

In Vivo Mapping of Hydrogen Peroxide and Oxidized Glutathione Reveals Chemical and Regional Specificity of Redox Homeostasis

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SUMMARY

The glutathione redox couple (GSH/GSSG) and hydrogen peroxide (H₂O₂) are central to redox homeostasis and redox signaling, yet their distribution within an organism is difficult to measure. Using genetically encoded redox probes in *Drosophila*, we establish quantitative in vivo mapping of the glutathione redox potential (E_{GSH}) and H₂O₂ in defined subcellular compartments (cytosol and mitochondria) across the whole animal during development and aging. A chemical strategy to trap the in vivo redox state of the transgenic biosensor during specimen dissection and fixation expands the scope of fluorescence redox imaging to include the deep tissues of the adult fly. We find that development and aging are associated with redox changes that are distinctly redox couple-, subcellular compartment-, and tissue-specific. Midgut enterocytes are identified as prominent sites of age-dependent cytosolic H₂O₂ accumulation. A longer life span correlated with increased formation of oxidants in the gut, rather than a decrease.

INTRODUCTION

Reactive oxygen species, oxidative stress, and antioxidant levels are widely believed to play key roles in health and disease. For example, oxidants and oxidative changes have been linked to inflammation (Nathan and Ding, 2010) and aging (Finkel and Holbrook, 2000). Oxidants are suspected of causing or exacerbating various disease states either through the damage they may inflict or by inducing alterations in redox-sensitive cell signaling pathways (Houstis et al., 2006; Lin and Beal, 2006). Unfortunately, our knowledge of the redox changes that actually occur in the physiological whole-body context of living animals is extremely limited. We know very little about the redox differences that exist naturally between the different tissues and cell types within the body. It also remains unclear to what

extent in vivo redox states are influenced by behavioral and environmental factors, e.g., physical activity, nutrition, or infection. Our lack of knowledge is especially obvious with regard to the exact chemical nature of these changes, their subcellular origin, their spatio-temporal distribution, and their biological relevance.

Most previous redox measurements in animal model organisms have either relied on indirect evidence, e.g., late-stage markers of cellular damage, or have been based on disruptive methods. Oxidative damage markers are considered questionable because damage levels may reflect changes in repair and turnover rather than differential oxidant levels (Murphy et al., 2011). On the other hand, disruptive methods, e.g., the determination of GSH/GSSG ratios by extracting whole organisms or tissues, average over cell types and subcellular compartments. It is, however, very likely that the consideration of tissue-, cell type-, and subcellular compartment-specific redox differences and changes is crucial for our understanding of redox biology.

Another important issue is that no single redox couple or reactive species can be considered representative of all redox processes that occur in a cell at a given instant of time. Cells harbor a variety of redox couples, many of which are far from being in equilibrium with each other, and most have distinct roles and regulation. For example, an increase in glutathione disulfide (GSSG) inside the mitochondrial matrix is likely to have different causes and consequences (and therefore biological meaning) than an increase in cytosolic H₂O₂ concentration. Consequently, it seems expedient to analyze the various redox species specifically and separately, with subcellular resolution on various timescales.

Recent advances in probe development, based on redox-sensitive GFPs (roGFPs) (Dooley et al., 2004; Hanson et al., 2004), now make it possible to work toward the goal of redox couple-specific in vivo imaging at subcellular resolution in model organisms. RoGFPs contain an engineered dithiol/disulfide switch on their surface. The redox equilibrium of the engineered cysteines is associated with measurable ratiometric fluorescent changes. Importantly, the redox-dependent fluorescence ratio exhibited by roGFP2 is insensitive to pH changes in the physiological range (Schwarzlander et al., 2008). It has been established that endogenous cellular

glutaredoxins facilitate equilibration of roGFPs with the glutathione pool (Meyer et al., 2007). Subsequently, we showed that roGFP2 can be directly fused to glutaredoxin (Grx), which increases specificity for glutathione, enhances the kinetics of equilibration between the glutathione and roGFP redox couples, and effectively allows measurement of the glutathione redox potential (E_{GSH}) (Gutscher et al., 2008). Moreover, the fusion to Grx makes the roGFP2 response independent of endogenous Grx. This independence is important because the expression and/or activity of endogenous Grx may differ between cell types, subcellular compartments, developmental stages, and environmental conditions. Importantly, we also recognized that roGFPs can be converted into specific probes for H_2O_2 by coupling them to the microbial H_2O_2 sensor oxidant receptor peroxidase 1 (Orp1) instead of to Grx (Gutscher et al., 2009).

The goal of this work was to undertake steps toward establishing a measuring system that allows monitoring of the actual in vivo status of chemically defined redox species (i.e., the GSH/GSSG redox couple and H_2O_2 , respectively) in defined subcellular compartments (i.e., cytosol and mitochondria, respectively) with cellular resolution for the whole body of an animal. To this end, we generated transgenic *Drosophila melanogaster* using four different redox probes to allow measurement of E_{GSH} in the cytosol (cyto-Grx1-roGFP2) and in the mitochondrial matrix (mito-roGFP2-Grx1), and for measuring H_2O_2 in the cytosol (cyto-roGFP2-Orp1) and in the mitochondrial matrix (mito-roGFP2-Orp1). In order to obtain an organism-wide overview, we chose to express these probes ubiquitously through the tubulin promoter. The parallel use of probes for E_{GSH} and H_2O_2 can be considered complementary: while the glutathione redox pair represents a major protection system against oxidants, H_2O_2 is the major oxidant species involved in protein thiol oxidation and redox regulation.

In this study, we present the following findings: First, live imaging of biosensor-transgenic larvae reveals natural redox differences that exist between different cells and tissues. The imaging of living larvae also identifies localized redox changes that occur during physiological transitions such as the cessation of feeding behavior. Second, live redox imaging reveals that the authentic in vivo redox state of the probes can be dependably trapped and conserved by a chemical procedure. Thus, fluorescence-based redox imaging can be applied to dissected animals and becomes available to otherwise optically inaccessible tissues. Third, we identify adult tissues that show either age-dependent or age-independent oxidation. A key insight is that aging-dependent pro-oxidative changes exist but are highly restricted to particular tissues and regions, instead of affecting the whole organism. Midgut enterocytes are major sites of cytosolic H_2O_2 accumulation during aging. Increased life span was accompanied by increased formation of oxidants, rather than by a decrease. Fourth, our measurements suggest that the two oxidant species GSSG and H_2O_2 can change independently of each other. They appear to carry different kinds of biological information and therefore must be measured specifically and separately. Fifth, redox imaging allows investigation of the in vivo influence of individual gene products and pharmacological agents. N-acetyl cysteine (NAC) did not show obvious antioxidative effects in vivo but

rather induced an increase in mitochondrial H_2O_2 in various tissues.

RESULTS

Generation of Biosensor-Transgenic Flies

To enable the observation of in vivo redox processes, we created flies broadly expressing either a cytosolic E_{GSH} probe (Tubulin-Gal4, UAS-Grx1-roGFP2), a mitochondrial E_{GSH} probe (Tubulin-Gal4, UAS-mito-roGFP2-Grx), a cytosolic H_2O_2 probe (Tubulin-roGFP2-Orp1), or a mitochondrial H_2O_2 probe (Tubulin-mito-roGFP2-Orp1). In these constructs, Grx1 mediates roGFP2 oxidation by GSSG, and Orp1 mediates roGFP2 oxidation by H_2O_2 , respectively (Figures 1A and 1B). The correct subcellular targeting to either cytosol or mitochondria was confirmed by confocal microscopy (see Figure S1 available online). The general in situ response of each probe was tested by exposure of freshly prepared imaginal discs to exogenous reductant (dithiothreitol, DTT) or oxidant (diamide, DA) for 2 min (Figures 1A' and 1B'). In each case the observed dynamic range between fully reduced and fully oxidized tissues was between 5 and 7 (Figures 1A'' and 1B'').

