Loss of Glial Lazarillo, a homolog of Apolipoprotein D, reduces lifespan and stress resistance in Drosophila

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Summary

The vertebrate Apolipoprotein D (ApoD) is a lipocalin secreted from subsets of neurons and glia during neural development and aging [1-3]. A strong correlation exists between ApoD overexpression and numerous nervous system pathologies as well as obesity, diabetes and many forms of cancer [4, 5]. However, the exact relationship between the function of ApoD and the pathophysiology of these diseases is still unknown. We have generated loss-of-function Drosophila mutants for the Glial Lazarillo (GLaz) gene [6], a homolog of ApoD in the fruitfly, mainly expressed in subsets of adult glial cells. The absence of GLaz reduces the organism’s resistance to oxidative stress and starvation, and shortens male lifespan. The mutant flies exhibit a smaller body mass due to a lower amount of neutral lipids stored in the fat body. Apoptotic neural cell death increases in aged flies or upon paraquat treatment, which also impairs neural function as assessed by behavioral tests. The higher sensitivity to oxidative stress and starvation and the reduced fat storage revert to control levels when a GFP-GLaz fusion protein is expressed under the control of the GLaz natural promoter. Finally, GLaz mutants have a higher concentration of lipid peroxidation products, pointing to a lipid peroxidation protection or scavenging as the mechanism of action for this lipocalin. In agreement with Walker et al. [7], analyzing the effects of over-expressing GLaz, we conclude that GLaz has a protective role in stress situations, and that its absence reduces lifespan and accelerates neurodegeneration.
Results and discussion

Flies lacking GLaz are more sensitive to oxidative and starvation stress

We generated two loss-of-function alleles for GLaz by imprecise excision of a nearby EP-element located 1425 bp upstream of the transcription initiation site (see Figure 1S). Flies homozygous for the two alleles, GLaz^{A1} and GLaz^{A2} (both null at the RNA level), are viable and fertile, suggesting that GLaz is not essential for embryonic or postembryonic development. Since oxidative stress is one of the common factors to the many situations in which ApoD is overexpressed, we tested the sensitivity of the null GLaz mutants to various stressors. GLaz mutant adults are in fact more sensitive to oxidative (Figure 1A,C) and starvation stress (Figure 1D). These effects are seen both in males and females, but only male data will be considered here. Sensitivity to oxidative stress was still observed after placing the GLaz^{A2} allele over a deficiency uncovering 22 genes around the GLaz locus, discarding the possibility that deleterious mutations elsewhere in the genome could contribute to the observed phenotype (Figure 2S). A putative contribution of the neighboring gene Spt1 was also rejected, since transheterozygous flies with the lethal allele Spt1^{l(2)Sh1626} [8] and GLaz^{A2} show survival curves similar to the control GLaz^{A2/+} or GLaz^{+/+} flies upon exposure to paraquat or H2O2 (see Figure 2S and supplementary text). Moreover, Spt1 expression levels show no significant changes in GLaz^{A2} mutants (results not shown), as assessed by quantitative RT-PCR using the ProbeLibrary technology (Exiqon) and 18S as endogenous control. Together, these results suggest that GLaz protects the organism against damage caused by environmental stress. Transgenic flies overexpressing GLaz in nervous and muscular tissues have been independently generated by Walker et al. [7].
An excess of GLaz results in an increased resistance to oxidative stress and starvation, strongly supporting a protective role for GLaz.

**The absence of GLaz reduces lifespan**

Oxidative damage to macromolecules is also one of the major causes of the aging process [9], and increased longevity is correlated with resistance to several forms of stress [10]. In accordance with these relationships, GLaz mutant adults have a reduced lifespan. The same results are obtained with the GLaz mutation placed in two genetic backgrounds. The survival curves of age-synchronized cohorts of male flies reared at 25ºC show a reduction in both the median and the maximum lifespan (Figure 1E,F), reflecting an early onset of senescence. Mirroring this phenotype, GLaz overexpression [7] results in an extended male longevity.

**GLaz expression sites in the adult fly: the nervous system and hemocytes**

In order to understand how a lipocalin, an extracellular lipid-binding protein, can contribute to the regulation of oxidative damage in general and of aging in particular, we analyzed the expression pattern of GLaz in adult flies (Figure 2). We generated a reporter gene construct that drives the expression of GFP under the GLaz promoter (GFP-R3rd line; see Figure 1S and supplementary text). The expression of the reporter gene is mostly seen in the nervous system, where it is restricted to a subset of cells in the perineurial sheath surrounding brain and ganglia, some cells in the optic system, (Figure 2A) and in the mushroom body (not shown). Perineurial cells surrounding the peripheral nerves and motoneuron terminal branches also show expression (Figure 2B). In addition to their stereotyped position, the lack of co-expression with neuronal markers (Figure 2A) and coincidence with expression sites of the REPO glial marker (not shown) [11] suggest these are glial cells. This expression is consistent with both the
developmental expression of GLaz in Drosophila [6], and with the main glial expression of vertebrate ApoD [1-3]. Both, sequence comparisons and exon-intron structure [12, 13] already point to a close GLaz-ApoD kinship. Expression similarities suggest that regulatory regions might be conserved as well. Outside the nervous system, expression is observed only in the proventriculus (not shown) and in cells located in the hemocoel which are probably hemocytes (Figure 2C).

