

# Enhancing autophagy by redox regulation extends lifespan in *Drosophila*

## Authors

Helena M. Cochemé<sup>1,2,3\*</sup>, Ivana Bjedov<sup>4</sup>, Sebastian Grönke<sup>5</sup>, Katja E. Menger<sup>6</sup>,  
Andrew M. James<sup>6</sup>, Jorge Ivan Castillo Quan<sup>3</sup>, Andrea Foley<sup>1,2</sup>, Claudia Lennicke<sup>1,2</sup>,  
Marcela Buricova<sup>1,2</sup>, Jennifer Adcott<sup>3</sup>, Filipe Cabreiro<sup>1,2</sup>, Michael P. Murphy<sup>6</sup>,  
Linda Partridge<sup>3,5\*</sup>

## Affiliations

<sup>1</sup> MRC London Institute of Medical Sciences, Du Cane Road, London W12 0NN, UK.

<sup>2</sup> Institute of Clinical Sciences, Imperial College London, Hammersmith Hospital Campus,  
Du Cane Road, London W12 0NN, UK.

<sup>3</sup> Institute of Healthy Ageing and GEE, University College London, Gower Street, London  
WC1E 6BT, UK.

<sup>4</sup> UCL Cancer Institute, 72 Huntley Street, London WC1E 6DD, UK.

<sup>5</sup> Max Planck Institute for Biology of Ageing, Joseph-Stelzmann-Str. 9b, 50931 Cologne,  
Germany.

<sup>6</sup> MRC Mitochondrial Biology Unit, University of Cambridge, Cambridge Biomedical Campus,  
Hills Road, Cambridge CB2 0XY, UK.

\* correspondence: [helena.cocheme@lms.mrc.ac.uk](mailto:helena.cocheme@lms.mrc.ac.uk) [partridge@age.mpg.de](mailto:partridge@age.mpg.de)

22 **Redox signalling is an important modulator of diverse biological pathways and processes, and**  
23 **operates through specific post-translational modification of redox-sensitive thiols on cysteine**  
24 **residues <sup>1-4</sup>. Critically, redox signalling is distinct from irreversible oxidative damage and**  
25 **functions as a reversible ‘redox switch’ to regulate target proteins. H<sub>2</sub>O<sub>2</sub> acts as the major**  
26 **effector of redox signalling, both directly and through intracellular thiol redox relays <sup>5,6</sup>.**  
27 **Dysregulation of redox homeostasis has long been implicated in the pathophysiology of many**  
28 **age-related diseases, as well as in the ageing process itself, however the underlying**  
29 **mechanisms remain largely unclear <sup>7,8</sup>. To study redox signalling by H<sub>2</sub>O<sub>2</sub> *in vivo* and explore**  
30 **its involvement in metabolic health and longevity, we used the fruit fly *Drosophila* as a model**  
31 **organism, with its tractable lifespan and strong evolutionary conservation with mammals <sup>9</sup>.**  
32 **Here we report that inducing an endogenous redox-shift, by manipulating levels of the H<sub>2</sub>O<sub>2</sub>-**  
33 **degrading enzyme catalase, improves health and robustly extends lifespan in flies,**  
34 **independently of oxidative stress resistance and dietary restriction. We find that the catalase**  
35 **redox-shifted flies are acutely sensitive to starvation stress, which relies on autophagy as a**  
36 **vital survival mechanism. Importantly, we show that autophagy is essential for the lifespan**  
37 **extension of the catalase flies. Furthermore, using redox-inactive knock-in mutants of Atg4a,**  
38 **a major effector of autophagy, we show that the lifespan extension in response to catalase**  
39 **requires a key redox-regulatory cysteine residue, Cys102 in Atg4a. These findings**  
40 **demonstrate that redox regulation of autophagy can extend lifespan, confirming the**  
41 **importance of redox signalling in ageing and as a potential pro-longevity target.**

42  
43 To explore the role of endogenous redox signalling *in vivo*, we used the UAS/GAL4  
44 expression system to up-regulate catalase in wild-type (WT) flies. Global up-regulation of catalase  
45 under control of the *daughterless* promoter (da-GAL4>UAS-cat) extended the median and  
46 maximum lifespan of female flies (typically by ~10-15%; Fig. 1a). Importantly, for these  
47 experiments we used the *white Dahomey* (*w<sup>Dah</sup>*) background, which is a long-lived and outbred WT,  
48 hence we are extending healthy lifespan and not rescuing a short-lived defect. Catalase was over-  
49 expressed ~5-fold at the mRNA level in whole flies (Fig. S1a). Lifespan was not extended in males  
50 (Fig. 1a), despite similar catalase over-expression (Fig. S1a), however interventions modulating  
51 nutrient-sensing in *Drosophila* often show gender-specific effects on survival in females, that are  
52 absent or marginal in males <sup>10</sup>.

53 The da-GAL4>UAS-cat flies were mildly delayed in eclosing, without affecting the  
54 proportion of larvae surviving to adulthood (Fig. S1b). To exclude developmental effects, we  
55 showed that the lifespan extension could be fully recapitulated using the inducible GeneSwitch  
56 system, with over-expression from d2 of adulthood onwards (da-GS>UAS-cat ±RU; Figs. 1b, S1a).

57 Varying the dose of the inducer drug RU (50-400  $\mu$ M) still did not extend lifespan in males and  
58 had only marginal effects on lifespan extension in females (Fig. S1c), suggesting that the catalase  
59 benefits are at maximum potential. Over-expressing catalase under the control of an alternative  
60 ubiquitous driver (actin5c-GAL4>UAS-cat) also extended lifespan (Fig. S1d). Furthermore,  
61 catalase-mediated lifespan extension was independent of *Wolbachia* status (Figs. S1e,f), which can  
62 influence fly longevity and physiology<sup>11,12</sup>. In addition to lifespan extension, the catalase females  
63 also exhibited increased healthspan<sup>13</sup>, as inferred from their enhanced climbing ability with age  
64 (Fig. 1c). To explore effects on age-specific mortality, trajectories derived from the survival curves  
65 revealed a shift in the intercept, but not the slope (Fig. 1d), indicating that catalase over-expression  
66 decreased the overall risk of death, rather than slowing its rate of increase with age<sup>14</sup>. Therefore,  
67 the catalase over-expressing flies were healthier for longer.

68 Using the inducible GeneSwitch system showed that induction of catalase from middle-age  
69 (d28 and d42) or old-age (d56) was sufficient to extend lifespan (Figs. 1e, S1g), although not to the  
70 full extent from induction at d2. By d56, the -RU control flies had already started dying, yet  
71 switching to +RU treatment even at this late stage still enhanced survival. This implies that for full  
72 benefits the redox shift needs to occur early in life, yet late-onset still offers protection. The level  
73 of catalase over-expression induced by RU was equivalent at all ages, as were the levels of  
74 endogenous catalase in the controls, eliminating any contribution from changes in RU consumption  
75 or endogenous catalase expression with age (Fig. S1h). We conclude that ubiquitous up-regulation  
76 of catalase improves healthspan and extends lifespan in female WT flies. Interestingly, tissue-  
77 specific catalase up-regulation using a range of drivers (e.g. tubule, gut, fat body, neuronal; Figs.  
78 S1i-m), did not recapitulate the strong lifespan extension obtained by the ubiquitous drivers,  
79 suggesting that catalase is either acting in an untested tissue (or combination of tissues), or  
80 alternatively is required at a more global organismal level.

