSS may function through reacting with H_2O_2 to decrease the production of $\text{HO}^\bullet$, or
 358 scavenging the produced $\text{HO}^\bullet$. We examined the Fe^{3+} production from the Fenton reaction
 359 with or without the addition of S_8 . The Fe^{3+} production from the $\text{Fe}^{2+} + \text{H}_2\text{O}_2 + \text{S}_8$ solution
 360 was not decreased but slightly higher than that without S_8 (Fig. 5D), suggesting that S_8 did not
 361 react with H_2O_2 in the Fenton solution. The slight increase in Fe^{3+} production in the presence
 362 of S_8 favorably argues that S_8 reacts $\text{HO}^\bullet$ and drives the Fenton reaction forward. We also
 363 determined the rate constant of the $\text{S}_8 + \text{H}_2\text{O}_2$ reaction to be $1.04 \text{ M}^{-1} \cdot \text{s}^{-1}$ (supplementary
 364 material, Figure S4), much lower than the reported rate constant of the Fenton reaction, $\sim 10^3$
 365 $\text{M}^{-1} \cdot \text{s}^{-1}$ [31], further supporting that S_8 does not react with H_2O_2 in the Fenton solution. GSH
 366 and L-ascobic acid are well-known cellular antioxidants [32,33], but neither of them showed
 367 SC DNA protection at similar concentrations to RSS ($20 \mu\text{M} \sim 300 \mu\text{M}$, Fig. 5E and 5F). Thus,
 368 S_8 likely scavenges $\text{HO}^\bullet$ to protect SC.

369 H_2S was recognized as an endogenous antioxidant in a profound study that led the tide of
 370 H_2S related research [34]. However, we found that it promoted pDNA cleavage in the Fenton
 371 solution (Fig. 5G), indicating it stimulates the Fenton reaction. To reveal the underlying
 372 mechanism, we mixed Fe^{3+} (instead of Fe^{2+}), H_2O_2 , and pDNA with or without H_2S . The
 373 pDNA cleavage was observed in the presence of H_2S , but not in its absence (Fig. 5H).
 374 Without iron, the H_2O_2 solution with H_2S showed no pDNA cleavage (Fig. 5I). These results
 375 suggested that H_2S functions through reducing Fe^{3+} to Fe^{2+} to facilitate the Fenton reaction.
 376 For verification, we analyzed the Fe^{3+} production from the Fenton reaction with or without
 377 H_2S , and much higher Fe^{3+} was detected from the Fenton reaction without H_2S than with H_2S

(Fig. 5D). Black precipitate (FeS) was temporary produced in the $\text{Fe}^{2+} + \text{H}_2\text{O}_2 + \text{H}_2\text{S}$ solution, but it disappeared in 2 min, indicating that S^{2-} in FeS is still available for reacting with H_2O_2 and Fe^{3+} . These results suggest that H_2S enhances the Fenton reaction.

We then measured the rate constants of sulfur-containing compounds reacting with $\text{HO}^\bullet$. H_2S slightly descended the absorbance ratio (A_0/A) of the $\text{HO}^\bullet$ probe, suggesting that it actually enhanced the production of $\text{HO}^\bullet$ (from Fenton reaction) other than scavenging it (Fig. 6A). GSH modestly reacted with $\text{HO}^\bullet$, represented by a relative flat curve of the absorbance ratio; whereas GSSH and S_8 rapidly reacted with $\text{HO}^\bullet$ with 13.32×10^9 and 8.15×10^9 rate constants, respectively (Fig. 6B). These results, again, indicated that RSS was efficient $\text{HO}^\bullet$ scavenger.

RDL2 expresses and functions in post-logarithmic phase

The $\Delta rdl2$ strain released less H_2S than wt strain, detected by lead acetate papers (Fig. 7A). We checked the transcription levels (obtained from the transcriptome data) of enzymes involved in H_2S production pathways. Cbs (encoding gene is *CYS3*) and Cse (encoding gene is *CYS4*) that are responsible for H_2S production from organic substrate (mainly cysteine) were up-regulated in $\Delta rdl2$ strain (Fig. 7B). The enzymes responsible for H_2S production from inorganic substrates (sulfate and sulfite) were not significantly changed. These results suggested that $\Delta rdl2$ strain actually has a higher H_2S productivity than wt strain, but most of the produced H_2S may be consumed in Fe^{3+} reduction, leading to a less release portion.