We also generated a construct that drives the expression of a GLaz-GFP fusion protein under the same native GLaz promoter (see Figure 1S and supplementary text). The construct uses the genomic GLaz sequence, including introns, so all the regulatory regions within the locus are expected to be present. Two independent insertions of this construct (on the 2nd and X chromosome) yield an expression pattern (not shown) similar to the transcriptional reporter construct. Thus, a supply of GLaz within the nervous system is provided by secretion from glial cells, while secretion from hemocytes could generate a systemic supply of GLaz.

The effects of GLaz mutation on motor activity impairment are enhanced under oxidative stress conditions

The expression pattern within the nervous system led us to evaluate whether the mutants show an age- and oxidative stress-dependent nervous system functional impairment. Locomotor behavior has been used extensively to evaluate neurodegeneration and age-related functional decline [14]. We found no differences in running, climbing or flying abilities of young mutant and control flies (not shown). We then tested 21 days old flies, when the mortality risk has increased only slightly for the mutant males, and found that GLaz mutant males significantly reduce their climbing ability (Fig. 3A). Although senescence of all motor activities tested does occur in females, no effect of genotype
was observed (not shown). These data are compatible with a mild acceleration of motor activity senescence in males lacking GLaz.

After 24h of exposure to paraquat, GLaz mutant flies of both sexes perform significantly worse than controls in all tests (Fig. 3B). An increased level of apoptotic death has been reported in flight and leg muscles upon treatment with oxidative stressors and during normal aging in wild type flies [15]. This apoptotic pattern supports the notion that the normal decline of locomotor functions with age is due to accumulation of oxidative damage disturbing neuromuscular function. The enhancement of locomotor decline in GLaz mutants suggests that this damage is intensified when GLaz is not present. However, can the observed effects be causally linked to a nervous system defect or to a more widespread damage affecting other tissues?

**Apoptotic cell death increases within and outside the nervous system in GLaz mutants upon aging or under oxidative stress treatment**

In agreement with Zheng et al. [15], we detect low signs of apoptosis within the nervous system of aged wild type flies (as detected by TUNEL, 8±4 cells/section), but a significant increase in apoptotic cell death is observed in the aged GLaz^A2/A2^ brain (25±6 TUNEL-positive cells/section, t-Test \( p=0.003 \)). Apoptosis in neural cells also increases when young GLaz^A2/A2^ flies are exposed to paraquat (95±35 TUNEL-positive cells/section; Figure 2E and inset), while levels in the wild-type nervous system remain low (33±6 TUNEL-positive cells/section, t-Test \( p=0.007 \); Figure 2D). These differences in patterns of apoptosis reveal that, in normal conditions, GLaz contributes to the higher tolerance to oxidative damage of the nervous system. Therefore, at least part of the decrement in locomotor performance observed in aged and paraquat-treated mutant flies
can arise from a diminished nervous system function due to the death of irreplaceable cells.

Nevertheless, the nervous system is not the only tissue in which apoptosis levels rise. The number of TUNEL-positive nuclei in the pericerebral fat body of old $GLaz^{\Delta2/\Delta2}$ flies (10±3 TUNEL-positive cells/section) increases compared to a negligible control level (3±2 TUNEL-positive cells/section, p=0.002). Upon paraquat feeding, also described by Zheng et al. [15]), apoptotic nuclei are commonly observed in the digestive system and the abdominal and pericerebral fat body (30±23 vs. 8±4 TUNEL-positive cells/section of $GLaz^{\Delta2/\Delta2}$ and wild-type flies respectively, t-Test $p=0.01$; Figure 2F-G). Thus, the loss of GLaz function seems to be altering other tissues in addition to the nervous system.

**Fat body physiology and morphology is greatly altered in GLaz mutant flies**

Because changes in body size have also being linked to changes in longevity and stress resistance [16], we explored whether the weight of GLaz mutant flies differs from wild type controls. Both sexes of $GLaz^{\Delta2/\Delta2}$ flies show significantly lower wet and dry body weight (Fig. 3C; male data are shown).

Protein content does not differ between genotypes (not shown, t-Test $p=0.23$). Therefore, most of the body weight variations observed can be accounted for by a marked reduction in relative fat content of $GLaz^{\Delta2/\Delta2}$ flies (Fig. 3D; male data are shown). Further analyses show that the relative content of triglycerides (Fig. 3D) in both young and old adults is significantly decreased when GLaz is absent. These biochemical parameters correlate well with the observed morphology of the fat body: both pericerebral and abdominal fat body tissue of GLaz mutants are markedly reduced in size (Figure 4A-B, I). Young adult $GLaz^{\Delta2/\Delta2}$ flies of both sexes show fewer and smaller
lipid bodies (Figure 4C-D) and aging worsens this effect with a reduction in the fat body mass (Figure 4E-F). Moreover, the limited lipid bodies present in old GLazΔ2/Δ2 flies are accompanied by increased eosinophilia due to protein aggregates, a morphological indicator of forthcoming cell death (Figure 4H).

These data suggest that the absence of GLaz provokes (i) a profound reorganization in the lipid storage/lipid metabolism balance, (ii) an acceleration of age-dependent neural cell death in the CNS, and (iii) intense degeneration in both fat and neural tissues upon experimental oxidative stress.