Tissue-Specific Redox Differences and Changes in Living Larvae

To obtain information about basal redox states and endogenous redox differences between tissues, we performed live imaging of third-instar larvae. We found the cytosolic E_{GSH} probe to be highly reduced throughout the whole larva (Figures 2A and 2A'), close to the lower end of the measuring range, corresponding to -300 mV or less (Meyer and Dick, 2010). There was little variation within and between tissues, as exemplified by measurements on five major tissues (muscle, hemocytes, gut, Malpighian tubules, and fat) (Figures 2A and 2A' and Figure S2A). In contrast, mitochondrial E_{GSH} was clearly heterogeneous within and between different tissues (Figures 2B and 2B'): while gut enterocytes and muscles were consistently found in a highly reduced state, hemocytes, Malpighian tubules, and fat body tissues showed variation toward a much more oxidized mitochondrial glutathione pool (Figures 2B and 2B' and Figure S2B, and representative detailed Figure 2C). The cytosolic and mitochondrial H_2O_2 probes also showed significant variation between tissues (Figures 2D, 2D', 2E, and 2E'). For instance, in both compartments Malpighian tubules exhibited higher H_2O_2 levels than did muscle tissue. Having observed steady-state redox differences in living larvae, we then asked if changes in larval physiology lead to changes in either E_{GSH} or endogenous H_2O_2 levels. To investigate a physiological transition that occurs naturally during larval development, we compared feeding larvae to those that had just stopped feeding and entered the wandering stage. In a highly reproducible manner, the wandering larvae, but not the feeding larvae, exhibited pronounced cytosolic H_2O_2 production within a delimited patch of anterior adipose tissue (Figure 2F, Figures S2C and S2C'). Only minor changes were reported by the mitochondrial H_2O_2 probe and no changes at all by the two E_{GSH} probes (Figures S2D, S2D', and S2E). Together, these observations show that physiological redox differences and changes can be seen in living larvae and that they are distinct in terms of location, tissue type, subcellular compartment, and redox chemistry.

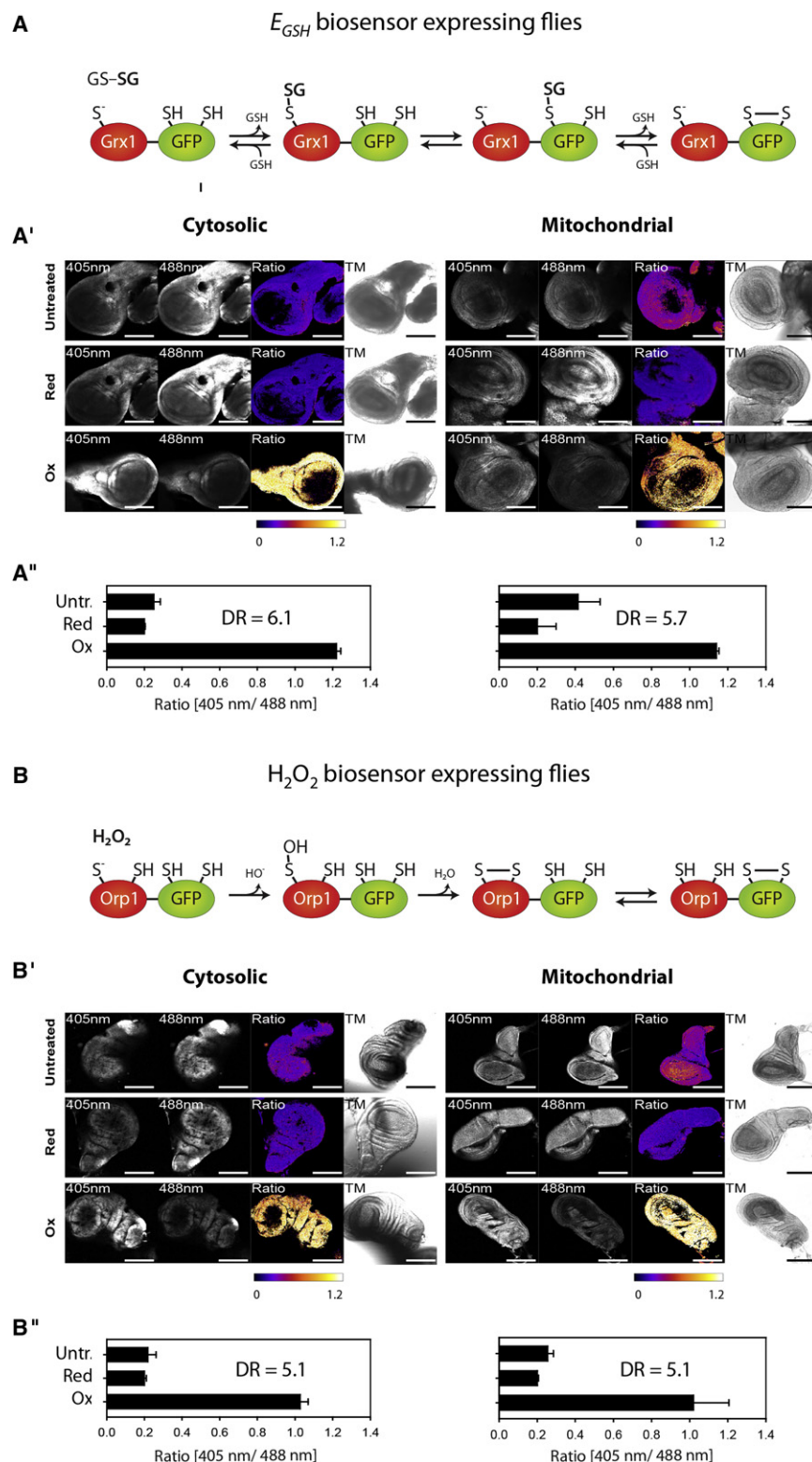


Figure 1. Generation of Biosensor-Transgenic Flies

(A and B) Molecular mechanism of GSSG sensing in Grx1-roGFP2 transgenic flies (A) and of H_2O_2 sensing by roGFP2-Orp1 transgenic flies (B). (A' and B') Transgenic lines expressing E_{GSH} probes (A') or H_2O_2 probes (B') in either the cytosol or the mitochondrial matrix were tested for in situ responsiveness. All four lines responded as expected to exogenously applied reductant (DTT) and oxidant (DA) as shown for larval wing discs of third-instar larvae. Scale bars, 200 μ m (A') and 250 μ m (B'). (A'' and B'') The corresponding dynamic range (DR) is indicated for each probe. Error bars represent the SD of triplicates.

on adult flies. Therefore, we asked if flies can be dissected and their tissues fixed without perturbing the endogenous state of the redox probes. We expected that from the moment of disruption of organismal integrity (i.e., dissection) tissues will progressively show signs of oxidation over time. However, it was fundamentally unclear how fast the process of tissue oxidation would actually proceed after dissection. To address this question, we imaged larvae before, during, and after dissection. Exemplified by the response of cyto-Grx1-roGFP2 in the midgut (Figure 3A), we generally found a very slow but continuous increase in sensor oxidation that became evident about 10 min after the initiation of dissection. A more detailed time course (Figure 3A') showed that the redox state in the intact organism (0 min) does not significantly change during and immediately after dissection. Very similar behavior was recorded for mito-roGFP2-Grx1 and both Orp1-based probes. These observations demonstrated that alterations of the sensor redox state after dissection (i.e., in tissues that are not directly damaged) are not immediate but rather require several minutes to show effect. This suggested to us the opportunity to fully prevent artificial probe oxidation by using fast-acting tissue-permeating alkylating agents during the dissection and fixation processes. We observed previously that thiol alkylation of roGFP-based probes fully conserves their redox state and protects them against subsequent oxidation (Gutscher et al., 2008). We reconfirmed in vitro that

Chemical Conservation of the Biosensor Redox State during Dissection and Fixation

It is important to be able to perform in situ redox measurements not only on the transparent stages of early development but also

N-ethyl maleimide (NEM) is a highly effective roGFP2-thiol-blocking agent (Figure 3B) that acts almost instantaneously (Figure 3C) and also protects against thiol oxidation mediated by short- or long-term incubation with paraformaldehyde (PFA)