81 The catalase over-expressors were exceptionally resistant to multiple modes of oxidative  
82 stress - by exogenous H<sub>2</sub>O<sub>2</sub> (Fig. 1f), the redox cyler paraquat both upon feeding (Fig. 1g) and  
83 injection (Fig. 1h), as well as hyperoxia (Figs. 1i, S1n). However, this enhanced oxidative stress  
84 resistance is unlikely to explain the lifespan extension in females, because catalase over-expression  
85 protected males to a similar extent against oxidative stress without increasing longevity.

86 To explore the mechanism underlying the catalase-mediated lifespan extension, we  
87 examined its relationship to dietary restriction (DR), which is a robust and evolutionary conserved  
88 nutritional intervention known to have health and longevity benefits<sup>15</sup>. We measured the lifespan  
89 response of catalase over-expressor females to DR by varying the yeast content (i.e. protein source)  
90 in the food, while maintaining the sugar content constant<sup>16</sup>. This generated a typical tent-shaped  
91 response (Fig. 2a), with lifespan decreased at very low yeast levels (0.1x), highest under restricted

92 conditions (0.5x), then gradually shortened towards more fully-fed conditions (1.5x). The lifespan  
93 of the catalase flies was enhanced relative to controls at all yeast levels (Fig. 2a), while fecundity  
94 increased with yeast content throughout the 0.1-1.5x range for both the control and catalase females  
95 (Fig. S2a). Therefore, the catalase over-expressor females exhibit a normal DR response, and the  
96 lifespan extension upon catalase up-regulation is not mediated by the activation of DR pathways.

97 While the DR experiment revealed improved survival compared to control at a range of  
98 yeast concentrations (Fig. 2a), including extremely poor nutritional conditions (0.1x-yeast; Fig. 2b),  
99 we unexpectedly observed that the catalase over-expressing females, but not males, were acutely  
100 sensitive to complete starvation (Fig. 2c). There was no difference in triacylglyceride (TAG) levels  
101 at d7 (t=0 in Fig. 2d) and during a starvation time course (Fig. 2d). Similarly, the levels of glycogen  
102 storage and mobilisation were the same in control and over-expressor females (Fig. 2e). Therefore,  
103 the starvation sensitivity of the catalase flies was not due to differences in metabolic energy reserves  
104 or their mobilisation.

105 Autophagy is a known longevity assurance process, involved in the response to nutritional  
106 challenges such as starvation<sup>17-22</sup>. Furthermore, there is evidence for redox-regulation of  
107 autophagy<sup>23</sup>. Therefore, we next explored the involvement of autophagy in the differential  
108 starvation response and longevity of the catalase flies. To monitor autophagy status *in vivo* directly,  
109 we quantified the levels of Atg8 (LC3 in mammals), a major autophagosome marker. The levels of  
110 both the de-lipidated (Atg8-I) and lipidated (Atg8-II) forms were strongly elevated in the catalase  
111 over-expressors (Fig. 2f), indicating that autophagy is induced. Indeed, levels of autophagy are  
112 physiologically fine-tuned, with both autophagy inhibition and excessive activation shown to  
113 induce starvation sensitivity *in vivo*<sup>24,25</sup>. Furthermore, similarly to the starvation stress, the catalase  
114 flies were also sensitive to treatment with the autophagy inhibitor chloroquine (Fig. 2g), confirming  
115 that autophagy is affected.

116 To test the involvement of autophagy in the longevity of the catalase flies, we down-  
117 regulated autophagy by RNAi of Atg5 (Figs. S2b). Atg5 knock-down enhanced sensitivity to  
118 starvation (Fig. S2c)<sup>24</sup>, but did not affect the lifespan of control females under fed conditions (da-  
119 GS>UAS-Atg5RNAi ±RU; Fig. 2h). Importantly, the lifespan extension by catalase over-  
120 expression was abolished in an Atg5-RNAi background (da-GS>UAS-Atg5RNAi+UAS-cat ±RU;  
121 Fig. 2i). Therefore, autophagy is required for the enhanced longevity by catalase up-regulation.

122 Redox regulation of autophagy has been described for Atg4 in the context of starvation-  
123 induced ROS production *in vitro*<sup>26</sup>. Atg4 is the only cysteine peptidase amongst the autophagy  
124 components, and is essential for autophagosome biogenesis<sup>23</sup>. Atg4 regulates autophagy by  
125 processing Atg8 at two critical stages: 1) the initial cleavage of Atg8, mediated by the redox-  
126 insensitive catalytic cysteine of Atg4, therefore this first step promoting Atg8 lipidation is redox-

127 independent; and 2) the subsequent redox-dependent de-lipidation of Atg8, which is selectively  
128 inactivated upon oxidation of an adjacent redox-regulatory cysteine in Atg4. Therefore, under  
129 oxidising conditions, lipidated Atg8 accumulates due to the redox-mediated suppression of de-  
130 conjugation by Atg4, thereby enhancing autophagosome biogenesis and promoting Atg4-mediated  
131 autophagy (Fig. 3a).

132 We recently showed that fasting for 24 h is associated with a strong oxidising shift of bulk  
133 cysteine residues in *Drosophila in vivo*<sup>27</sup>. We therefore hypothesised that the starvation sensitivity  
134 of the da-GAL4>UAS-cat females may be attributed to such thiol redox changes. To explore the  
135 effects of catalase up-regulation on global thiol redox state, we applied the same redox proteomic  
136 technique, OxICAT<sup>27</sup>, to the catalase over-expressing females. In OxICAT, samples undergo  
137 differential labelling of cysteine residues according to redox status, followed by trypsin proteolysis  
138 and enrichment for cysteine-containing peptides, and finally detection by tandem mass  
139 spectrometry. This allows both the identification of redox-responsive cysteine residues, as well as  
140 determination of their redox state. The bulk redox state of cysteines in control flies does not change  
141 with age, with the majority remaining at ~10-15% oxidised<sup>27</sup>. In contrast, the catalase over-  
142 expressors displayed an oxidising shift in cysteine redox state relative to controls with increasing  
143 age (Figs. 3b-d, S3a-d, Table S2). This finding is counter-intuitive, since we are up-regulating an  
144 antioxidant enzyme. Therefore, we tested the hypothesis that by quenching H<sub>2</sub>O<sub>2</sub>, catalase over-  
145 expression blocks H<sub>2</sub>O<sub>2</sub> redox signals that up-regulate other antioxidant systems and redox couples.  
146 The Keap1/Nrf2 signalling pathway is an appealing candidate for this process, as it is an oxidative  
147 stress response pathway that enhances the expression of a range of redox processes and is known  
148 to be redox-regulated in *Drosophila*<sup>28</sup>. To assess this pathway, we used a transgenic reporter for  
149 Keap1/Nrf2 activity (gstD-GFP). This pathway was up-regulated with age in controls, but not in  
150 the long-lived catalase flies (Fig. S3e), suggesting that catalase over-expression prevented the  
151 induction of Keap1/Nrf2 signalling with age and thus the induction of a range of redox processes.  
152 Altogether, we have shown that catalase flies undergo an unexpected global oxidising thiol redox  
153 shift with age. This oxidation is consistent with the enhancement of autophagy via redox-regulation  
154 of Atg4.

155 The protein sequence of Atg4 is evolutionarily conserved, with both the catalytic cysteine  
156 (Cys98 in *Drosophila* Atg4a) and the adjacent redox-regulatory cysteine (Cys102 in *Drosophila*  
157 Atg4a) present in flies and mammals (Figs. 4a, S4a). To dissect the physiological role of Atg4 redox  
158 regulation *in vivo*, we generated a transgenic knock-in fly line by CRISPR, where the regulatory  
159 cysteine in endogenous Atg4a was replaced by a redox-inactive serine residue (C102S mutant).  
160 Basal levels of autophagy were not affected under control conditions (Fig. 4b, UAS-cat/+, Atg4a-  
161 C102S), whereas autophagy induction by catalase over-expression was fully abolished (Fig. 4b, da-

162 GAL4>UAS-cat, Atg4a-C102S). Therefore, this redox-regulatory cysteine in Atg4 is required for  
163 autophagy induction by redox signalling *in vivo*, as previously reported *in vitro* <sup>26</sup>.