**Restoring GLaz expression under its native promoter returns oxidative stress sensitivity and fat body physiology back to control levels**

When a GLaz-GFP fusion protein construct (GLaz-Fx line, see Figure 1S and supplementary text) is placed in a GLazΔ2/Δ2 background, the survival upon paraquat treatment returns to control levels (Fig. 1B). Starvation sensitivity is also recovered, with a 66.2 % increase in median survival time when comparing GLaz-Fx:GLazΔ2/Δ2 to GLazΔ2/Δ2 (p < 0.00001 log-rank test). The size and morphology of the fat body is restored as well (Figure 4I-L, N=15 flies/genotype). These results confirm that the observed phenotypes are due to the lack of GLaz protein, with negligible contributions, if any, from neighboring genes (see supplementary text for discussion).

The native regulatory regions used here to reinstall GLaz expression in the null mutants seem to control the level of expression within physiological levels. Interestingly, as described in the accompanying paper [7], strong overexpression in the nervous system does not provide beneficial effects on longevity. The amount of secreted GLaz might therefore be a critical factor.
We can conclude from our data that GLaz expression in a subset of cells within the nervous system and in hemocytes is sufficient to fulfill the protective function of GLaz against oxidative damage, starvation resistance, and its influence on fat body physiology (regulating lipid stocks). This also suggests that the effects seen in the fat body are exerted non-autonomously. Whether a systemic supply of GLaz provided by hemocytes is sufficient for these effects, or the GLaz protein produced within the nervous system has direct or indirect influences on lipid management by fat body cells can not be discerned. However, it is interesting that brain-fat body regulatory neurohormonal circuits are proposed to control nervous system and somatic senescence, which in turn are linked to the response to nutrient levels and environmental stressors [see 17 for a review].

**Lipid peroxidation protection or scavenging as the mechanism of action for the lipocalin GLaz**

With a few exceptions, lipocalin function has been related to the binding of hydrophobic ligands. This biochemical property is put to work in different cellular and organismal contexts, generating a panoply of physiological roles, such as lipid transport [18], regulation of the innate immune system [19], regulation of cell proliferation and apoptosis [20], anti-inflammatory and antibacterial function [21] as well as scavenging of toxic molecules, like heme [22] or lipid peroxidation products [23]. In addition, some lipocalins have enzymatic activities, including antioxidant activities: alfa-1-microglobulin is able to protect tissues from pro-oxidant molecules, inhibit oxidation and remove oxidation products [22, 24].

The data presented so far strongly suggest that GLaz is part of a defense system acting in situations of unbalanced oxidative stress, provoked exogenously from
environmental conditions or endogenously by aging or pathological situations. GLaz could act as a general scavenger of toxic products (most probably peroxidated lipids) and/or exert a protective role by preventing oxidation of lipids, which is known to occur in the CNS and fat body of aged and parquat-treated flies [25].

This hypothesis predicts that the levels of peroxidated lipids should be higher in the GLaz mutants. That is in fact the case: the concentration of free malondialdehyde (MDA), the main product derived from unstable lipid peroxides, is more than two times higher in GLaz^2/2 flies than in control flies (Fig. 3E). The levels of MDA therefore represent a measure of the degree of oxidative damage in the tissue.

**How can a molecule protecting against oxidative stress modify lipid stocks in the fat body?**

If GLaz function is exerted by lipid peroxidation protection or scavenging, then the absence of GLaz would mimic a stress condition. This would result in an accelerated senescence and neurodegeneration. Fat body cells also show more cell death upon aging in GLaz null mutants. However, the depletion of lipid bodies is a more generalized phenomenon within this tissue, and the lower resistance to starvation could be directly linked to this effect. Possibilities to be considered are that GLaz might exert a direct signaling function on fat body cells; or that it might perform a transport function that controls the supply of lipids in a rate-limiting fashion. However, a direct interaction with the fat body is not a requirement, since the stress provoked by the absence of a protective agent (GLaz) would secondarily become a feedback loop reverberating on the stress response signaling networks. In this scenario, depletion of lipids from the fat body in GLaz null mutant flies would be the result of the metabolic activation triggered, for example, by biogenic amines (dopamine and octopamine) in response to the unfavorable
conditions [26]. Interestingly, ApoD has been identified as a direct target of the liver X receptors in adipose tissue [27], pointing to a role of this lipocalin in the regulation of lipid homeostasis in vertebrate fat tissue as well.

**Functional significance of GLaz/ApoD function in disease and aging**

In recent years data coming from different model organisms (*C. elegans*, Drosophila and mouse [see 17 for a review]) suggest that the general scaffold of the neurohormonal and signaling pathways controlling stress responses and aging has been evolutionarily conserved. The loss-of-function (this work) and the gain-of-function [7] analyses of *GLaz* in Drosophila clearly suggest that this lipocalin has a protective function both during experimental (mimicking disease) or endogenous (aging) oxidative stress. Although co-options to new functions and multifunctionality [28] can not be ruled out, the GLaz function is most likely conserved in ApoD, the vertebrate lipocalin closer to the insect-vertebrate node in the gene family tree.

Evidence pointing to a role for ApoD in aging and disease has accumulated over the years. The results presented here suggest that GLaz/ApoD is an active, non-dispensable, element that is predicted to influence the time course and the severity of degenerative diseases and cancer as well.