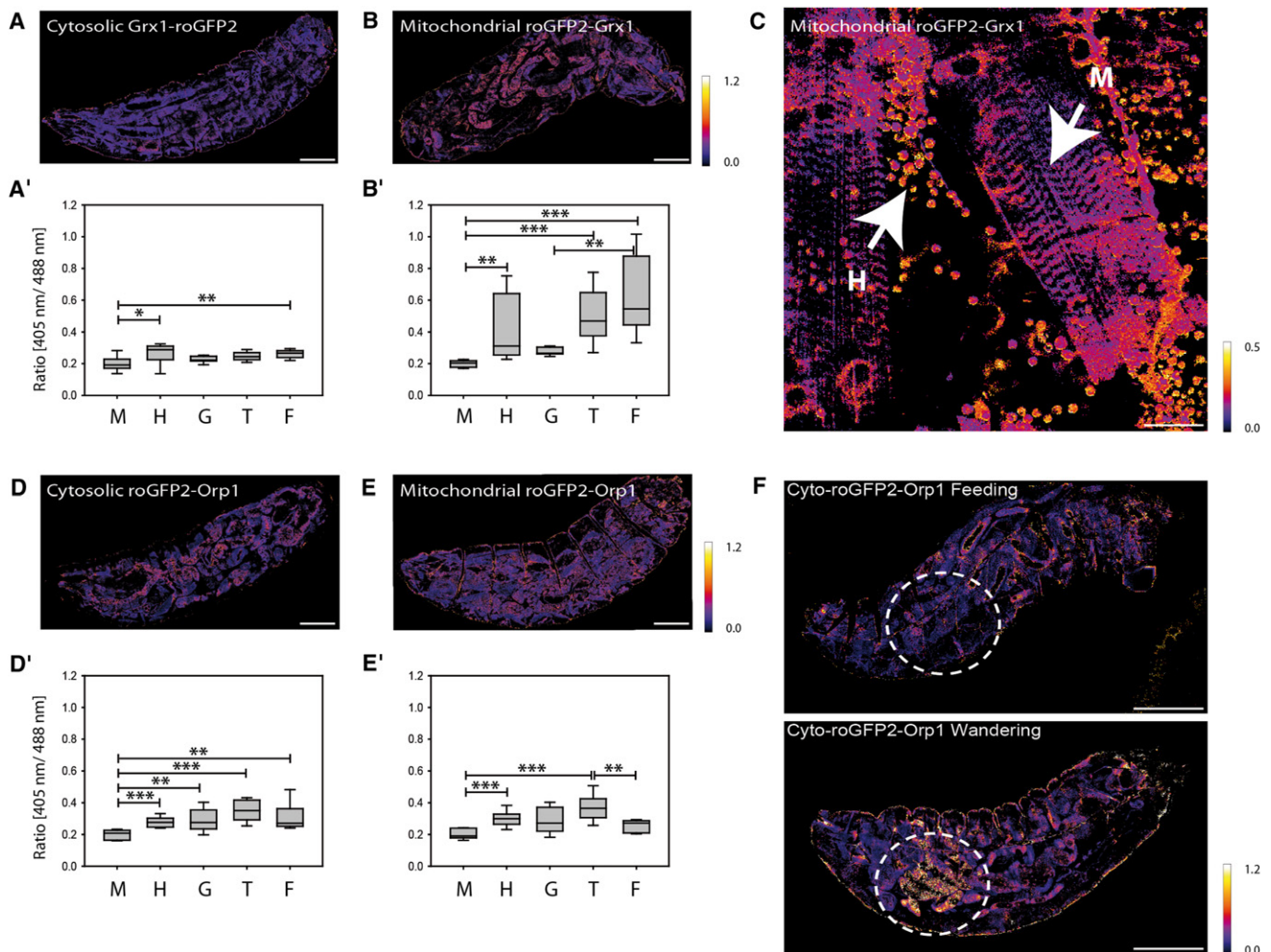


Figure 2. Tissue-Specific Redox Differences and Redox Changes in Living Larvae

(A, B, D, and E) Ratio images of whole intact larvae expressing cyto-Grx1-roGFP2 (A), mito-roGFP2-Grx1 (B), cyto-roGFP2-Orp1 (D), and mito-roGFP2-Orp1 (E). Probe responses in selected tissues were quantified in ten individual third-instar larvae for each biosensor (A', B', D', and E'). Boxes, lower/upper quartile; whiskers, 5th/95th percentile. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. M, muscle; H, hemocytes; G, gut; T, (Malpighian) tubules; F, fat. Scale bars, 600 μm . (C) A representative detailed image to highlight tissue-specific differences evident in mito-roGFP2-Grx1-expressing larvae. Arrows indicate hemocytes (H) and muscle tissue (M). Scale bar, 60 μm . (F) Feeding (top) and wandering (bottom) third-instar larvae expressing the cytosolic roGFP2-Orp1 probe. Scale bars, 600 μm .

(Figure 3D). Protection against PFA-mediated oxidation was also confirmed in cell culture experiments (Figure 3E). NEM was then shown to prevent sensor oxidation during tissue preparation and PFA fixation (Figure 3F), the probe redox state being stable even after challenge with high concentrations of the strong thiol oxidant diamide (Figure 3G). In conclusion, dissection in the presence of at least 2 mM NEM is both necessary and sufficient to prevent oxidation artifacts and to allow study of endogenous redox states in dissected larval and adult flies.

Age-Related Redox Changes in the Adult Fly

To address the question of how tissue redox states differ in adult flies and if tissues display pro-oxidative changes during aging, we used the NEM-based fixation technique described in the previous section to analyze various tissues from flies of different age. On the one hand, we found redox differences that exist in young individuals and remain unchanged with increasing age.

In particular, abdominal fat tissue is segregated into regions of different morphology (Figures S3A and S3B) which show pronounced differences in mitochondrial H_2O_2 levels in both younger (Figure 4A) and older flies (Figure 4B). No corresponding differences were seen with the mitochondrial E_{GSH} probe (Figure 4C and Figure S3B). On the other hand, age-dependent pro-oxidative changes do exist as well; however, they are not uniformly distributed but rather restricted to particular tissues. Most prominent among the examined tissues, midgut enterocytes exhibit a pronounced age-dependent increase in cytosolic H_2O_2 levels (Figures 4D and 4D'). In this case, a corresponding change in cytosolic E_{GSH} is also detectable which is much less pronounced, yet significant (Figure S3C). We conclude that age-related redox changes are highly nonuniform within the organism and that their detection depends critically on the measured redox species/redox pair, the subcellular compartment, and the tissue/cell type investigated.

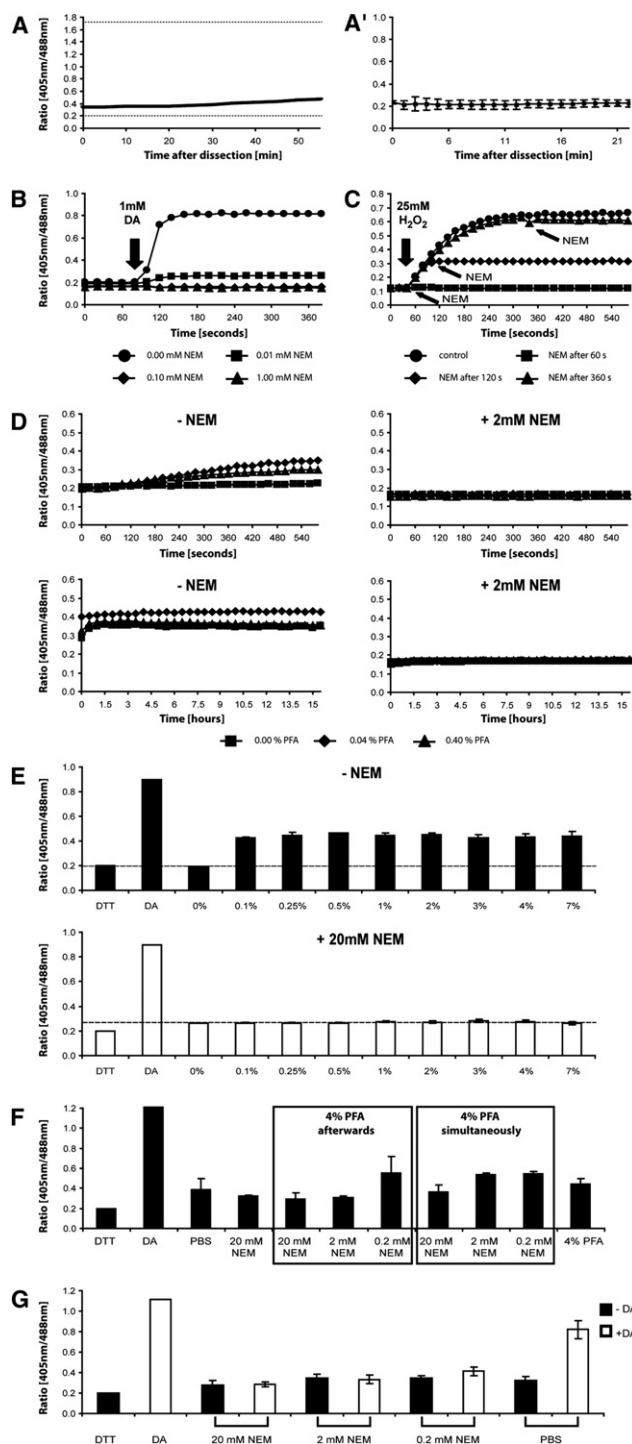


Figure 3. Chemical Conservation of the Biosensor Redox State during Dissection and Fixation

(A) (A and A') The gut of a third-instar larva expressing cyto-Grx1-roGFP2 was dissected in PBS, then imaged over 55 min (A). Reference lines indicate complete probe reduction (ratio, 0.2) and oxidation (ratio, 1.67), as determined by in situ calibration with DTT and DA, respectively. To obtain a more detailed time course for the first minutes following dissection, larval guts expressing cyto-Grx1-roGFP2 were imaged in intact living larvae (0 min) and then in the same larvae every minute up until 22 min after dissection (A'). Error bars represent the SD (n = 3).