The intracellular ROS levels of wt and $\Delta rdl2$ strains were compared using the DCFH-DA probe. Wt strain contained constantly lower ROS level than $\Delta rdl2$ strain and both showed

400 increasing ROS levels with growth time expanding (Fig. 7C). When H₂O₂ was added, ROS
401 level in wt strain was moderately increased; whereas, ROS level in $\Delta rdl2$ strain was sharply
402 increased (Fig. 7C), indicating that RSS is critical for controlling the level of intracellular
403 ROS.

404 The Rdl2-GFP expressing strain was treated with H₂O₂, and the expression of Rdl2-GFP
405 was only slightly increased in high H₂O₂ condition (> 5 mM/OD₆₀₀ cell) (Fig. 7D), indicating
406 that the expression of Rdl2 might not be ROS-induced. We analyzed the expression level of
407 Rdl2-GFP in different growth periods by flow cytometry, and observed that Rdl2 mainly
408 expressed in post-logarithmic phases (after 12 h in YPD medium) (Fig. 7E). For confirmation,
409 we inoculated cells of 8 h-culture (in which Rdl2 had not been expressed) onto the
410 H₂O₂-containing agar medium, wt cells barely grew and $\Delta rdl2$ cells totally did not grow.
411 When cells of 24 h-culture were inoculated (in which Rdl2 had been expressed), wt cells grew
412 much better while $\Delta rdl2$ cells also showed little growth (Fig. 7F). These results indicated that
413 the expression of Rdl2 is mainly controlled by growth related system(s) and its expression is
414 concurrent with the accumulation of intracellular ROS.

415

416 Discussion

417 In this study, we verified that Rdl2 is the main RSS-generating enzyme in *S. cerevisiae*
418 mitochondria. Rdl2 deletion resulted in RSS decrease in the mitochondria. Further, we
419 observed that Rdl2 deletion leads to morphology change of mitochondria even without the
420 treatment of H₂O₂ (Fig. 3). When facing H₂O₂ stress, Rdl2 deletion strain is more fragile with
421 obvious changes in mitochondria. To understand the underlying mechanism, we studied the

422 Rdl2 involved reactions and found that it generates RSS via releasing sulfane sulfur atoms
 423 from relatively stable sulfane sulfur carriers (Thiosulfate and RS_nR , Fig. 2). More importantly,
 424 the RSS product of Rdl2 can scavenge $HO^\bullet$ and hence protects mitochondria from $HO^\bullet$
 425 induced damages, such as loss of mitDNA, impair of mitochondria membrane, and
 426 disturbance of iron homeostasis.

427 Although we detected that HS_nH is the main product of Rdl2 *in vitro*, considering the
 428 abundance of intracellular GSH, the main product should be GSSH *in vivo* ($GSH + S_n \rightarrow$
 429 $GSSH + S_{n-1}$). It is reported that RSSH is an excellent H-atom ($H^\bullet$) donor, which easily gives
 430 its $H^\bullet$ to alkoxyl ($RO^\bullet$) with a $\sim 10^9 M^{-1}\cdot s^{-1}$ rate constant [35]. RSSH then becomes $RSS^\bullet$
 431 radical, and two $RSS^\bullet$ radicals dimerize to form one more stable RSSSSR with a $5 \times 10^9 M^{-1}\cdot s^{-1}$
 432 rate constant [35,36]. In contrast, the rate constant of $HO^\bullet$ reacting with DNA is at $10^8 M^{-1}\cdot s^{-1}$
 433 level, at least one magnitude lower than that of RSSH giving H-atom reaction. Thus, RS_nH
 434 and HS_nH chemicals have the potential capability of scavenging $HO^\bullet$ before it can damage
 435 DNA *in vivo*. For S_n , it is unknown how it scavenges $HO^\bullet$. Possibly via donating an electron
 436 to $HO^\bullet$ and forming $S_n^\bullet$ itself, then two $S_n^\bullet$ radicals form S_{2n} that has a longer chain. Further
 437 studies are required to elucidate the exact mechanism. It is noteworthy that although we
 438 observed that GSH and L-ascorbic acid did not protect pDNA from the Fenton reaction even
 439 when their concentrations were 6-fold higher than that of Fenton solution (Fig. 5E and 5F), a
 440 previous study demonstrated that GSH did show DNA protection effect at much higher
 441 concentration (>25 fold of Fenton solution). The authors suggested that GSH may also react
 442 with $HO^\bullet$ through H-atom transfer mechanism [37]. Nonetheless, GSH is a much less efficient
 443 H-atom donor than RSS as verified previously [35,36].