Since the Drosophila adult is essentially a non-regenerating post-mitotic organism, functional conservation with the vertebrate ApoD is expected especially in tissues that are also mainly post-mitotic, e.g., the nervous system. If genetic manipulations in vertebrate model organisms confirm this prediction, the potential use of ApoD as a therapeutic agent in neurodegenerative disorders should become a research priority.
Figure Legends

Figure 1. The absence of GLaz reduces resistance to oxidative stress and starvation, and shortens lifespan. Sensitivity to stress is rescued if normal GLaz expression is restored. Data in A-D are the average ± SEM from three independent experiments. Male data are shown. Log-rank test was used for statistical analysis. Flies still alive at the end of the experiment are assigned a censoring value of 1 and their survival time was considered as a minimum survival time.

(A) Survival of GLaz^{Δ2/Δ2} flies compared to wild type flies upon exposure to a ROS generator (paraquat, 20 mM) supplied in the food (N=100/genotype); \( p < 0.00001 \).

GLaz^{Δ2/Δ2} mutants were outcrossed with the Canton-S (CS) strain. Line G2, homozygous for the GLaz^{Δ2} allele, is compared to line G10, homozygous for the GLaz^{Δ+} allele.

(B) The sensitivity to paraquat of GLaz^{Δ2/Δ2} mutant flies is reverted back to control levels when a GFP-GLaz fusion protein is expressed under the control of the GLaz natural promoter (N=75/genotype); \( p < 0.00001 \). Paraquat was supplied in Whatman 3M filter papers soaked with 1 ml of a 10% sucrose, 20 mM paraquat solution (see methods and supplementary text).

(C) Survival analysis upon feeding 1% H₂O₂ on day 1 and 5% H₂O₂ on subsequent days to the GLaz mutant and control flies (N=150/genotype); \( p < 0.00001 \). H₂O₂ was supplied daily in Whatman 3M filter papers soaked with 1 ml of a 10% sucrose solution with H₂O₂.

(D) Effect of starvation on survival of GLaz^{Δ2/Δ2} compared to GLaz^{+/+} flies (N=75/genotype); \( p < 0.00001 \). Humidity was supplied daily in Whatman 3M filter papers soaked with 1 ml of water.
(E) Lifespan determination of $GLaz^{A2/A2}$ compared to wild type males (CS). Median survival time is reduced by 19.3% in the $GLaz$ mutant (log-rank test, $p<0.0001$, $N=65$/genotype) and maximum survival time is reduced by 10.9% (Gehan´s Wilcoxon test, $p=0.0013$).

(F) Reduction of lifespan is maintained after outcrossing the $GLaz^{A2}$ mutant allele with the CS strain (see supporting text) ($N=225$/genotype). Median survival time is reduced by 18.1% (log-rank test: $p<0.00001$). Maximum survival time is reduced by 12.7% (Wilcoxon test: $p<0.0001$).

**Figure 2.** (A-C) Expression of $GLaz$ in the adult fly. GFP reporter expression was assayed by anti-GFP immunohistochemistry on horizontal paraffin sections of 3 days-old adult flies carrying the GFP reporter construct R3rd (red labeling in A-C). Monoclonal antibody 22C10 was used as a neuronal marker in A,B (green labeling). Anterior is up.

(A) In the brain, the transcriptional reporter is expressed in the perineurial sheath glia (arrows) and in subsets of cells in the medulla neuropil, the lamina, and the lobula and lobula plate in the optic lobe (triangles). The location of the $GLaz$ driven GFP expression in these cells suggests its glial origin.

(B) In muscle fibers (m), peripheral nerves labeled by 22C10 are surrounded by GLaz-GFP positive perineurial glia.

(C) $GLaz$-reported labeling in cells in the hemocoel.

(D-G) Patterns of apoptotic cell death upon loss of $GLaz$ function in adult young flies after exposure to experimental oxidative stress. See text for quantitative analysis of TUNEL-positive cells. Pericerebral fat body (white triangle in E) as well as cells in the brain, show apoptotic cell death in paraquat treated $GLaz$ mutants. Apoptotic cells are
observed in the brain cortex (arrows in D,E, inset in E), and in the brain neuropil (probably glial cells, black triangles in D,E).

(F,G) TUNEL-positive cells are found in the digestive tract (black triangle in G) and the abdominal fat body (white triangles in G).

Abbreviations; br: brain fb: fat body; g: gut; La: lamina; Lo: lobula; Lp: lobula plate; m: muscle; Me: medulla; OL: optic lobe; pfb: pericerebral fat body; Re: retina. Bars represent 50\(\mu\)m (C) or 25\(\mu\)m (A,B,D-G).

**Figure 3.**

Age-related (A) and oxidative stress-triggered (B) functional decline of locomotor activities. Geotropism and phototaxis responses were evaluated as described by Palladino et al. [29]. Flies collected and reared as for the longevity analyses (see Supporting Online Material) were tested in groups of 10 at 3-4 days (not shown) or 20-21 days of age. Male data are shown. Scores are represented as 1/t to facilitate visual comparison with other tests results. A better performance gets a higher score in all tests.