Having observed pronounced age-dependent pro-oxidative changes in midgut enterocytes, we asked about their relationship to life span. First, we compared male and female flies. The median life span of males was shorter by 12 days (Figure 4D'). Despite their shorter life span, males exhibited much less H₂O₂ accumulation in the gut (Figures 4D and 4D'). Second, we examined *chico*^{1/+} flies, which carry a mutation in the insulin/IGF signaling pathway that leads to life span extension (Clancy et al., 2001). We confirmed that the heterozygous *chico*^{1/+} genotype leads to an extension of median life span in females (8 days, Figure 4E), yet for males we did not record a significant difference (Figure 4F). We observed that *chico*^{1/+} females showed an accelerated accumulation of cytosolic H₂O₂ in the gut (Figure 4E), while *chico*^{1/+} males showed a delayed accumulation (Figure 4F). Thus, the increase in female life span afforded by *chico*^{1/+} was accompanied by increased rather than decreased formation of oxidants. In conclusion, the generation of H₂O₂ in gut enterocytes does not seem to be a factor in the limitation of life span.

In Vivo Redox Changes Caused by Gene Silencing

To explore the possibilities of combining redox imaging with reverse genetics, we created flies expressing biosensors ubiquitously under the tubulin promoter together with dsRNA constructs expressed specifically in the posterior compartment of the wing disc using engrailed-GAL4. This procedure allows imaging of redox differences side by side between the posterior compartment of the wing disc where a gene of choice is knocked down, compared to the control anterior compartment. Gene silencing of thioredoxin reductase (TrxR) led to increased H₂O₂ levels and increased glutathione oxidation in both the cytosol and mitochondria, as measured with the corresponding probes (Figure 5A). This result is expected, because in *Drosophila* TrxR also acts as the main glutathione reductase (Kanzok et al., 2001) and is targeted to both cytosol and mitochondria (Missirlis et al., 2002). Silencing of ND42, a component of respiratory complex I, led to increased mitochondrial, but not cytosolic,

(B) Reduced recombinant Grx1-roGFP2 was incubated with different NEM concentrations for 80 s and subsequently challenged with 1 mM DA.

(C) H₂O₂ (25 mM) was injected into a solution of reduced recombinant Grx1-roGFP2 after 40 s. NEM (20 mM) was injected after 40, 100, or 340 s.

(D) Different PFA concentrations were injected after 120 s to a solution of reduced Grx1-roGFP2 which was preincubated with either PBS (left panels) or 2 mM NEM (right panels). The Grx1-roGFP2 redox state was monitored for 10 min (upper panels) and for 16 hr following PFA addition (lower panels).

(E) HeLa cells expressing Cyto-Grx1-roGFP2 were preincubated with PBS (upper panel) or 20 mM NEM (lower panel) for 5 min and consecutively fixed with various concentrations of PFA (0%–7%). The resulting redox state of Cyto-Grx1-roGFP2 was analyzed by flow cytometry. The reference lines indicate the ratio of the non-PFA treated control. Data are means ± SD (n = 2).

(F) Freshly dissected guts expressing cytosolic Grx1-roGFP2 were treated with different NEM concentrations (0.2, 2, and 20 mM) for 10 min. PFA (4%) was applied simultaneously with NEM or afterwards. The resulting in situ redox state of the probe was determined by microscopy. Data are means ± SD (n = 3).

(G) Larvae were dissected in the presence of different concentrations of NEM (20, 2, 0.2, and 0 mM). Subsequently, prepared guts were challenged with 5 mM DA to quantify the degree of protection against probe oxidation. The resulting in situ redox state of the probe was determined by microscopy. Data are means ± SD (n = 3).

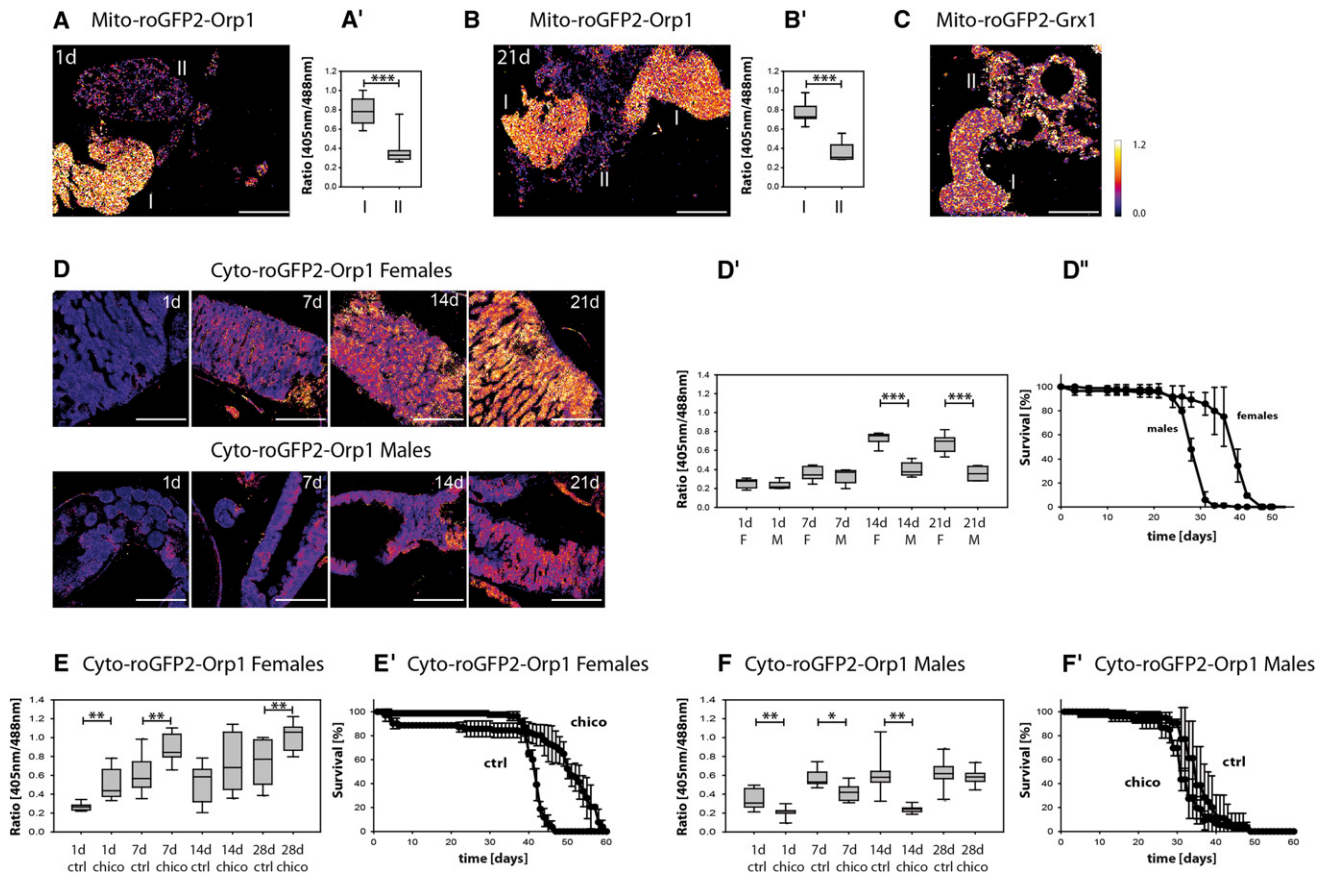


Figure 4. Age-Related Redox Changes in the Adult Fly

(A–C) Fat body tissue of young (A) and old flies (B) expressing mito-roGFP2-Orp1 shows significant age-independent heterogeneity in mitochondrial H_2O_2 levels. No corresponding E_{GSH} difference is reported by the mito-roGFP2-Grx1 probe (C). The two types of fat tissue (labeled I and II) are indicated. Scale bars, 75 μm . *** $p < 0.001$. The false color scale applies to all ratio images in the figure.