444 Mitochondria antagonize ROS at three levels. The scavenging of $O_2^{\bullet-}$ is conducted by SOD
445 and the scavenging of H_2O_2 is performed by GPX enzymes (Fig. 7). It is reported that the rate
446 constants of GPX reactions with H_2O_2 are in the range of $10^6 \sim 10^7 M^{-1} \cdot s^{-1}$ [38,39]. Metal iron
447 mediated-Fenton reaction is the main origin of $HO^{\bullet}$ in mitochondria with a rate constant of
448 $\sim 10^3 M^{-1} \cdot s^{-1}$ [31]. Considering the physiological concentrations of H_2O_2 and Fe^{2+} are ~ 20 nM
449 and ~ 500 nM, respectively [8], and the rate constants of $RSS + H_2O_2$ reactions are only 1.04
450 $M^{-1} \cdot s^{-1}$ — $23.76 M^{-1} \cdot s^{-1}$ [40], RSS should have no chance to react with H_2O_2 in mitochondria.
451 Thus, RSS plays antioxidation function mainly through scavenging $HO^{\bullet}$, as we observed that
452 it reacts with $HO^{\bullet}$ more rapidly than GSH. This may represent the third ROS-antagonizing
453 level in mitochondria (Fig. 8).

454 On the other hand, H_2S has been deemed as an antioxidant in many reports [34,41,42],
455 further studies including ours found that its reaction with H_2O_2 is slow with 2nd rate constant
456 between $0.46 M^{-1} \cdot s^{-1}$ — $0.76 M^{-1} \cdot s^{-1}$ [40]. Considering the physiological concentration of H_2S is
457 much lower than that of RSS [15], the reaction between H_2S and H_2O_2 may not happen either
458 *in vivo*. Herein, we found that H_2S has promoting effect on the Fenton reaction, indicating that
459 H_2S has the pro-oxidation function other than antioxidation.

460 In terms of the whole eukaryotic kingdom, there are probably four routes for mitochondria
461 to obtain reactive sulfane sulfur: exporting from cytoplasm, generating from H_2S via Sqr,
462 generating from cysteine via Mst or Crs2, and generating from sulfane sulfur stocks via
463 rhodanese (Fig. 8). These routes may function with different efficiencies and in different
464 conditions. Sqr-mediated route might be the most efficient one; however, Sqr activity is ETC
465 (electron transport chain) dependent, hence it may function mostly in hypoxic condition.

466 Rhodanese-mediated route is oxygen independent, they may function in all conditions. For
467 cells having no Sqr (such as *S. cerevisiae*) or whose Sqr is not active (such as neuron), the
468 rhodanese-mediated RSS biogenesis represents a general strategy for protecting cells from
469 HO[•]-induced damage.

470 In conclusion, our study has three important observations:

- 471 i. RSS has HO[•] scavenging function, highlighting the unparallel role of RSS among all
472 known natural anti ROS agents.
- 473 ii. H₂S stimulates the Fenton reaction, suggesting that it may promote oxidative damage,
474 instead of being an antioxidant.
- 475 iii. Rhodaneses are critical for maintaining mitochondrial health, adding a new function for
476 them in anti ROS besides sulfur metabolism.