(A) No difference between genotypes is observed in phototaxis, but \(GLa_2^{\Delta2/\Delta2}\) aged males get a lower score in geotropism since they take significantly longer to climb the same distance (N=50/genotype). Flight performance was scored following Benzer [30]. No difference between genotypes is observed in flying ability (N=150/genotype).

(B) After 24 h of feeding with 20 mM paraquat supplied in filter paper, locomotor activities in the three behavioral tests were significantly impaired in \(GLa_2^{\Delta2/\Delta2}\) mutants as compared to paraquat-treated control flies. Phototaxis was evaluated as above. Methods imposing a less strenuous exercise to the flies were chosen to evaluate geotropism and flying abilities. N=20/genotype. Female data are shown. Percent
number of flies climbing over a 6 cm line in 15 seconds is recorded in 4 consecutive trials. Spontaneous number of flights over 4 intervals of 2 minutes is recorded.

(C-E) Absence of GLaz results in a smaller body mass due to a lower amount of neutral fats, and in accumulation of lipid peroxidation products.

(C) Body weight differs significantly between $GLaz^{Δ2/Δ2}$ and $GLaz^{+/+}$. Mutant males weight less than $GLaz^{+/+}$ flies (N=200/genotype).

(D) Relative fat content in $GLaz^{Δ2/Δ2}$ flies is significantly smaller than in control flies (N=200/genotype). Levels of triglycerides normalized to protein content also differ significantly between genotypes in young and aged flies (N=70/genotype).

(E) Levels of free malondialdehyde (MDA), the most abundant product derived from unstable lipid peroxides, are highly increased in $GLaz$ null mutants compared to control flies. Results are shown normalized to protein content. Experiments performed in triplicate (N=150/genotype).

Data are represented as mean ± SD. Statistical differences assayed by unpaired, two-sided Student’s t-test.

Figure 4. Lack of GLaz profoundly alters the morphology of the fat body tissue. The effect is reverted when a GLaz-GFP fusion protein is expressed under the control of $GLaz$ natural promoter.

Standard hematoxilin-eosin histochemistry was performed on paraffin (C-L) or resin (A-B) sections. N=15 flies/sex/genotype/condition.

(A-B) Horizontal brain sections showing the pericerebral fat body (triangles) of control and mutant 7-days old flies. Mutant fat body tissue is markedly reduced in size.
(C-D) Abdominal fat body of 3-days old control and $GLaz^{Δ2/Δ2}$ flies showing the reduction in size an number of lipid bodies (triangles) in mutant tissue. The same effect is observed in the pericerebral fat body (not shown).

(E-H) Aging enhances this effect as shown in the pericerebral fat body of aged control (E, G) and $GLaz^{Δ2/Δ2}$ (F, H) flies. The same effect is observed in the abdominal fat body (not shown). G and H panels are magnifications of E and F respectively. Arrow in H points to an eosinophilic cell.

(I-L) $GLaz^{Δ2/Δ2}$ fat body size reduction and decrease in lipid bodies (I, K) is restored to normal levels in mutant flies expressing the GLaz-GFP fusion protein (J, L; Fx construct) in brain and hemocytes (see Figure 3). Abdominal fat body (triangles) is shown in I-J, while pericerebral fat body is shown in K-L (triangles point to lipid bodies).

Abbreviations; Re: retina. Bars represent 50µm (A-B, I-J), 50µm (E-F) or 20µm (C-D,G-H, K-L).

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References


**Supporting Online Material**

**Loss of Glial Lazarillo, a homolog of Apolipoprotein D, reduces lifespan and stress resistance in Drosophila**

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**Materials and Experimental Procedures**

**Fly strains and husbandry.**

Flies were grown at 25ºC under a 12h-12h light cycle. Food recipe: yeast 84 g/l, sugar 84 g/l, NaCl 3.3 g/l, agar 10 g/l, wheat flour 42 g/l, apple juice 167 ml/l, propionic acid 5 ml/l.

\[ GLaz^{Δ2} \text{ and } GLaz^{Δ1} \text{ mutant alleles were obtained in a } w^{1118} \text{ background by imprecise excisions of the P-element (P}\{\text{EP}\}EP2383) \text{ (Figure1S). The fly line EP-2383 was isogenized before excision. Homozygous } GLaz^{Δ2/Δ2} \text{ where outcrossed into Canton-S (CS) background (see supporting text). Two lines, G2(GLaz^{Δ2/Δ2}) and G10(GLaz^{+/+}), were chosen for further characterization.} \]

Transheterozygous flies \[ GLaz^{Δ2} Spt1^{+/+}/GLaz^{+} Spt1^{l(2)Sh1626} \text{ or } GLaz^{+} Spt1^{+/+}/GLaz^{+} Spt1^{l(2)Sh1626} \text{ were generated by crossing homozygous virgin females from the outcrossed lines G2(GLaz^{Δ2/Δ2}) or G10(GLaz^{+/+}) with Spt1^{l(2)Sh1626}/CyO males. The } Spt1^{l(2)Sh1626}/CyO \text{ line was kindly provided by S.X. Hou [8].} \]

The deficiency Df(2R)Exel 8057 which uncovers 22 genes (from CG13323-CG4630, including GLaz) was obtained from the Bloomington Stock center (BL7871).
GLaz$^{A2}$ allele was placed over the deficiency by crossing homozygous virgin females with Df(2R)Exel 8057/CyO males.