164 To interrogate the role of Atg4a Cys102 in mediating the longevity of the catalase flies, we  
165 performed survival assays with the Atg4a-WT CRISPR control line, and reproduced the catalase  
166 lifespan extension in this background (Fig. 4c). The Atg4a-C102S point mutation did not affect  
167 survival of control flies, confirming that this knock-in alone is not deleterious (Fig. 4d). Critically,  
168 in contrast to the Atg4a-WT control, the lifespan extension upon catalase up-regulation was fully  
169 abolished in the Atg4a-C102S mutant background (Fig. 4d). Therefore, redox-regulation of  
170 autophagy via Atg4a-Cys102 mediates the longevity upon catalase over-expression. Enhancing  
171 autophagy is an evolutionarily conserved intervention associated with health and survival benefits,  
172 and here we demonstrate that selective redox-mediated up-regulation of autophagy can extend  
173 lifespan.

174 Many attempts have been made to extend lifespan in model organisms by enhancing their  
175 antioxidant capacity, notably through the over-expression of antioxidant enzymes, including  
176 catalase <sup>29-32</sup>. These trials have been largely unsuccessful, casting doubt on the causative role of  
177 ROS and oxidative damage in ageing <sup>33</sup>. Therefore, our finding that catalase over-expression  
178 extends lifespan was at first surprising. The original study over-expressing catalase in *Drosophila*  
179 found no effect on lifespan and only modest resistance to oxidative stress by H<sub>2</sub>O<sub>2</sub> <sup>29</sup>. However, the  
180 study used an extra chromosomal copy under its endogenous promoter, resulting in far lower over-  
181 expression of catalase (~1.75-fold at the mRNA level and ~1.5-fold increased enzyme activity).  
182 The degree of catalase over-expression is therefore likely to be important for the lifespan extension.  
183 Furthermore, the earlier study used only males, while our findings show a robust effect specifically  
184 in females. The results of the two studies are therefore not discordant with one another.

185 Our redox proteomic analysis has revealed that the catalase flies undergo a thiol oxidising  
186 shift in bulk cysteine redox state. Interestingly, this pattern is similar to our earlier observations in  
187 WT flies under starvation stress <sup>27</sup>. Nutrient deprivation can directly affect redox homeostasis by  
188 depleting the provision of important reducing equivalents, such as NADPH and glutathione,  
189 mediating an intracellular oxidising shift <sup>34</sup>. Therefore, we suggest that the catalase flies undergo a  
190 thiol oxidising shift that is perceived as an internal state of starvation, which triggers the induction  
191 of autophagy as a protective response (Fig. 4e). Autophagy plays a fundamental role in healthy  
192 physiology, such as cellular differentiation, tissue remodelling, and mitochondrial homeostasis, as  
193 well as in the response to stress and the clearance of cellular damage <sup>22</sup>. Consequently, enhanced  
194 autophagy is a common denominator of many evolutionary conserved interventions that extend  
195 lifespan <sup>21,22</sup>, both genetically, such as down-regulation of insulin signalling <sup>35</sup>, and  
196 pharmacologically, for instance rapamycin treatment <sup>36</sup>. Furthermore, direct up-regulation of



197 autophagy has been shown to exert health benefits and extend lifespan in a range of model  
198 organisms including worms, flies and mice<sup>37-39</sup>. Several components of the autophagy pathway are  
199 known to be redox regulated, including Atg3 and Atg7<sup>40</sup>, as well as the focus of our study Atg4<sup>26</sup>.  
200 Overall, we have shown that shifting the *in vivo* redox state of *Drosophila* through over-expression  
201 of catalase extends lifespan and healthspan in females through redox regulation of autophagy via a  
202 key redox-regulatory cysteine in Atg4a. Our findings further emphasise the importance of fine-  
203 tuning autophagy in health and disease, and demonstrate how manipulation of redox signalling *in*  
204 *vivo* can ameliorate the effects of ageing. Furthermore, our data are consistent with a growing view  
205 in the ageing field that many effects of ROS on longevity are likely to be through alterations in  
206 redox signalling rather than through lessening of oxidative damage<sup>7,8</sup>.

207

## 208 **Methods**

209 **Fly strains and husbandry.** The *white Dahomey* ( $w^{Dah}$ ) strain of *Drosophila melanogaster* was  
210 used as the WT background. The *Dahomey* stock was collected in 1970 in Dahomey (presently the  
211 Republic of Benin), and maintained since then as large population cages, ensuring outbreeding and  
212 overlapping generations. The  $w^{Dah}$  stock was derived by incorporation of the  $w^{1118}$  mutation into  
213 the outbred *Dahomey* background by back-crossing. Flies were either negative ( $w^{Dah}$ ) or positive  
214 ( $w^{Dah+}$ ) for the bacterial cytoplasmic endosymbiont *Wolbachia*, with infection status confirmed by  
215 PCR using published primers against *wsp*<sup>11</sup>. The  $w^{Dah}$  stock was originally achieved by tetracycline  
216 treatment of  $w^{Dah+}$ <sup>11</sup>. All transgenic lines were back-crossed into the appropriate  $w^{Dah}$  background  
217 for at least 6-10 generations. See the Supplementary Information for details of all fly strains.  
218 Experimental flies were incubated at 25°C on a 12 h light:12 h dark cycle with 65% humidity.

219

220 **Fly food.** Flies were raised on standard sugar-yeast-agar medium (SYA) consisting of: 5% w/v  
221 sucrose (granulated sugar, Tate & Lyle), 10% w/v yeast (#903312, MP Biomedicals), 1.5% w/v  
222 agar (A7002, Sigma), supplemented with nipagin (Sigma H5501; 30 mL/L of 10% w/v nipagin in  
223 95% EtOH) and propionic acid (Sigma P1386; 0.3% v/v) as mould inhibitors, added once the food  
224 had cooled down to ~60°C<sup>16</sup>. Expression via the inducible GeneSwitch system was achieved by  
225 addition of the drug RU (RU486/mifepristone; M8048, Sigma) to standard SYA once cooled down  
226 to ~60°C, typically at 200  $\mu$ M from a 0.1 M stock in EtOH. For dietary restriction (DR)  
227 experiments, the yeast content was varied to give 1% (0.1x), 5% (0.5x), 7.5% (0.75x), 10% (1x =  
228 SYA) or 15% (1.5 SYA) w/v yeast<sup>41</sup>.

229

230 **Experimental flies.** For all experiments, eggs were collected over a defined period (<24 h) to  
231 ensure a synchronous population and reared at constant density in 200 mL bottles with SYA<sup>41</sup>.

232 Eclosing adults of a defined age were kept as a mixed population for ~48 h to allow mating, then  
233 separated into males and females under mild CO<sub>2</sub> anaesthesia, and maintained as separate sexes  
234 from then on.