(D) Comparison of midgut enterocytes in young (1 day), middle-aged (7 and 14 days), and old flies (21 days) expressing cyto-roGFP2-Orp1 shows a significant age-dependent increase in cytosolic H_2O_2 levels, with females showing a stronger accumulation than males. Quantification using five flies per age and gender (D'), and corresponding life span curve (D''). Scale bars, 150 μm . Boxes, lower/upper quartile; whiskers, 5th/95th percentile. *** $p < 0.001$.

(E and F) Comparison of the age-dependent increase of H_2O_2 levels in gut enterocytes of wild-type and *chico*^{1/+} females (E), and wild-type and *chico*^{1/+} males (F) (1, 7, 14, and 28 days), using ten flies per age for quantification. Boxes, lower/upper quartile; whiskers, 5th/95th percentile. * $p < 0.05$, ** $p < 0.01$. Life span curve of wild-type and *chico*^{1/+} females (E'), and of wild-type and *chico*^{1/+} males (F').

glutathione oxidation (Figure 5B). In contrast, silencing of Jafrac1, the major cytosolic 2-Cys peroxiredoxin, did not show any effects in the posterior wing disc (Figure 5C). However, when Jafrac1 was silenced ubiquitously, using tubulin-GAL4, highly tissue-specific effects were observed, in particular cytosolic accumulation of H_2O_2 and GSSG in the Malpighian tubules (Figures 5D and 5D'). To a lesser extent, mitochondria were also affected (Figure S4). This suggests that silencing of Jafrac1 increases steady-state H_2O_2 levels only in certain tissues and/or physiological conditions. We conclude that redox imaging can be readily combined with genetic approaches to explore the role of gene products on cellular redox states throughout the organism.

In Vivo Redox Changes Caused by Pharmacological Agents

Biosensor transgenic flies offer the opportunity to examine the in vivo influence of pharmacological agents considered to act

as oxidants or antioxidants. As an example, we fed larvae with antimycin A, an inhibitor of respiratory complex III, and observed the larval response by live imaging. As expected, in various tissues throughout the larva antimycin A induced mitochondrial (Figure 6A'), but not cytosolic (Figure 6A), probe oxidation. While antimycin A is well understood as an inducer of mitochondrial oxidants, it is much less established if or how small molecule antioxidants influence tissue redox states in vivo. We therefore examined the effect of NAC, one of the most widely used compounds, on larvae and adult flies. Given the common notion that NAC acts as a generic antioxidant, we examined larvae expressing TrxR dsRNA under the engrailed promoter (compare Figure 5A) and asked if NAC feeding would mitigate the oxidative load in the posterior wing disk. However, no significant influence could be detected with either of the two cytosolic probes (Figures S5A and S5A'). We then looked for redox changes in whole larvae by live imaging and also included the

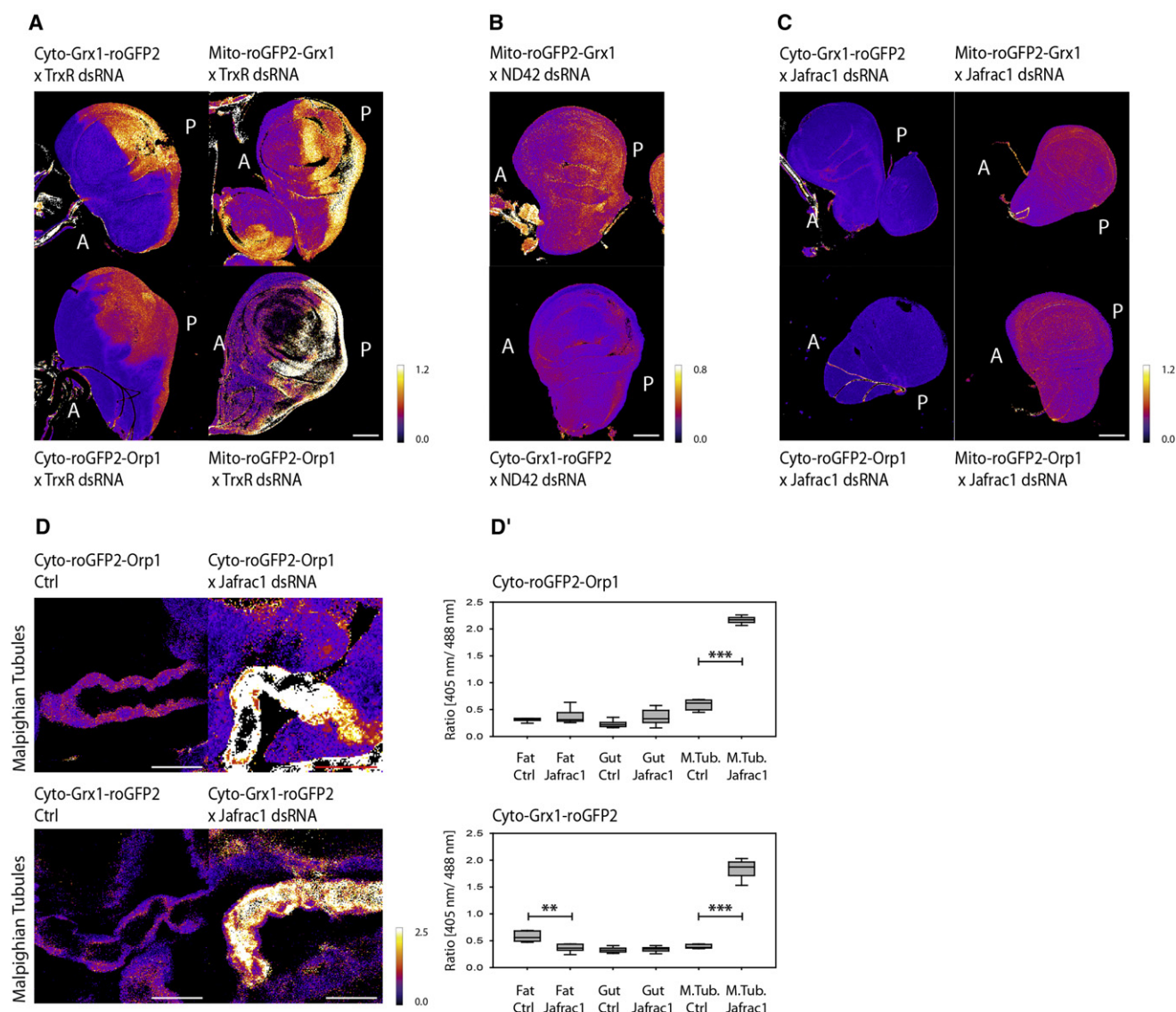


Figure 5. In Vivo Redox Changes Caused by Gene Silencing

(A) Silencing of TrxR in the posterior (P) compartment of the wing disc mediates a pro-oxidative response of all four probes relative to the anterior (A) compartment. Scale bar, 150 μ m.

(B) Silencing of complex I subunit ND42 leads to mitochondrial but not cytosolic glutathione oxidation in the posterior part of the wing disc. Scale bar, 150 μ m.

(C) Silencing of Jafrac1 in the posterior (P) compartment of the wing disc does not lead to measurable redox changes. Scale bar, 150 μ m.

(D) Ubiquitous silencing of Jafrac1 leads to increased cytosolic H_2O_2 and elevated E_{GSH} in Malpighian tubules (M. Tub.). Quantification using five larvae per condition (D'). Scale bars, 75 μ m. Boxes, lower/upper quartile; whiskers, 5th/95th percentile. ** $p < 0.01$, *** $p < 0.001$.

mitochondrial probes. The only significant ($p < 0.01$) NAC-dependent change was an increase in mitochondrial H_2O_2 in various tissues (exemplified for fat and gut tissue in Figure 6B'). The only possible indication of an antioxidant effect, a lowering of cytosolic H_2O_2 levels in the gut (Figure 6B), was of marginal significance ($p = 0.05$). We also extended the NAC feeding experiments to adult flies, which did not show significant changes, except for increased mitochondrial H_2O_2 and GSSG in the Malpighian tubules (Figure S5B). In conclusion, under the conditions tested we did not find convincing evidence for an in vivo antioxidant activity of NAC.