A transcriptional reporter construct, containing 1876 bp of the genomic sequence upstream of GLaz gene plus the 5’UTR of GLaz (45 pb) followed by the green fluorescent protein (GFP) coding sequence, was used for P-element mediated transformation of $w^{1118}$ flies. An insertion on the 3rd chromosome (GLaz-R-3rd) was chosen for further characterization (Figure 1S F).

A fusion protein reporter construct was also used to drive the expression of GLaz-GFP under the same upstream sequences used in GLaz-R-3rd (Figure 1S G). The upstream sequence was followed by the entire genomic GLaz sequence up to the last coding nucleotide. The stop codon was removed and the GFP coding sequence was placed in frame. Two independent P-element mediated insertions were analyzed, one on the second chromosome (GLaz-F-2nd) and one on the X chromosome (GLaz-Fx).

**Molecular characterization of GLaz mutants and reporter lines.**

Initial selection and posterior genotyping of the GLaz alleles was carried out by PCR amplification of genomic DNA. The primers 5’ ATGGAGGAACAGTGGAATATG 3’ and 5’ TGGTTGTACCGCTCAAAACTGAAA 3’ that lie outside the deleted region were used to show the presence and size of the deletion (Figure 1S C). The absence of a GLaz mRNA in the mutant flies was tested by RT-PCR using the primers 5’ TGTGTACGCTCAAACACTGAAA 3’ and 5’ ATTTGCTGGGACAGATGCCTAC 3’ (Figure 1S D), and by Northern analysis of total RNA probed with the complete GLaz cDNA (Figure 1S E). Total RNA was extracted from adult flies using Trizol. 20 µg of RNA was electrophoresed in a formaldehyde-agarose gel and blotted to a nylon membrane, which was hybridized in Ultrahyb solution (Ambion) for 18 hours at 65 ºC
to the \textit{GLaz} radiolabeled probe. The membranes were then washed at 65 °C, and exposed to film.

Expression of GFP under the control of \textit{GLaz} promoter in reporter lines (selected by the presence of \textit{w}+) was first assayed by fluorescence microscopy of dissected brains and thoracic ganglia. Since fluorescence signal was very low (not shown), we tested for the expression of GFP by immunoblot on crude protein extracts from heads and thorax of transgenic flies. Levels of expression are relatively high (not shown), suggesting that the low fluorescence signal could be due to some conformational change in the GFP protein that deters its normal fluorescence emission.

Quantitative RT-PCR experiments were performed using the Drosophila ProbeLibrary technology (Exiqon) and analyzed in an ABI PRISM 7700 sequence detection system (Applied Biosystems). Primers of equal amplification efficiency designed for Spt1 and for 18S (endogenous control). The amplifications were performed on three independent RNA Trizol extractions for each genotype (\textit{GLaz}^{A2/A2} / \textit{GLaz}^{+/+}). RNA was quantified by Nanodrop technology and the reverse transcription reaction was primed with random hexamers. Triplicate PCR reactions were performed for each RNA extraction.

**Lifespan analysis.**

Flies were collected within 24 h of eclosion and were allowed time for mating (2-3 days after adult emergence). They were then separated by sex under brief CO$_2$ anesthesia and housed in groups of 25. They were raised at 25°C under a 12h-12h light cycle and transferred to fresh food vials every 2-3 days.
Body weight and fat content.

Five groups of 40 flies (3 days old) were weighed in a precision balance (Sartorius® ultra microbalance) after killing them by a short exposure to cold (–80°C). Flies were then dried in glass vials at 65°C for 36 h and re-weighed to calculate dry body weight. The fat-free dry weight value was calculated after extraction of fat with diethyl ether for 24 h (with 2 changes of 4 ml). Flies were dried again at 65°C for 24 h and re-weighed. Relative fat content was calculated by dividing absolute fat content by dry weight.

Triglycerides content was determined by an enzymatic colorimetric test (TG, Roche) in pools of 50 flies. Measures were carried out in triplicate and were referenced to the amount of total protein per sample estimated by the Bradford’s assay.

Lipid peroxidation levels.

A spectrophotometric assay was used to determine the concentration of free malondialdehyde (MDA-586, Bioxytech). Groups of 50 flies/genotype were homogenized in 100 mM phosphate buffer (pH 7.2) in the presence of an antioxidant (butylated hydroxytoluene, BHT) to prevent new lipid peroxidation during homogenization. Half of each lysate was used to perform sample blanks. Absorbance at 586 nm of experimental samples was subtracted from blank samples and referred to a standard curve. Three independent experiments with measurements in triplicate were performed.

Behavioral assays.

Flies for all behavioral assays were collected as described for the longevity analysis. The tests were performed when flies were 3-4 days old or after aging in the same conditions as for the lifespan assay (see above). The same groups of flies were subjected
sequentially to the running and climbing tests. Different samples were used for the flight performance measures. Brief CO₂ anesthesia was used to separate flies in groups. Tests were performed following a minimum of 24 hours after anesthesia.