235

236 **Lifespan & stress assays.** Lifespan assays were set up as above, typically with n~10-15 flies per  
237 vial and a total of n~100-250 flies per condition. Flies were transferred to fresh food without gassing  
238 every ~2-3 days, with deaths and censors recorded. Stress assays were performed on d7 flies  
239 (typically n>100 per condition in groups of ~15-20 flies per vial), with deaths scored regularly  
240 following initiation of treatment. See Table S1 for full survival assay information. For H<sub>2</sub>O<sub>2</sub>  
241 resistance, flies were transferred onto medium containing 5% v/v H<sub>2</sub>O<sub>2</sub> (Sigma H1009), 5% w/v  
242 sucrose, 1.5% w/v agar. For paraquat stress, flies were either transferred onto standard SYA food  
243 supplemented with 20 mM paraquat (Sigma 856177), or injected with 75 nL of 1 mg/mL paraquat  
244 in Ringers buffer (3 mM CaCl<sub>2</sub>, 182 mM KCl, 46 mM NaCl, 10 mM Tris base, pH 7.2 HCl) and  
245 maintained on standard SYA<sup>42</sup>. Starvation stress was assayed by transferring flies to 1.5% w/v agar  
246 medium, which lacks nutrients but allows hydration. Chloroquine (10 mM, Sigma C6628) was  
247 prepared in 5% w/v sugar, 1.5% w/v agar. Hyperoxia was performed by incubating flies on standard  
248 SYA vials in a glove box chamber set at 90% O<sub>2</sub> using a ProOx controller (BioSpherix). The  
249 majority of lifespans (Figs. 1a-b, 2b,h-i, 4c-d, S1d-f, S2c) and stress assays (Figs. 1f-g, 2c,g) were  
250 repeated at least twice as independent biological experiments, except Figs. 1h-i and S1c,g,i-m,  
251 which were performed once.

252

253 **Development time.** Eggs were collected from flies in cages onto grape juice agar plates over a  
254 defined time window (~4 h). After ~24 h, the resulting L1 larvae were picked onto SYA food at a  
255 density of 50 per vial (n=500 total per genotype), and the time to adult eclosion was monitored.

256

257 **Climbing assay.** Climbing ability (negative geotaxis) was assayed essentially as described<sup>43</sup>.  
258 Briefly, groups of 15 flies were transferred to a sawn-open 25 mL serological pipette (35 cm long,  
259 1.5 cm diameter), with the base sealed by parafilm. The flies were tapped down within the column  
260 and observed during 45 s, after which their location was recorded. The column was separated into  
261 three sections: top 10 cm, middle, bottom 3 cm. Each cohort was evaluated 3 times, using 5 groups  
262 per genotype. The climbing performance index was calculated as:  $1/2 (n^{\text{total}} + n^{\text{top}} - n^{\text{bottom}}/n^{\text{total}})$ .

263

264 **Metabolic and molecular assays.** Flies for molecular experiments were rapidly transferred to pre-  
265 chilled microtubes via a small plastic funnel and snap frozen in liquid nitrogen, then stored at -80°C  
266 until required. Flies were always frozen at approximately the same time of day to minimise any



267 circadian variation. For some assays, frozen flies were separated into body segments using forceful  
268 manual impact <sup>44</sup>.

269

270 **Western blotting.** Frozen fly samples were homogenised directly into 2X Laemmli loading buffer  
271 (Bio-Rad) supplemented with 5% v/v  $\beta$ -mercaptoethanol (Sigma) using a pellet pestle and motor  
272 (usually 5/10 females into 100/200  $\mu$ L) and separated by standard SDS-PAGE. The following  
273 primary antibodies were used at the indicated dilutions: anti-actin (AbCam Ab1801; 1:1,000), anti-  
274 Atg8 (a generous gift from K. Köhler <sup>45</sup>; 1:1,000), anti-catalase (Sigma C0979; 1:10,000), anti-GFP  
275 (Cell Signaling #2955; 1:1,000). Blots were developed using standard ECL, followed by analysis  
276 with FIJI (ImageJ) software.

277

278 **Energy storage assays.** Whole body triacylglyceride (TAG) and glycogen levels were measured  
279 in d7 females (n=5 flies per sample, n=6-8 replicates per genotype) under control (fed) conditions  
280 and in response to starvation. For the TAG assay, flies were homogenised in 0.05% v/v Tween-20  
281 and assayed using the Triglyceride Infinity Reagent (Thermo-Scientific TR22421) in a 96-well  
282 plate measuring absorbance at 540 nm. For the glycogen assay, flies were homogenised in saturated  
283 sodium sulphate, then the subsequent pellet was resuspended in anthrone reagent (Sigma 319899)  
284 and assayed in a 96-well plate measuring absorbance at 620 nm <sup>46</sup>.

285

286 **OxICAT.** To measure the redox state of protein cysteine residues, we performed redox proteomics  
287 using OxICAT, with protein isolation, cysteine-residue labelling, peptide preparation and LC-  
288 MS/MS analysis performed exactly as described previously <sup>27</sup>. See the Supplementary Information  
289 for further details.

290

291 **Statistical analysis.** Lifespan and stress assays were plotted as cumulative survival curves, and  
292 statistical analysis was performed by Log-Rank test. Other data were analysed by Student's t-test  
293 or ANOVA as appropriate in GraphPad Prism 8.

294

## 295 **References**

- 296 1. Sena, L. A. & Chandel, N. S. Physiological roles of mitochondrial reactive oxygen species.  
297 *Mol. Cell* **48**, 158-167 (2012).
- 298 2. Holmstrom, K. M. & Finkel, T. Cellular mechanisms and physiological consequences of  
299 redox-dependent signalling. *Nat. Rev. Mol. Cell Biol.* **15**, 411-421 (2014).
- 300 3. Collins, Y. *et al.* Mitochondrial redox signalling at a glance. *J. Cell Sci.* **125**, 801-806 (2012).
- 301 4. Paulsen, C. E. & Carroll, K. S. Cysteine-mediated redox signaling: chemistry, biology, and  
302 tools for discovery. *Chem Rev* **113**, 4633-4679 (2013).

- 303 5. Murphy, M. P. How mitochondria produce reactive oxygen species. *Biochem. J.* **417**, 1-13 (2009).
- 304 6. Stocker, S., Van Laer, K., Mijuskovic, A. & Dick, T. P. The Conundrum of Hydrogen Peroxide  
305 Signaling and the Emerging Role of Peroxiredoxins as Redox Relay Hubs. *Antioxid. Redox*  
306 *Signal.* **28**, 558-573 (2018).
- 307 7. Orr, W. C., Radyuk, S. N. & Sohal, R. S. Involvement of redox state in the aging of *Drosophila*  
308 *melanogaster*. *Antioxid. Redox Signal.* **19**, 788-803 (2013).
- 309 8. Jones, D. P. Redox theory of aging. *Redox Biol.* **5**, 71-79 (2015).
- 310 9. Piper, M. D. W. & Partridge, L. *Drosophila* as a model for ageing. *Biochim. Biophys. Acta*  
311 *Mol. Basis Dis.* **1864**, 2707-2717 (2018).
- 312 10. Austad, S. N. & Fischer, K. E. Sex Differences in Lifespan. *Cell Metab.* **23**, 1022-1033 (2016).
- 313 11. Toivonen, J. M. *et al.* No influence of Indy on lifespan in *Drosophila* after correction for  
314 genetic and cytoplasmic background effects. *PLOS Genet.* **3**, e95 (2007).
- 315 12. Ikeya, T., Broughton, S., Alic, N., Grandison, R. & Partridge, L. The endosymbiont Wolbachia  
316 increases insulin/IGF-like signalling in *Drosophila*. *Proc. Biol. Sci.* **276**, 3799-3807 (2009).
- 317 13. Lopez-Otin, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The hallmarks of  
318 aging. *Cell* **153**, 1194-1217 (2013).
- 319 14. Mair, W., Goymer, P., Pletcher, S. D. & Partridge, L. Demography of dietary restriction and  
320 death in *Drosophila*. *Science* **301**, 1731-1733 (2003).
- 321 15. Fontana, L. & Partridge, L. Promoting health and longevity through diet: from model  
322 organisms to humans. *Cell* **161**, 106-118 (2015).
- 323 16. Grandison, R. C., Wong, R., Bass, T. M., Partridge, L. & Piper, M. D. Effect of a standardised  
324 dietary restriction protocol on multiple laboratory strains of *Drosophila melanogaster*. *PLOS*  
325 *One* **4**, e4067 (2009).
- 326 17. Madeo, F., Tavernarakis, N. & Kroemer, G. Can autophagy promote longevity? *Nat. Cell Biol.*  
327 **12**, 842-846 (2010).
- 328 18. Rubinsztein, D. C., Marino, G. & Kroemer, G. Autophagy and aging. *Cell* **146**, 682-695 (2011).
- 329 19. Kaushik, S. & Cuervo, A. M. Proteostasis and aging. *Nat. Med.* **21**, 1406-1415 (2015).
- 330 20. Vilchez, D., Saez, I. & Dillin, A. The role of protein clearance mechanisms in organismal  
331 ageing and age-related diseases. *Nat. Commun.* **5**, 5659 (2014).
- 332 21. Madeo, F., Zimmermann, A., Maiuri, M. C. & Kroemer, G. Essential role for autophagy in life  
333 span extension. *J. Clin. Invest.* **125**, 85-93 (2015).
- 334 22. Hansen, M., Rubinsztein, D. C. & Walker, D. W. Autophagy as a promoter of longevity:  
335 insights from model organisms. *Nat. Rev. Mol. Cell Biol.* **19**, 579-593 (2018).
- 336 23. Scherz-Shouval, R. & Elazar, Z. Regulation of autophagy by ROS: physiology and pathology.  
337 *Trends Biochem. Sci.* **36**, 30-38 (2011).