477

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482

483 **Author Contributions**

484 H. Liu and L. Xun designed the research and made plans for the experiments; Q. Wang, Z.
485 Chen, and Y. Xin performed Rd12 related experiments; X. Zhang performed Crs1 related
486 experiments. Y. Xia helped in data interpretation.

487

488 **Competing interests**

489 The authors declare no competing interests.

490

491 **Data availability**

492 The data that support the findings of this study are available from the corresponding author

493 upon request

494

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642 **Figure legends**

643 **Fig. 1. Activity and localization analyses of the enzymes pertaining to mitochondrial RSS**

644 **generation in *S. cerevisiae* BY4742.** A) SSP4 analysis of RSS generated by Crs1 *in vitro*.
 645 B&C) SSP4 analysis of intracellular RSS of BY4742 containing the *opo* plasmid (as control)
 646 and the Crs1 expression plasmid. D) SSP4 analysis of intracellular RSS of the CEN CEN.PK2
 647 wt (*crs1*^{+/+}) and *CRS1* knock-out (*crs1*^{-/-}) strains. E) Localization analysis of Rdl1 and 2.
 648 CMSRos is the Mito Tracker Red dye. Data were from three independent repeats and
 649 represented as average $\pm$ s.d.

650

651 **Fig. 2. *In vitro* and *in vivo* analysis of the Rdl2 contribution to mitochondrial RSS.** A)

652 BY4742 wt and Δ *rdl2* strains harboring mit-psGFP were cultured in YPD medium until
 653 OD600 reached 0.6, and then 408/488 excitation ratio of the cells was examined. B)
 654 Mitochondria harboring mit-psGFP were isolated from BY4742 wt strain, and were treated
 655 with 400 μ M different chemicals at 30°C for 1 h, and then 408/488 excitation ratio was
 656 examined. The higher the ratio, the higher the RSS. C) Mitochondria harboring mit-psGFP
 657 were isolated from BY4742 Δ *rdl2* strain, and were treated with 400 μ M different chemicals at
 658 30°C for 1 h, and then 408/488 excitation ratio was examined. D&E) *E. coli* wt and *rdl2*
 659 expressing strains were treated with thiosulfate and MeSSSMc. SSP4 was used to to examine
 660 the intracellular reactive sulfane sulfur. F&G) Purified Rdl2 was mixed with its substrates and
 661 the products were analyzed using SSP4. Data were from three independent repeats. *
 662 represents difference (p<0.05) and ** represents significant difference (p<0.01) in two-sided
 663 t-test. Data were from three independent repeats and represented as average $\pm$ s.d

664

665 **Fig. 3. Examination of the role of Rdl2 in maintaining mitochondrial health under ROS**

666 **stress.** A) BY4742 wt and $\Delta rdl2$ strains were cultivated in SD or YPG agar plates. 2 mM
667 H_2O_2 was added. B&C) BY4742 wt and $\Delta rdl2$ strains were cultivated in SD or YPG liquid
668 medium. 2 mM H_2O_2 was added. D) Mitochondrial morphology analysis of BY4742 wt and
669 $\Delta rdl2$ strains. The strains containing Cit1-GFP were cultivated in YPD medium to log phase
670 without H_2O_2 treatment. Images were captured with the laser confocal microscope LMS900.
671 E) Mitochondrial membrane potential analysis of wt and $\Delta rdl2$ strains. F) Oxygen
672 consumption analysis of wt and $\Delta rdl2$ strains. G) mit-DNA analysis of wt and $\Delta rdl2$ strains.
673 Embedded figure: the strains were treated with 2 mM H_2O_2 . Data were calculated from three
674 independent repeats.

675

676 **Fig. 4. Transcriptomics and targeted metabolomics analysis of BY4742 wt and $\Delta rdl2$**

677 **strains under the stress of 2 mM H_2O_2 .** A-F) Genes changed at transcriptional level. G&H)
678 Concentration changed metabolites. I) intracellular iron analysis. Transcriptomics and
679 targeted metabolomics analysis were performed with six parallel biological samples. Iron
680 analysis data were calculated from three independent repeats.