Running ability:
Running ability was estimated following Palladino et al. method [29]. Groups of 10 flies were transferred without anesthesia to a 10 ml serological pipette truncated and sealed with wax film at the conical end. Aluminum foil was used to cover 20 cm from the bottom. Flies were knocked down to the bottom by gentle tapping, and the pipette was immediately placed horizontally so that the uncovered area was illuminated by a fiber-optic lamp. The time taken for half of the sample to get to the illuminated area was recorded four times, and the inverse of the time was used as performance score. This way, the better performers get a higher score, facilitating comparisons with all other behavioral tests.

Geotaxis and climbing ability:
Climbing ability was monitored as in Palladino et al. [29]. Groups of 10 flies were scored four times by measuring the time taken by half of the sample to traverse the 150 ml line (13 cm) in a 250 ml graduated cylinder. The cylinder was illuminated from the top with a fiber-optic lamp and covered with wax film to prevent escapes. As for the running ability test, the inverse of the time taken was used as performance score.

When climbing ability was tested after 24 h of paraquat treatment the following test was performed. Flies were selected among the survivors by gentle aspiration of the flies moving or flying on the upper part of the vials. Flies with extremely low mobility were discarded. Groups of 10 flies housed in vials were recorded in video. After a gentle tap of the vial, the number of flies climbing above 6 cm in 15 seconds were counted. The cycle was repeated four times per vial and the average performance calculated.
**Flight ability:**

Flight performance was evaluated according to Benzer [30]. Groups of flies (n>200/genotype) were tested for flight ability in a graduated cylinder with oil-coated walls. The position (measured from the bottom of the cylinder) at which each fly gets stacked after they are dumped into the cylinder is measured and plotted in 10 intervals of 2.5 cm each. Overall performance was estimated by averaging the height-interval of each group of flies.

An alternative flight ability measurement was performed after 24 h of paraquat treatment. Selection of survivors was carried out as for the climbing alternative test. A gentle tap of the vial was followed by a 2 min. period of video recording in which number of spontaneous flights were counted. The cycle was repeated four times per vial and the average performance calculated.

**Oxidative stress toxicity and starvation stress.**

Flies collected as described for the longevity analysis were separated by sex in groups of 25 when they were 3 days old. Application of different stressors was performed as follows.

*Paraquat treatment:*

After a period of dry starvation (3 hours) flies were transferred to vials with Instant Food (Carolina Biological Supplies) with or without 20 mM paraquat (Sigma). Incubation proceeded in the absence of light to delay paraquat degradation. Deaths were scored every 4-8 h.

Alternatively, paraquat was supplied in filter papers soaked with 1 ml of a 10% sucrose-20 mM paraquat solution, or with sucrose alone. See supporting text for a comparison of the effects of paraquat depending on the method used.
**H$_2$O$_2$ treatment:**

Starting at age of 3 days, flies were transferred daily to vials with filter papers soaked with 1 ml of a 10% sucrose solution with or without H$_2$O$_2$. 1% H$_2$O$_2$ was used for the first 24 h and 5% for the subsequent days. Dead flies were scored every 4-8 h.

**Wet starvation treatment:**

Starting at age of 3 days, flies were transferred to vials with filter papers soaked with 1 ml of water. Dead flies were scored every 4-8 h.

**Histology, immunohistochemistry, and TUNEL assay.**

Adult flies were fixed with 4% paraformaldehyde, dehydrated in ethanol, and included in paraffin. Paraffin sections (7 µm) were dewaxed and rehydrated in an ethanol series. Alternatively, Technovit resin (Kulzer) was used according to the manufacturer instructions. Histochemical staining with hematoxylin and eosin was performed according to standard procedures. Anti-GFP rabbit serum (Invitrogen), and mAb 22C10 (DSHB) were used as primary antibodies for immunohistochemistry. Alexa-594 or FITC-conjugated secondary antibodies (Molecular probes) were used for immunofluorescent labeling. A biotin-conjugated secondary antibody and the ABC kit (Vector Labs) were used for samples developed for light microscopy. The labeled sections were observed with a Nikon (Eclipse 80i) microscope, photographed with a Jenoptik camera, and processed with Adobe Photoshop (version 5.5). Apoptotic cell death was assayed in paraffin sections of flies with the TUNEL labeling kit (Roche) following the manufacturer protocols. Quantification of TUNEL-positive cells was performed by sampling one every five 8 µm-sections (n=2 flies/genotype).
Supporting text

Changing the genetic background of GLaz mutations does not significantly alter their effect on stress sensitivity or longevity.

The \( GLaz^{\Delta2/\Delta2} \) line, generated five years ago, was kept as a homozygous stock and hence might have accumulated deleterious or compensating mutations that could affect longevity and stress resistance. Therefore we outcrossed the mutant line with our CS stock. Ten independent lines were PCR tested for the presence of the wild type or mutant allele of \( GLaz \). We selected several sister lines with the mutant allele \( GLaz^{\Delta2} \) or the wild type allele \( GLaz^{+} \) for further characterization. The lines G2, homozygous for the \( GLaz^{\Delta2} \) allele, and G10, homozygous for the \( GLaz^{+} \) allele were chosen as representatives for in depth characterization.