- 338 24. Scott, R. C., Schuldiner, O. & Neufeld, T. P. Role and regulation of starvation-induced  
339 autophagy in the *Drosophila* fat body. *Dev. Cell* **7**, 167-178 (2004).
- 340 25. Kang, C., You, Y. J. & Avery, L. Dual roles of autophagy in the survival of *Caenorhabditis*  
341 *elegans* during starvation. *Genes Dev.* **21**, 2161-2171 (2007).
- 342 26. Scherz-Shouval, R. *et al.* Reactive oxygen species are essential for autophagy and specifically  
343 regulate the activity of Atg4. *EMBO J.* **26**, 1749-1760 (2007).
- 344 27. Menger, K. E. *et al.* Fasting, but Not Aging, Dramatically Alters the Redox Status of Cysteine  
345 Residues on Proteins in *Drosophila melanogaster*. *Cell Rep.* **11**, 1856-1865 (2015).
- 346 28. Sykiotis, G. P. & Bohmann, D. Keap1/Nrf2 signaling regulates oxidative stress tolerance and  
347 lifespan in *Drosophila*. *Dev. Cell* **14**, 76-85 (2008).
- 348 29. Orr, W. C. & Sohal, R. S. The effects of catalase gene overexpression on life span and  
349 resistance to oxidative stress in transgenic *Drosophila melanogaster*. *Arch. Biochem. Biophys.*  
350 **297**, 35-41 (1992).
- 351 30. Orr, W. C., Mockett, R. J., Benes, J. J. & Sohal, R. S. Effects of overexpression of copper-zinc  
352 and manganese superoxide dismutases, catalase, and thioredoxin reductase genes on longevity  
353 in *Drosophila melanogaster*. *J. Biol. Chem.* **278**, 26418-26422 (2003).
- 354 31. Doonan, R. *et al.* Against the oxidative damage theory of aging: superoxide dismutases protect  
355 against oxidative stress but have little or no effect on life span in *Caenorhabditis elegans*. *Genes*  
356 *Dev* **22**, 3236-3241 (2008).
- 357 32. Perez, V. I. *et al.* The overexpression of major antioxidant enzymes does not extend the lifespan  
358 of mice. *Aging Cell* **8**, 73-75 (2009).
- 359 33. Gems, D. & Doonan, R. Antioxidant defense and aging in *C. elegans*: is the oxidative damage  
360 theory of aging wrong? *Cell Cycle* **8**, 1681-1687 (2009).
- 361 34. Filomeni, G., De Zio, D. & Cecconi, F. Oxidative stress and autophagy: the clash between  
362 damage and metabolic needs. *Cell Death Differ.* **22**, 377-388 (2015).
- 363 35. Toth, M. L. *et al.* Longevity pathways converge on autophagy genes to regulate life span in  
364 *Caenorhabditis elegans*. *Autophagy* **4**, 330-338 (2008).
- 365 36. Bjedov, I. *et al.* Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila*  
366 *melanogaster*. *Cell Metab.* **11**, 35-46 (2010).
- 367 37. Simonsen, A. *et al.* Promoting basal levels of autophagy in the nervous system enhances  
368 longevity and oxidant resistance in adult *Drosophila*. *Autophagy* **4**, 176-184 (2008).
- 369 38. Lapierre, L. R. *et al.* The TFEB orthologue HLH-30 regulates autophagy and modulates  
370 longevity in *Caenorhabditis elegans*. *Nat. Commun.* **4**, 2267 (2013).
- 371 39. Pyo, J. O. *et al.* Overexpression of Atg5 in mice activates autophagy and extends lifespan. *Nat.*  
372 *Commun.* **4**, 2300 (2013).

- 373 40. Frudd, K., Burgoyne, T. & Burgoyne, J. R. Oxidation of Atg3 and Atg7 mediates inhibition of  
374 autophagy. *Nat. Commun.* **9**, 95 (2018).
- 375 41. Bass, T. M. *et al.* Optimization of dietary restriction protocols in *Drosophila*. *J. Gerontol. A*  
376 *Biol. Sci. Med. Sci.* **62**, 1071-1081 (2007).
- 377 42. Kerr, F. *et al.* Direct Keap1-Nrf2 disruption as a potential therapeutic target for Alzheimer's  
378 disease. *PLOS Genet.* **13**, e1006593 (2017).
- 379 43. Rogers, I. *et al.* Ageing increases vulnerability to A $\beta$ 42 toxicity in *Drosophila*. *PLOS One* **7**,  
380 e40569 (2012).
- 381 44. Cochemé, H. M. *et al.* Using the mitochondria-targeted ratiometric mass spectrometry probe  
382 MitoB to measure H<sub>2</sub>O<sub>2</sub> in living *Drosophila*. *Nat. Protoc.* **7**, 946-958 (2012).
- 383 45. Barth, J. M., Szabad, J., Hafen, E. & Kohler, K. Autophagy in *Drosophila* ovaries is induced  
384 by starvation and is required for oogenesis. *Cell Death Differ.* **18**, 915-924 (2011).
- 385 46. Castillo-Quan, J. I. *et al.* Lithium Promotes Longevity through GSK3/NRF2-Dependent  
386 Hormesis. *Cell Rep.* **15**, 638-650 (2016).

387

## 388 **Acknowledgements**

389 This work was funded by the BBSRC, Wellcome Trust and Max Planck Society to LP. HMC is  
390 supported by the Medical Research Council UK (MC-A654-5QB90). Work in the MPM lab is  
391 supported by the Medical Research Council UK (MC-U105663142) and by a Wellcome Trust  
392 Investigator Award (110159/Z/15/Z). We are grateful to Pirrko Salmiheimo and Mumtaz Ahmad  
393 for help with preparation of fly media, Jigna Patel for early technical support, and Mike Harbour  
394 and Ian Fearnley for assistance with redox proteomics. We thank members of the Partridge and  
395 Cochemé labs for helpful discussions. We acknowledge the Bloomington *Drosophila* Stock Center  
396 and the Vienna *Drosophila* Resource Center (VRDC) for fly strains, and the BACPAC Resource  
397 Center for BAC clones.