DISCUSSION

In this study we used enzyme-coupled roGFP2-based probes to visualize the in vivo distribution of chemically defined redox species in a transgenic model animal. A key advantage of using genetically encoded redox probes is that they can be precisely targeted to distinct subcellular compartments. Furthermore, ratiometric probes have the benefit of being less prone to a range of common measurement artifacts. Among the genetically encoded ratiometric redox probes, the fluorescent reporter roGFP2 stands out, because its ratiometric signal is insensitive

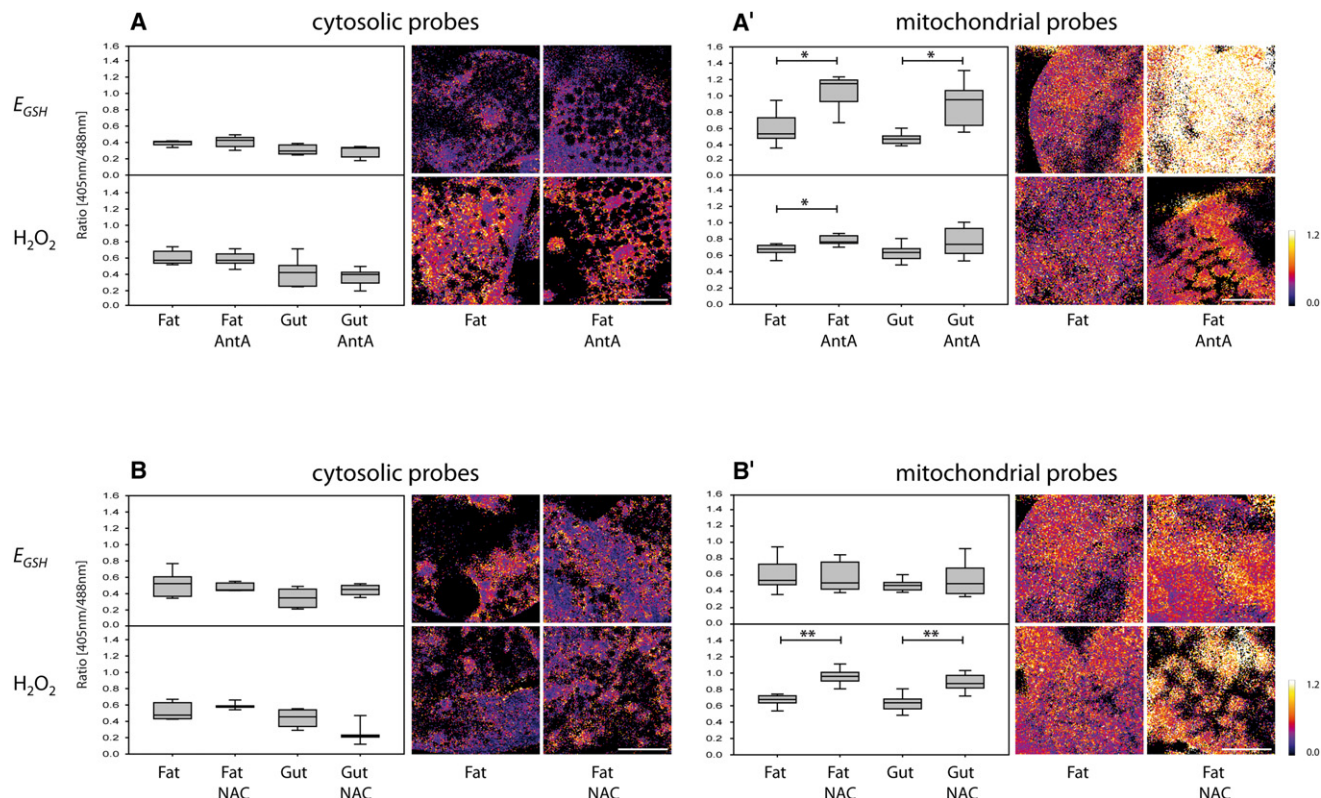


Figure 6. In Vivo Redox Changes Caused by Pharmacological Agents

(A) (A and A') Live imaging of third-instar larvae that were fed with 10 $\mu\text{g}/\text{mL}$ antimycin A for 3 hr. Quantification of all four probes in two representative tissues (fat and gut) using five larvae per condition. Representative images of fat tissue responses. Scale bars, 50 μm . Boxes, lower/upper quartile; whiskers, 5th/95th percentile. * $p < 0.05$.

(B) (B and B') Live imaging of third-instar larvae that developed from hatching to the L3 stage with food containing 0.1 mg/mL NAC. Quantification of all four probes in two representative tissues (fat and gut) using five larvae per condition. Representative images of fat tissue responses. Scale bars, 50 μ m. Boxes, lower/upper quartile; whiskers, 5th/95th percentile. * $p < 0.05$.

to pH in the range between 5.5 and 8.5 (Schwarzlander et al., 2008). Moreover, the response of roGFP2 can be made highly specific for chemically defined oxidants by application of a modular design principle that associates different redox species with the same fluorescent reporter: the oxidoreductase (Grx1)-based probe specifically and reversibly reports roGFP2 oxidation by GSSG, and the peroxidase (Orp1)-based probe specifically and reversibly reports roGFP2 oxidation by H_2O_2 . This concept allows comparison of different redox species in different subcellular compartments within the same transgenic system. We find that the two probes show distinct response patterns in vivo, underlining the critical role of the coupled redox enzyme in mediating a sensitive and specific response.

An important step in our study was the establishment and validation of a chemical blocking procedure to prevent artificial oxidation of the redox probes during specimen dissection and fixation, thereby expanding the scope of redox imaging to include the otherwise optically inaccessible parts of the model organism. The procedure is effective because the redox species under consideration (H_2O_2 and GSH/GSSG) do not change immediately upon dissection, but rather it takes several minutes until measurable effects take place. This time window can be exploited to trap the probe redox state with the alkylating agent

NEM, which is applied during dissection and permeates exposed tissues within seconds. A combined alkylation-fixation technique thus allows in situ analysis of the in vivo redox state in tissues otherwise inaccessible for direct optical imaging. Thus, the in vivo measurements reported in this study are based on a unique combination of probe features: genetic encoding with specific subcellular targeting, defined oxidant specificity, amenability to instant trapping of the redox state, and pH-insensitive ratiometric reporting.

The almost uniform probe expression obtained under a tubulin promoter allowed us to make a number of general biological observations which have implications regarding the robustness of redox homeostasis, redox compartmentalization, and the relationship between H_2O_2 and E_{GSH} . Throughout the living organism, the cytosolic E_{GSH} is very close to the lower end of the roGFP2 measuring range, which corresponds to a glutathione redox potential between -300 and -320 mV (Meyer and Dick, 2010). This value is very similar to that obtained for other eukaryotes, including yeast (Braun et al., 2010; Ostergaard et al., 2004), *Arabidopsis* (Meyer et al., 2007), and human cells (Gutscher et al., 2008). It is therefore likely that a cytosolic steady-state E_{GSH} in this range is a general feature of eukaryotes, possibly because a less negative potential would drive toxic levels of

cytosolic protein thiol oxidation (Lopez-Mirabal and Winther, 2007). We observed very little variation of cytosolic E_{GSH} in either larvae or in young or old adults. Thus, cytosolic glutathione redox homeostasis in vivo appears to be very robust and stably maintained with increasing age. The stability of cytosolic E_{GSH} in living *Drosophila* contrasts with the observation that cultured mammalian tumor cells show long-lived pro-oxidative changes in cytosolic E_{GSH} depending on cell density, nutrient availability, and other factors (Gutscher et al., 2008). These observations may indicate that cytosolic glutathione redox homeostasis is more stable or restricted in vivo than it is in a cell culture system under ex vivo conditions. However, in contrast to cytosolic E_{GSH} , mitochondrial E_{GSH} was found to be much more variable and dynamic, likely reflecting the fact that mitochondria are major generators of endogenous oxidants (Murphy, 2009). The observed in vivo differences between cytosolic and mitochondrial E_{GSH} are also compatible with the notion that cytosolic and mitochondrial glutathione pools are independently regulated (Hu et al., 2008).

The comparison between Grx- and Orp-based probes also supports the conjecture that changes in E_{GSH} and H_2O_2 levels are not necessarily coupled and concurrent. For example, the high levels of mitochondrial H_2O_2 within patches of adult adipose tissue do not translate into any measurable increases in mitochondrial E_{GSH} (Figures 4A–4C). Likewise, the local increase in cytosolic H_2O_2 that was evident in wandering larvae (Figure 2F) was not associated with a change in cytosolic E_{GSH} (Figure S2E). The explanation may be that in these situations H_2O_2 is predominantly metabolized by thioredoxin-coupled peroxidases, rather than being converted to GSSG. Alternatively, it may be that GSSG is so efficiently reduced (or excreted) that cytosolic E_{GSH} is maintained despite increased rates of glutathione oxidation. Taken together, it seems that H_2O_2 measurements do not necessarily inform about the state of the glutathione system and vice versa. Accordingly, different redox species likely convey different kinds of biological information, and this may hold true for most other oxidant species and redox couples, including $O_2^{\cdot -}$, NO, and $NAD^+/NADH$, suggesting that one needs to measure all these species separately and specifically with cellular and subcellular compartment resolution.