Lifespan differences of the original \( GLaz^{\Delta2/\Delta2} \) mutant and wild type (CS) flies (Fig. 1E) are very similar to those of the outcrossed lines (Fig. 1F). Likewise, sensitivity to paraquat was similar in both the \( GLaz^{\Delta2/\Delta2} \) original line and the outcrossed lines (results not shown), discarding any significant contribution of hybrid vigor to the lifespan or stress resistance of the outcrossed lines. Thus, the higher sensitivity to stressors and the reduction of lifespan observed in the lines carrying the \( GLaz^{\Delta2} \) allele are not due to deleterious mutations accumulated elsewhere in the genome.

\( GLaz \) phenotype can not be ascribed to alteration of other genes in the genome.

A deficiency uncovering the \( GLaz \) genomic region (22 genes) fails to complement the oxidative stress sensitivity phenotype of the \( GLaz \) null mutants (Fig 2S), discarding the possibility that mutations in other regions of the genome might contribute to the observed effects. We have also discarded a specific contribution of the nearest gene, Spt-1 (see Figure 1S A), to the oxidative stress sensitivity conferred by the \( GLaz^{\Delta2} \) allele.
(Figure 2S). Finally, the rescue of this phenotype (as well as the starvation sensitivity and fat body reduction phenotypes) by the GLaz-GFP fusion protein expressed under the control of the native promoter (Figures 1B and 4I-L) demonstrate that GLaz expression is sufficient to fulfill the stress protective function and the role in the control of lipid stocks. However, the deletion removing the beginning of GLaz gene is upstream of the essential Spt1 gene, and thus, it might contribute to its regulation. Our quantitative PCR experiments show no significant alterations in the level of expression of Spt1 in the null GLaz mutants, suggesting that Spt1 would not significantly influence other putative phenotypes.

**Comparison of paraquat feeding methods.**

We have observed that the method used to feed the flies with paraquat influences the amount of effect obtained. When paraquat is added to the food, the effect in survival is consistently lower than when it is supplied, at the same concentration, in filter papers (see methods section). To discard that the difference was due to variables related to the flies that might have changed over time (e.g. general health status) we performed an experiment with a batch of flies collected from the same bottle and treated with 20 mM paraquat supplied in the two forms. The difference was again observed (not shown). We conclude that some components in the food must have antioxidant properties that counterbalance the effect of paraquat. Therefore, the effective concentration of paraquat when supplied in the food is lower than when supplied in filter papers. Only experiments using one methodology are integrated in a given set for analysis.
Supporting figures

Figure 1S. Generation of GLaz loss-of-function mutants and GFP reporter line


(B) GLaz\textsuperscript{A1} and GLaz\textsuperscript{A2} are null alleles generated by imprecise excision of the P element P\{EP\}EP2383. Deletions start at \pm 12 bp from the EP insertion site and cover 2049 bp and 2794 bp respectively, eliminating the transcription initiation site, the first coding exon and part of the first intron of GLaz gene.

(C-E) Molecular analysis of GLaz alleles at the DNA and RNA levels. (C) Primers flanking the deletions were used to amplify fragments from genomic DNA. These fragments were sequenced to determine the exact break points of the deletions (not shown).

(D) Primers in exons 1 and 2 were used to amplify fragments from mRNA by RT-PCR. No band can be detected in the mutant alleles. Absence of genomic DNA contamination was proved by omitting the reverse transcriptase from the reaction mixture (RT- control lanes). The expected wild type genomic fragment was amplified in the same experiment for comparison.

(E) Northern analysis of total RNA probed with P\textsuperscript{32}-labeled GLaz cDNA. Hybridization shows the expected 0.8 kb band in wild type flies while no signal is detected in the RNA from GLaz\textsuperscript{A1/A1} or GLaz\textsuperscript{A2/A2} homozygous flies, confirming that both alleles are null alleles.

(F) The GFP transcriptional reporter construct.

(G) The GLaz-GFP fusion-protein reporter construct.
Figure 2S. Complementation studies of oxidative stress sensitivity phenotypes.

The neighboring gene Spt1 does not contribute to the sensitivity to oxidative stress of the GLazΔ2 allele. Transheterozygous flies GLazΔ2 Spt1+/GLaz+ Spt1l(2)Sh1626 are as resistant to paraquat (A) or H2O2 (B) as the controls heterozygous for either gene. When the GLazΔ2 allele is placed over a deficiency uncovering 22 genes in the GLaz locus area, the survival curve upon paraquat (A) or H2O2 exposure (not shown) is similar to the GLazΔ2/Δ2 homozygous flies, discarding mutations in distant areas of the genome as putative contributors to the GLaz null mutant phenotypes. In all cases the oxidative stressor was supplied in filter papers.
**Figure 1**

A. *Paraquat*

B. *Paraquat Rescue*

C. *H$_2$O$_2$*

D. *Wet Starvation*

E. *Longevity*  
GLaz($\Delta 2$)  
CS  
G10(wt)  
G2($\Delta 2$)

F. *Longevity*  
G10(wt)  
G2($\Delta 2$)
**Figure 3**

- **A** 21-days old flies
  - Phototaxis
  - Geotropism
  - Flight

- **B** Paraquat-treated 4-days old flies
  - Phototaxis
  - Geotropism
  - Flight

- **C** Body weight
- **D** Lipid content
- **E** Lipid peroxidation

Legend:
- G10(wt)
- G2(Δ2)

Statistical significance:
- p < 0.0001
- p = 0.001
- p = 0.022
- p = 0.038
- p = 0.027
Figure 4