398

## 399 **Author contributions**

400 Conceived the project: HMC and LP. Performed experiments: HMC, IB, FC, JICQ and CL.  
401 Provided technical support: AF, MB and JA. Generated the CRISPR mutants: SG. Conducted and  
402 analysed the redox proteomics: KEM and AMJ. Directed the work: HMC, MPM and LP. Wrote  
403 the manuscript: HMC, MPM and LP, with input from all the authors.

404

## 405 **Competing interests**

406 The authors declare no competing interests.

407 **Figure legends**

408 **Fig. 1 | Ubiquitous catalase over-expression extends lifespan in flies, independently of**  
409 **oxidative stress resistance. a**, Constitutive, ubiquitous catalase over-expression (da-GAL4>UAS-  
410 cat) extended the survival of female flies in a  $w^{Dah}$  (*white Dahomey*) WT background relative to the  
411 UAS-cat/+ ( $p=5.7 \times 10^{-7}$ ) and da-GAL4/+ ( $p=3.3 \times 10^{-6}$ ) controls. The control lines were not  
412 significantly different from each other ( $p=0.7504$ ). No effect was observed in males ( $p>0.05$  for all  
413 comparisons). Lifespans were performed with  $n=200$  flies per condition. **b**, Inducible catalase over-  
414 expression from early adulthood (d2) using the GeneSwitch system extended the lifespan of female  
415 flies (da-GS>UAS-cat  $\pm$ RU,  $p=1.1 \times 10^{-16}$ ). RU had no effect on the da-GS/+ control line  
416 ( $p=0.7161$ ). Lifespans were performed with  $n=225-300$  flies per condition. Inset: catalase over-  
417 expression assessed by Western blotting in whole d9 females (=d7 of RU induction), with actin as  
418 a loading control. **c**, Healthspan, inferred from climbing performance, was improved in catalase  
419 over-expressing females. Climbing was assayed on da-GS>UAS-cat females  $\pm$ RU to control for  
420 effects of eye colour on this behaviour. Data are presented as box-and-whisker plots (min/max error  
421 bars) of  $n=5$  replicates per condition, each with  $n=15$  flies per sample, analysed by unpaired  
422 Student's t-test. **d**, Mortality trajectories of the da-GS>UAS-cat  $\pm$ RU survival curves from **(b)**,  
423 fitted with a linear regression trendline (dotted line). **e**, Late onset over-expression of catalase using  
424 the inducible GeneSwitch system from either middle-age (d28 and d42) and old-age (d56) extended  
425 the lifespan of female flies ( $p=7.2 \times 10^{-8}$ ,  $p=1.0 \times 10^{-7}$  and  $p=1.4 \times 10^{-3}$ , respectively against the -RU  
426 control). Lifespans were performed with  $n=270$  flies per condition, and were plotted from point of  
427 RU induction relative to the remaining -RU control flies at that age (see Fig. S1g for the original  
428 survival data). **f**, Catalase over-expressing flies were strongly resistant to exogenous  $H_2O_2$  stress  
429 relative to controls (da-GAL4>UAS-cat v. UAS-cat/+;  $p=5.0 \times 10^{-61}$  females,  $p=3.2 \times 10^{-33}$  males).  
430  $H_2O_2$  treatment (5% v/v in sucrose/agar medium) was initiated at d7, with  $n=105$  males ( $n=75$  for  
431 da-GS>UAS-cat) and  $n=120$  females per condition. **g**, Catalase over-expressing flies were resistant  
432 to chronic dietary paraquat stress relative to control flies (da-GAL4>UAS-cat v. UAS-cat/+;  
433  $p=6.93 \times 10^{-31}$  females,  $p=7.99 \times 10^{-16}$  males). Paraquat treatment (20 mM in SYA food) was initiated  
434 at d7, with  $n=100$  flies per condition. **h**, Catalase over-expressing flies (da-GAL4>UAS-cat) were  
435 resistant to acute paraquat stress relative to controls (UAS-cat/+). d7 females were injected with  
436 75 nL of 1 mg/mL paraquat in Ringers buffer (+PQ,  $n=150$  flies) or mock injected with buffer alone  
437 (-PQ,  $n=120$  flies). **i**, Catalase over-expressing flies were resistant to environmental hyperoxia  
438 stress relative to controls (da-GAL4>UAS-cat v. UAS-cat/+;  $p=1.7 \times 10^{-8}$  females,  $p=8.1 \times 10^{-3}$   
439 males). Incubation at 90%  $O_2$  was initiated at d7, with  $n=120$  flies per condition (except  $n=90$  for  
440 UAS-cat/+ females). All survival assays (**a,b,e,f,g,i**) were analysed by Log-Rank test (see Table S1  
441 for full n numbers and p values). n/s,  $p>0.05$ ; \*,  $p<0.05$ ; \*\*,  $p<0.01$ ; \*\*\*,  $p<0.001$ .



442 **Fig. 2 | Catalase lifespan extension requires autophagy. a**, Catalase over-expressor (da-  
443 GAL4>UAS-cat) and control (UAS-cat/+) females displayed a normal response to dietary  
444 restriction (DR). Median lifespan is plotted against the yeast content of the diet, with 1x  
445 corresponding to standard SYA food. Data are the means  $\pm$ range of 2 independent lifespan  
446 experiments, each set up with n=150-160 flies per genotype, analysed by two-way ANOVA  
447 (Fisher's LSD). **b**, Survival curve on 0.1x-fold yeast from (**a**). The catalase over-expressor females  
448 (da-GAL4>UAS-cat) were longer-lived than controls (UAS-cat/+) under low yeast nutritional  
449 conditions ( $p=3.6 \times 10^{-10}$ , n=160 flies per genotype). **c**, Catalase over-expressor females (da-  
450 GAL4>UAS-cat) were sensitive to starvation stress relative to UAS-cat/+ and da-GAL4/+ controls  
451 ( $p=5.6 \times 10^{-5}$  and  $p=7.6 \times 10^{-7}$ , respectively). No difference was observed in males ( $p>0.05$  for all  
452 comparisons). Assays were performed at d7 with n=120 flies per condition (except n=80 for UAS-  
453 cat/+ females). **d-e**, Triacylglyceride (TAG, **d**) and glycogen (**e**) levels in whole females assayed at  
454 d7 (t=0) and depletion in response to starvation treatment. Data are presented as box-and-whisker  
455 plots (min/max error bars) of n=4-6 replicates per genotype, each with n=5 females per sample,  
456 analysed by unpaired Student's t-test ( $p>0.05$ ). **f**, Catalase over-expressor females (da-  
457 GAL4>UAS-cat) displayed enhanced autophagy induction compared to UAS-cat/+ controls at d7,  
458 assessed by Western blotting against Atg8, normalised to actin. Data are means  $\pm$ SD of n=6  
459 biological replicates, each with n=10 abdomens per sample, analysed by paired Student's t-test  
460 (\*,  $p<0.05$ ). Right, typical bands probed against Atg8 with actin as a loading control (see Fig. S4b  
461 for the full blot). **g**, Catalase over-expressor females (da-GAL4>UAS-cat) were sensitive to  
462 treatment with the autophagy inhibitor chloroquine (10 mM in sucrose/agar medium) relative to  
463 UAS-cat/+ and da-GAL4/+ controls ( $p=2.6 \times 10^{-5}$  and  $p=3.5 \times 10^{-9}$ , respectively). Assays were  
464 performed at d7 with n=160 flies per condition. **h**, Global Atg5 knock-down did not decrease  
465 lifespan in a WT background under control conditions (da-GS>UAS-Atg5RNAi  $\pm$ RU;  $p=0.4177$ ).  
466 Lifespan assays were performed on n=225-240 females. **i**, Lifespan extension upon catalase over-  
467 expression (da-GS>UAS-cat  $\pm$ RU;  $p=7.2 \times 10^{-15}$ ) was abolished in an autophagy-deficient  
468 background (da-GS>UAS-cat+UAS-Atg5RNAi  $\pm$ RU;  $p=0.1701$ ). Survival assays (**b,c,g,h,i**) were  
469 analysed by Log-Rank test (see Table S1 for full n numbers and p values). n/s,  $p>0.05$ ; \*,  $p<0.05$ ;  
470 \*\*\*,  $p<0.001$ .