681

682 **Fig. 5. Plasmid DNA cleavage and Fenton reaction assays performed with Rdl2 products**

683 **and different chemicals.** Upper figure shows the gel electrophoresis results of pDNA and
684 lower figure shows the percentage of pDNA in SC form. A) 1, untreated pDNA; 2, pDNA
685 treated with Fenton reagents (50 μM Fe^{2+} and 50 μM H_2O_2); 3, pDNA treated with Fenton

686 reagents and thiosulfate (300 μ M); 4, pDNA treated with Fenton reagents and products from
 687 Rdl2 + thiosulfate reaction (10 fold-dilution); 5, pDNA treated with Fenton reagents and
 688 products from Rdl2 + thiosulfate reaction (2.5 fold dilution); 6, pDNA treated with Fenton
 689 reagents and MeSSSMe (300 μ M); 7, pDNA treated with Fenton reagents and products from
 690 Rdl2 + MeSSSMe reaction (10 fold-dilution); 8, pDNA treated with Fenton reagents and
 691 products from Rdl2 + MeSSSMe reaction (2.5 fold-dilution). B,C,E,F,&G) 1, untreated
 692 pDNA; 2, pDNA treated with Fenton reagents; 3-n, pDNA treated with Fenton reagents and
 693 tested chemicals (20 μ M-300 μ M). D) Fe^{3+} production from the Fenton reaction. CK is the
 694 mixture of 500 μ M Fe^{2+} + 500 μ M H_2O_2 ; 500 μ M S_8 or H_2S was added. H) 1, untreated
 695 pDNA; 2, pDNA treated with 50 μ M Fe^{3+} + 50 μ M H_2O_2 ; 3-7, pDNA treated with 50 μ M Fe^{3+} ,
 696 50 μ M H_2O_2 , and H_2S (20 μ M-300 μ M). I) 1, untreated pDNA; 2, pDNA treated with 50 μ M
 697 H_2O_2 ; 3-7, pDNA treated with 50 μ M H_2O_2 and H_2S (20 μ M-300 μ M). The SC percentage
 698 data were calculated from three independent repeats.

699

700

701 **Fig. 6. Kinetic analysis of $\text{HO}^\bullet$ radical scavenging activity of certain compounds using**
 702 **the modified CUPRAC method.** A) Kinetic plots measured using the $\text{HO}^\bullet$ probe
 703 3,5-dimethoxybenzoate. B) Rate constants calculated from the kinetic plots. Details of the
 704 CUPRAC method was described in supporting material.

705

706 **Fig. 7. *In vitro* analysis of the interaction between Rdl2 and ROS.** A) H_2S release
 707 detection using lead acetate papers. The strains were cultivated in SD medium for 80 h. B)

708 Transcription levels of enzymes involved in H₂S production pathways. Data were from
 709 transcriptomic analysis. The bar represent the average readcount of 6 parallel biological
 710 samples. C) Analysis of the intracellular ROS of BY4742 wt and *Δrdl2* strains. D) The
 711 expression of Rdl2-GFP was only slightly increased in high H₂O₂ condition (5 mM). E) The
 712 expression of Rdl2-GFP was different at different growth phases. F) BY4742 wt cells of
 713 stationary phase were more resistant to H₂O₂ than mid-log phase cells. Data were from three
 714 independent repeats and represented as average ± s.d

715

716 **Fig. 8. Schematic representation of the mitochondrial RSS biogenesis routes and**
 717 **functions.** *S. cerevisiae* mitochondria contain no Sqr or Mst. Rdl2 is the only enzyme that
 718 generates RSS, which can scavenge HO[•].

719

720

A **B** **C** **D** **E**

B

HO• scavengers	Linear equation and correlation coefficient	Rate constant ($M^{-1} s^{-1}$)
GSSH ($10^{-3} M$)	$y = 2.8917x + 1.1652$ $r: 0.9966$	13.32×10^9
S_8 ($10^{-3} M$)	$y = 1.7703x + 1.1753$ $r: 0.991$	8.15×10^9
GSH ($10^{-3} M$)	$y = 0.5916x + 0.9015$ $r: 0.9552$	2.71×10^9
H_2S ($10^{-3} M$)	$y = -0.2338x + 1.0666$ $r: 0.2977$	-

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