Knowledge about defined oxidants in defined locations should help to address the complex relationships between oxidants and various other phenomena. A controversial issue is the nature of the relationship between oxidants and aging. It is generally believed that pro-oxidative changes occur during aging. Redox measurements performed on whole flies support the idea, for both GSH/GSSG (Rebrin et al., 2004) and H_2O_2 (Cocheme et al., 2011). However, it is not clear if such changes occur over the whole organism or if they are restricted to particular tissues, regions, and cell types. Our approach allows the identification of tissues, cells, and subcellular compartments which undergo oxidation during aging in vivo. For *Drosophila* we have found that age-dependent pro-oxidative changes are highly restricted to particular locations, with the most prominent change being an increase of cytosolic H_2O_2 in midgut enterocytes. Based on this observation, we asked if and how the rate of H_2O_2 accumulation would be different in flies exhibiting a different life span. In two separate experiments (*chico*^{1/+} females versus control females, and control females versus

control males), increased life span was accompanied by increased formation of oxidants, rather than by a decrease. Thus, in this particular setting our results do not support the idea that aging-related oxidative processes limit life span. It is nevertheless likely that the oxidative phenomena we observe are causally connected to some biological function, possibly to an innate immune response (Ha et al., 2005), driven by an increase in bacterial load in the gut known to accompany aging without life span trade-off (Ren et al., 2007). In this case the observed increase in H_2O_2 levels in enterocytes would probably represent an epiphenomenon of aging rather than a driving force for the aging process. It is generally conceivable that age-related oxidant increases are mostly or largely epiphenomena (Gems and Doonan, 2009).

To proceed beyond correlations and to uncover causal relationships and biological meaning, it will be important not just to observe and quantify particular redox changes but also to manipulate (i.e., prevent/enhance) them in vivo, in a highly specific manner. The approach presented here allows the quantification of the actual in vivo influence of pharmacological or genetic interventions on local H_2O_2 levels or E_{GSH} . This information is crucial for the development of strategies to specifically manipulate in vivo redox states. However, our results also suggest that truly specific and predictable in vivo redox manipulations may be much more difficult to achieve than commonly acknowledged. For instance, silencing of the major cytosolic 2-Cys peroxiredoxin Jafra-1 had a pro-oxidative effect in some tissues but not in others. Moreover, NAC did not show obvious antioxidative effects in vivo, but rather induced elevated mitochondrial oxidant production in various tissues. Our experiments suggest that the widely held assumption that NAC acts as a generic antioxidant should be treated with caution. Nevertheless, it should be noted that measurements of E_{GSH} do not exclude the possibility that NAC increased total cytosolic GSH levels, and it is furthermore possible that NAC acts as a thiol redox modulator without acting as an antioxidant (Murphy et al., 2011; Parasassi et al., 2010). Taken together, the in vivo effects caused by genetic manipulation of individual redox enzymes or by treatment with small molecule antioxidants may not always meet expectations and may differ widely depending on tissue type, metabolic context, and other factors. Thus, it seems important to clarify if a chosen antioxidant actually reacts with the relevant oxidant in the relevant location inside the organism. Conversely, if an antioxidant shows a biological effect, it remains to be shown that it really lowers oxidant levels rather than acting through other mechanisms. It seems that a significant amount of work, exploiting in vivo probes, will be required before individual oxidant species and redox pairs can be specifically manipulated in vivo with some confidence. In conclusion, many of the observations we report could not have been predicted a priori and therefore highlight the importance of monitoring redox states in vivo.

EXPERIMENTAL PROCEDURES

Genetics

Transgenic *Drosophila melanogaster* were generated by germline injection into a w¹¹¹⁸ strain. Flies were raised at 25°C on standard medium. Gal4 driver strains were obtained from Bloomington (enGal4 #30564, tubGal4 #5138). In vivo gene knockdowns were performed using dsRNA lines from the Vienna

Drosophila RNAi Center (TrxR, GD 47308; ND42, GD 14444; Jafrac1, KK 109514). For dsRNA-mediated knockdown in the posterior wing disc and ubiquitous sensor expression, enGal4 (II); Tubulin-Sensor (III) lines were crossed with UAS-dsRNA flies. Ubiquitous sensor expression and ubiquitous Jafrac1 knockdown was achieved in flies simultaneously bearing Tubulin-GAL4, UAS-Sensor, and UAS-Jafrac1-dsRNA.

Aging Experiments

All aging experiments were performed at 29°C. To avoid influences of markers or balancers, biosensor-expressing males were crossed to w^{1118} virgins to obtain progeny heterozygous for the biosensor. First-instar larvae were collected from apple agar plates (20% apple juice, 80% PBS, 1% agar, 0.23% Tegosept) and transferred to vials containing food to ensure simultaneous eclosion. Male and female flies were housed together for the first 24 hr after eclosion. Subsequently, males and fertilized females were collected and housed separately. The flies were flipped every 2–3 days. To analyze flies of different ages (1, 7, 14, and 21 days) on the same day for side-by-side comparisons under identical microscopy conditions, separate populations of aging flies were set up under identical conditions with corresponding time delays. For aging experiments with *chico*^{1/+} flies, biosensor-expressing males were crossed to w^{1118} or *chico* virgin females to obtain sensor/+ or sensor/*chico*¹ flies, respectively. One-, seven-, fourteen-, and twenty-eight-day-old flies were compared side-by-side with the same microscope settings.

Feeding of Third-Instar Larvae and Adult Flies with Antimycin A and NAC

To study the influence of antimycin A, third-instar larvae were removed from the food vial, washed briefly with PBS, and transferred to a vial containing food supplemented with 10 µg/mL antimycin A. After 3 hr, living larvae were imaged as described below. To study the influence of NAC, egg-laying adult females were transferred to vials containing food with either 0.1 mg/mL or 1 mg/mL NAC. Third-instar larvae developing in the presence of 0.1 mg/mL NAC were analyzed by live imaging as described below. Likewise, third-instar larvae expressing UAS-TrxR-dsRNA with enGal4 were allowed to develop in food containing 1 mg/mL NAC. Wing discs were prepared and imaged immediately as described below. In addition, freshly eclosed adult flies were housed for 48 hr with food containing 1 mg/mL NAC, followed by dissection and fixation as described below.

Live Imaging of Third-Instar Larvae

Living third-instar larvae were removed from the food vial, cleaned briefly with PBS, and gently fixed with adhesive tape on a cover glass. Larvae were imaged immediately using a Leica TCS SP5 AOBS confocal microscope system (Leica Microsystems) with a HCX PL APO lambda blue 20× 0.7 NA objective (oil immersion) and Leica LAS AF software. Excitation of biosensor fluorescence by the 405 nm and 488 nm laser lines was performed sequentially and line by line. Emission was detected at 500–570 nm. For calibration of the biosensor response, larvae were subsequently dissected in PBS and salivary glands incubated with 10 mM DTT (5') or 1 mM DA (2') at room temperature, respectively. The ratio of fully reduced sample was set to 0.2.

Imaging of Freshly Dissected Tissue

To examine larval organs, e.g., the wing disc or salivary glands, organs were freshly dissected in PBS and transferred immediately to a Zeiss LSM 710 ConfoCor 3 microscope equipped with an EC Plan N DICl 40× 1.3 NA objective (oil immersion). Probe fluorescence was excited sequentially at 405 and 488 nm (line by line) and detected at 500–530 nm. TMRM staining was visualized by excitation at 561 nm and by emission at 580–650 nm. For freshly dissected wing discs, a 10× EN Plan N DICl 10×/0.3 NA dry objective was used. To fully reduce and oxidize the tissue, the dissected organs were incubated in 10 mM DTT (5') or 1 mM DA (2'), respectively. For consistency, we set the ratio of fully reduced organs to 0.2.

Conservation of the Biosensor Redox State with NEM

To conserve the redox state of roGFP2-based biosensors in dissected and fixed tissue, all dissections were performed in the presence of 20 mM NEM. Adult flies were briefly anaesthetized with N₂ and decapitated. The abdomen was cut and opened immediately in the presence of 20 mM NEM. All samples

were further incubated with NEM for 10 min at RT. Remaining NEM was removed by rinsing once with PBS. Afterward, samples were fixed with 4% PFA for 15 min at RT. Remaining PFA was removed by washing twice with PBS for 10 min. Samples were equilibrated in glycerol mounting medium (80% glycerol, 0.4% N-propyl gallate in PBS) overnight at 4°C and mounted the next day. Samples were stored horizontally at 4°C. For probe calibration, parallel measurements (side by side, keeping the exact same microscope settings) were performed on specimens that were fully reduced or fully oxidized (20 mM DTT or 2 mM DA for 10 min) during dissection and before being blocked with NEM. This allows determination of the in situ dynamic range and to infer the degree of oxidation of the probe in the actual samples.