471

472 **Fig. 3 | Catalase flies undergo an oxidising shift in global thiol redox state. a**, Scheme showing  
473 the dual function of Atg4 in autophagy: 1) initial redox-independent cleavage of Atg8, to expose a  
474 C-terminal glycine residue enabling lipidation by PE (phosphatidyl-ethanolamine) via the E1-like  
475 enzyme Atg7 and the E2-like enzyme Atg3. The conjugated Atg8-PE is involved in autophagosome  
476 elongation/closure; 2) redox-dependent de-lipidation of Atg8-PE, allowing interaction and fusion



477 of the autophagosome with the endosomal-lysosomal compartments, and recycling of cleaved  
478 Atg8. Oxidation of a redox-regulatory cysteine selectively inactivates the Atg8-PE de-conjugation  
479 activity of Atg4, promoting autophagosome biogenesis and therefore enhancing Atg4-mediated  
480 autophagy. **b**, Redox proteomic (OxICAT) analysis of d7, d28 and d56 catalase over-expressing  
481 females (da-GAL4>UAS-cat) compared to control (UAS-cat/+). Distribution of total cysteine  
482 residue oxidation levels, plotted as the proportion of the total number of peptides containing unique  
483 cysteine residues in each 5% quantile of percentage oxidation. Data are means  $\pm$ SEM of n=5  
484 biological replicates. **c-d**, Oxidation state of cysteine residues present, comparing control versus  
485 catalase over-expressor females at d7 (**c**) and d56 (**d**). Data points above the diagonal dotted line  
486 (slope=1) indicate cysteine residues more oxidised upon catalase up-regulation, with red symbols  
487 designating significance ( $p < 0.05$ ), assessed by unpaired two-tailed Student's t-test.

488

489 **Fig. 4 | Redox regulation of autophagy via Atg4a Cys102 extends lifespan.** **a**, Multiple sequence  
490 alignment of the Atg4a protein from *Drosophila* with the mouse and human orthologues, showing  
491 the catalytic cysteine residue (Cys98 in *Drosophila*) and the redox-regulatory cysteine (Cys102 in  
492 *Drosophila*). See Fig. S4a for the full sequence. **b**, Autophagy levels assessed by Western blotting  
493 against Atg8. The induction of autophagy in response to catalase over-expression was maintained  
494 in Atg4a-WT flies, but abolished in the Atg4a-C102S mutant background. See Fig. S4b for the full  
495 blots. Quantification of Atg8 levels by densitometry, normalised to actin as a loading control. Data  
496 are means  $\pm$ range of n=2 biological replicates, analysed by two-way ANOVA (Fisher LSD). **c-d**,  
497 Catalase over-expression extended lifespan in the Atg4a-WT control (**c**), but not in the redox-  
498 insensitive Atg4a-C102S knock-in background (**d**). Survival assays (**c,d**) were analysed by Log-  
499 Rank test (see Table S1 for full n numbers and p values). n/s,  $p > 0.05$ ; \*\*\*,  $p < 0.001$ . **e**, Scheme  
500 illustrating the mechanism underlying the lifespan extension upon redox regulation of autophagy.

# Figure 1

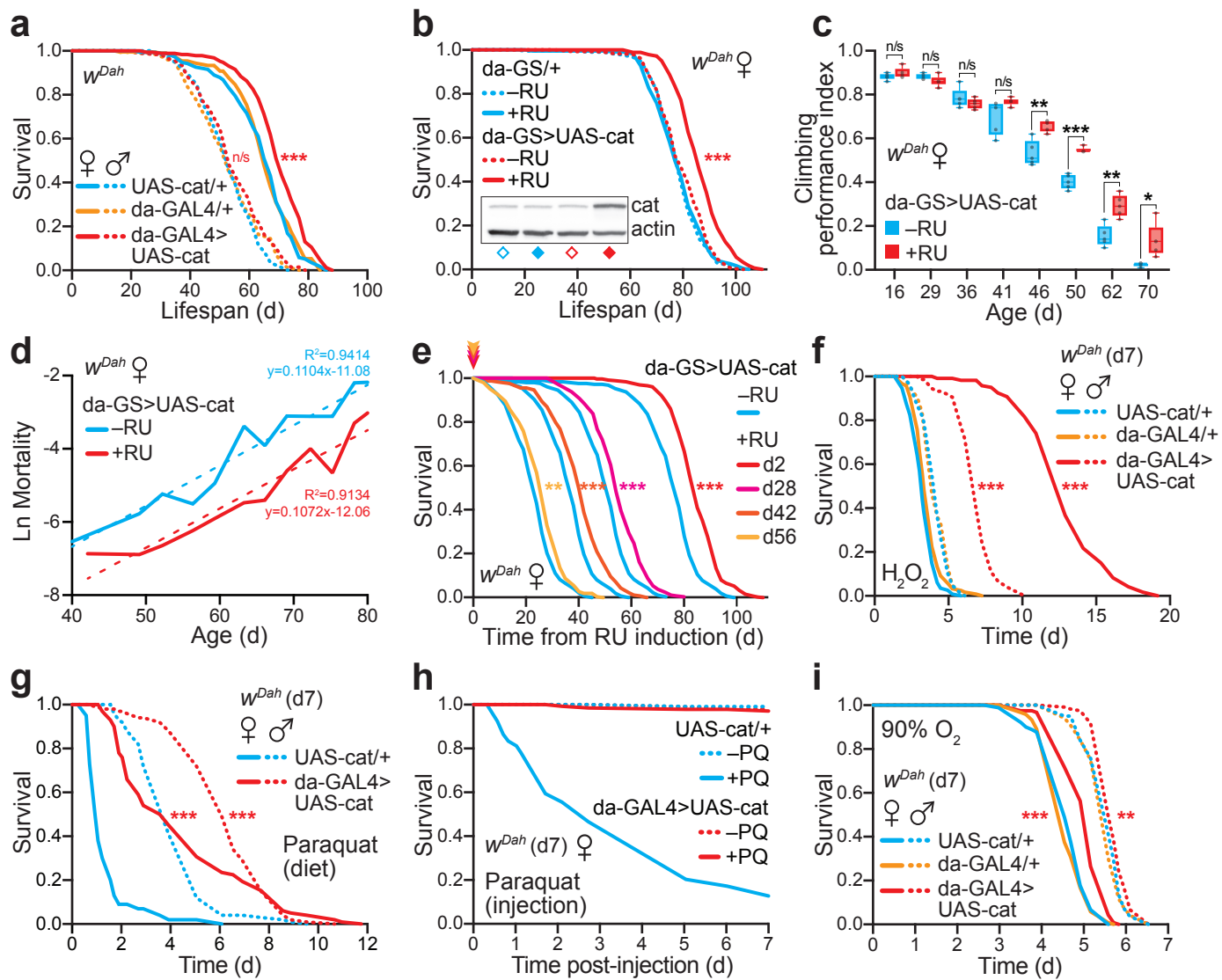


Figure 2

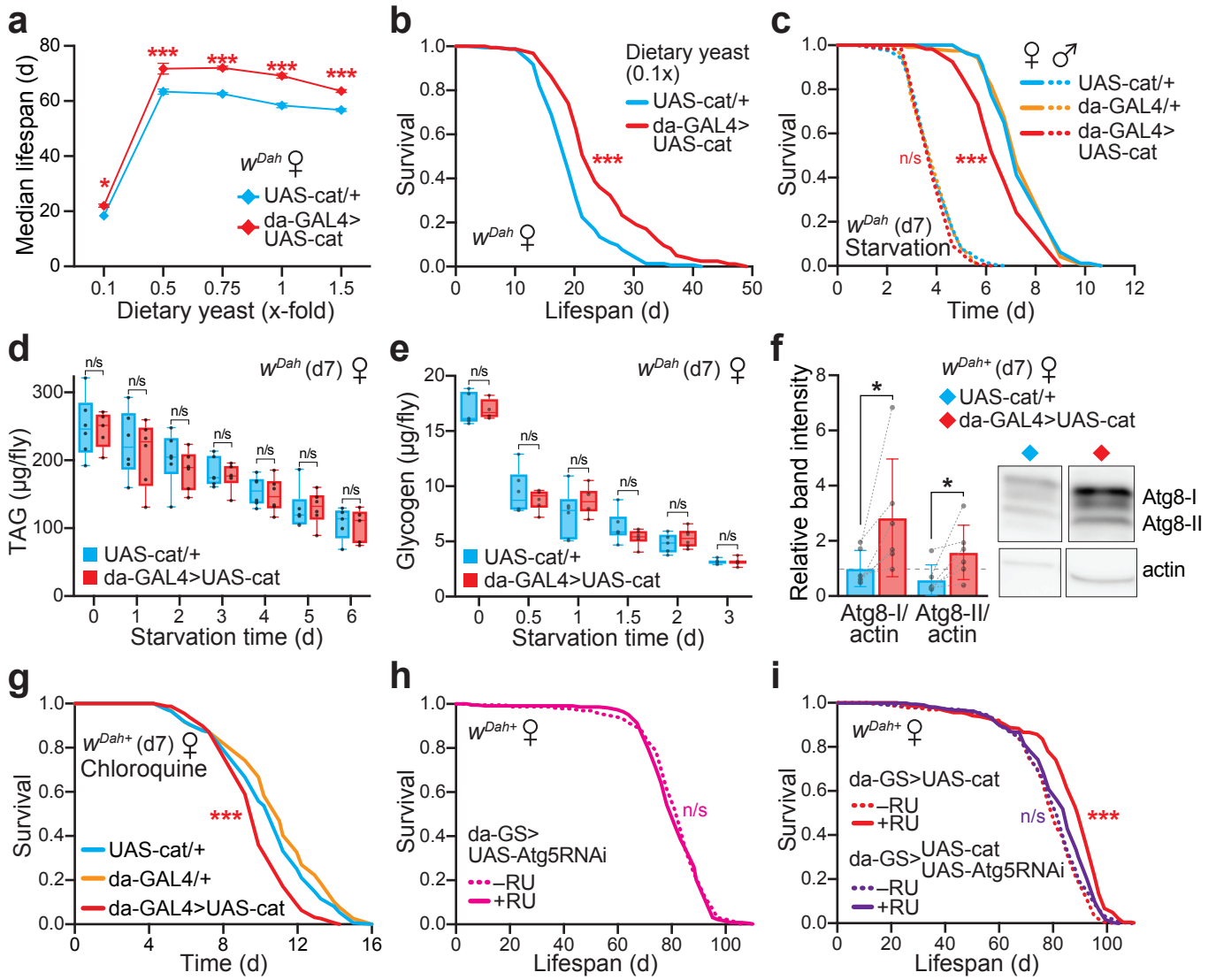
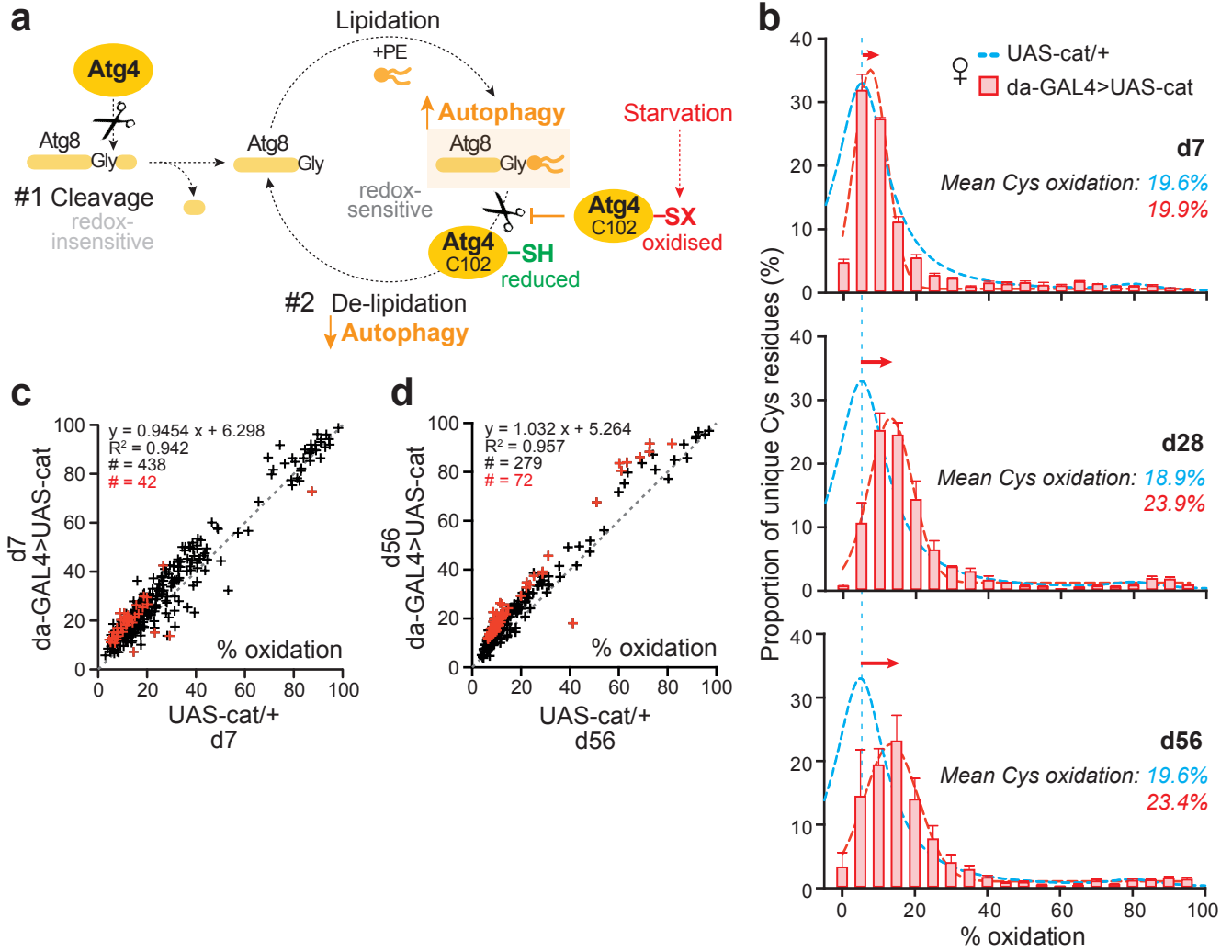


Figure 3



# Figure 4