Imaging of Fixed Tissue

Fixed tissues were imaged with a Zeiss LSM 710 ConfoCor 3 confocal microscope equipped with an EC Plan N DICl 40×, 1.3 NA objective (oil immersion). Probe fluorescence was excited sequentially at 405 and 488 nm (line by line) and detected at 500–530 nm. Nuclear stain To-Pro3 was excited at 633 nm and detected at 650–700 nm.

Image Processing

Images were saved as 16-bit tif files and processed by ImageJ. Background was subtracted using the rolling ball procedure set to 50 pixels. Pictures were then converted to 32-bit format. The intensities of the 488 nm image were thresholded, and values below threshold were set to “not a number” (NaN). A ratio image was created by dividing the 405 nm image by the 488 nm image pixel by pixel and displayed in false colors using the lookup table “Fire.”

In Vitro Experiments

Recombinant Grx1-roGFP2 and roGFP2-Grx1 were expressed in the *E. coli* strain BL21 (Stratagene) and purified via hexahistidine affinity chromatography. Purified recombinant proteins were desalted using Slide-a-Lyzer dialysis cassettes (Pierce). For measurements in a FLUOstar Omega platereader (BMG Labtech), the proteins were diluted into a degassed standard reaction buffer (100 mM phosphate, 5 mM EDTA [pH 7.0]) to a final concentration of 1 µM. RoGFP2 emission was detected (505–515 nm) after excitation at 390 and 480 nm. The ratio 390 nm/480 nm was calculated and plotted against time. For oxidation experiments, roGFP2 fusion proteins were first reduced with 10 mM DTT for 20 min on ice and desalted with Zeba Desalt spin columns (Pierce). To compare between experiments, the ratio of fully reduced protein was set to 0.1.

Cell Culture and FACS Analysis

HeLa cells were grown in DMEM (GIBCO) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (GIBCO). HeLa cells stably expressing Grx1-roGFP2 were generated by retroviral transduction and subsequent selection with 0.5 mg/ml puromycin. For FACS analysis, cells were pretreated with 20 mM NEM for 5 min at RT, washed once with PBS, and incubated for 5 min with different PFA concentrations. After washing twice with PBS, cells were stored at 4°C. We used a BD FACSAria flow cytometer equipped with 407 and 488 nm lasers for excitation and detected the emission with a standard GFP filter (515–545 nm).

Statistical Analysis

For multiple comparisons, data points were analyzed with one-way analysis of variance (ANOVA), in conjunction with the Tukey range test. To obtain p values for pairwise comparisons, a two-sided heteroscedastic t test was performed. Experimental data were visualized by box plot, showing lower quartile (bottom of box), upper quartile (top of box), median (band near the middle of the box), the fifth percentile (lower end of whisker), and the ninety-fifth percentile (upper end of whisker). Stars indicate p values as obtained from pairwise (t test) comparisons: *p < 0.05; **p < 0.01; ***p < 0.001.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.cmet.2011.10.010.

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Supplemental Information

In Vivo Mapping of Hydrogen Peroxide and Oxidized Glutathione Reveals Chemical and Regional Specificity of Redox Homeostasis

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Inventory of supplemental items

1. Figure S1 - relates to main Figure 1
2. Figure S2 - relates to main Figure 2
3. Figure S3 - relates to main Figure 4
4. Figure S4 - relates to main Figure 5
5. Figure S5 - relates to main Figure 6
6. Supplemental Experimental Procedures
7. Supplemental References

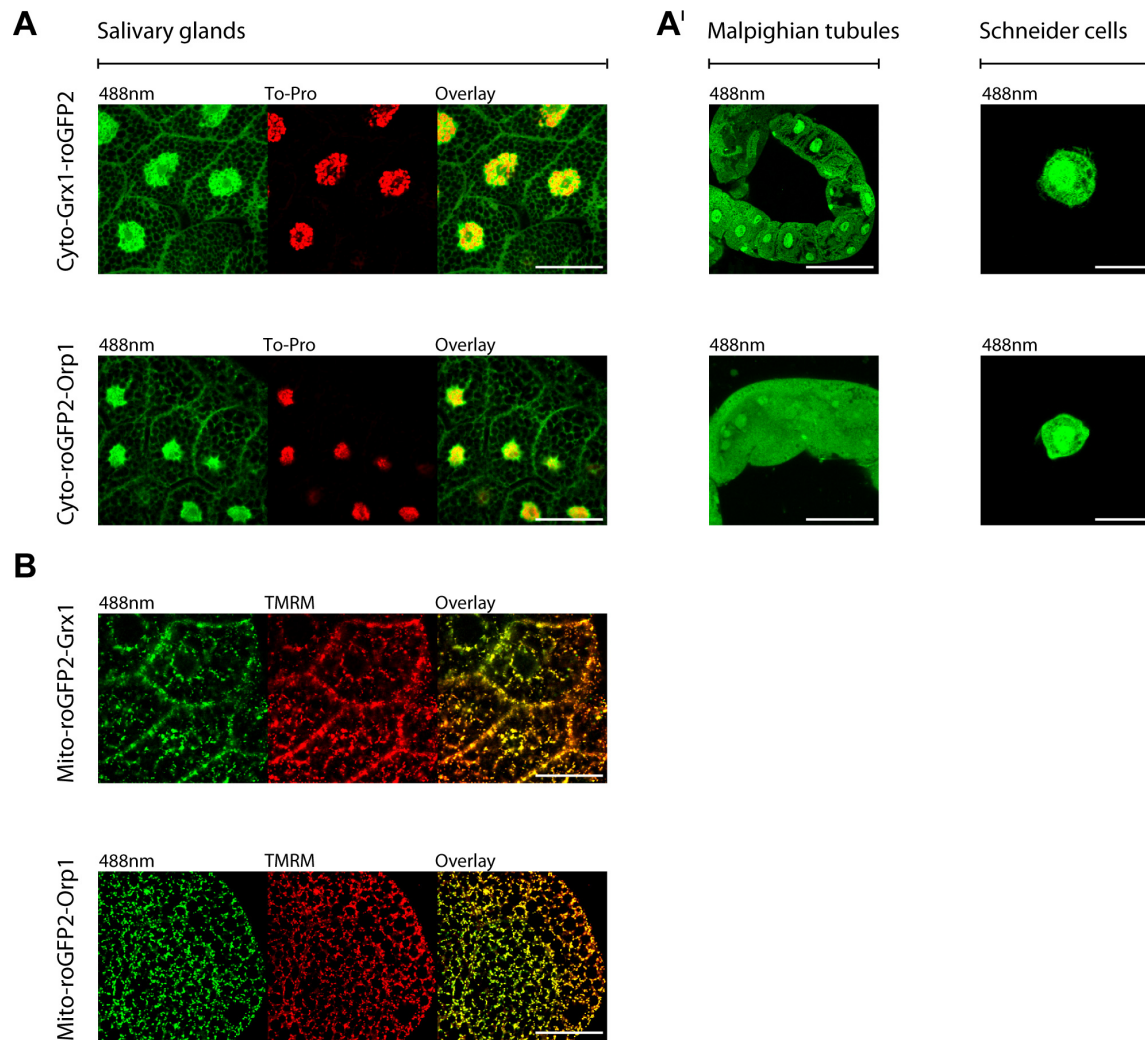


Figure S1: Subcellular localization of biosensors. (A) The cytosolic probes demonstrate nucleo-cytoplasmic localization. Imaging of salivary glands from third instar larvae reveals a reticular pattern that is typical for the cytosolic space of late L3 stage salivary gland epithelial cells. It is caused by densely packed mucin-containing granules occupying most of the cytoplasmic space, thus giving it a reticular appearance. Nuclei are counterstained with red-fluorescent To-Pro-3. Scale bars: 100 μ M. (A') Nucleo-cytoplasmic expression in Malpighian tubules (scale bars: 100 μ M) and Schneider cells (scale bars: 10 μ M). (B) The mitochondrially targeted probes as imaged in salivary glands of transgenic larvae demonstrate co-localization with TMRM. Scale bars: 100 μ M.

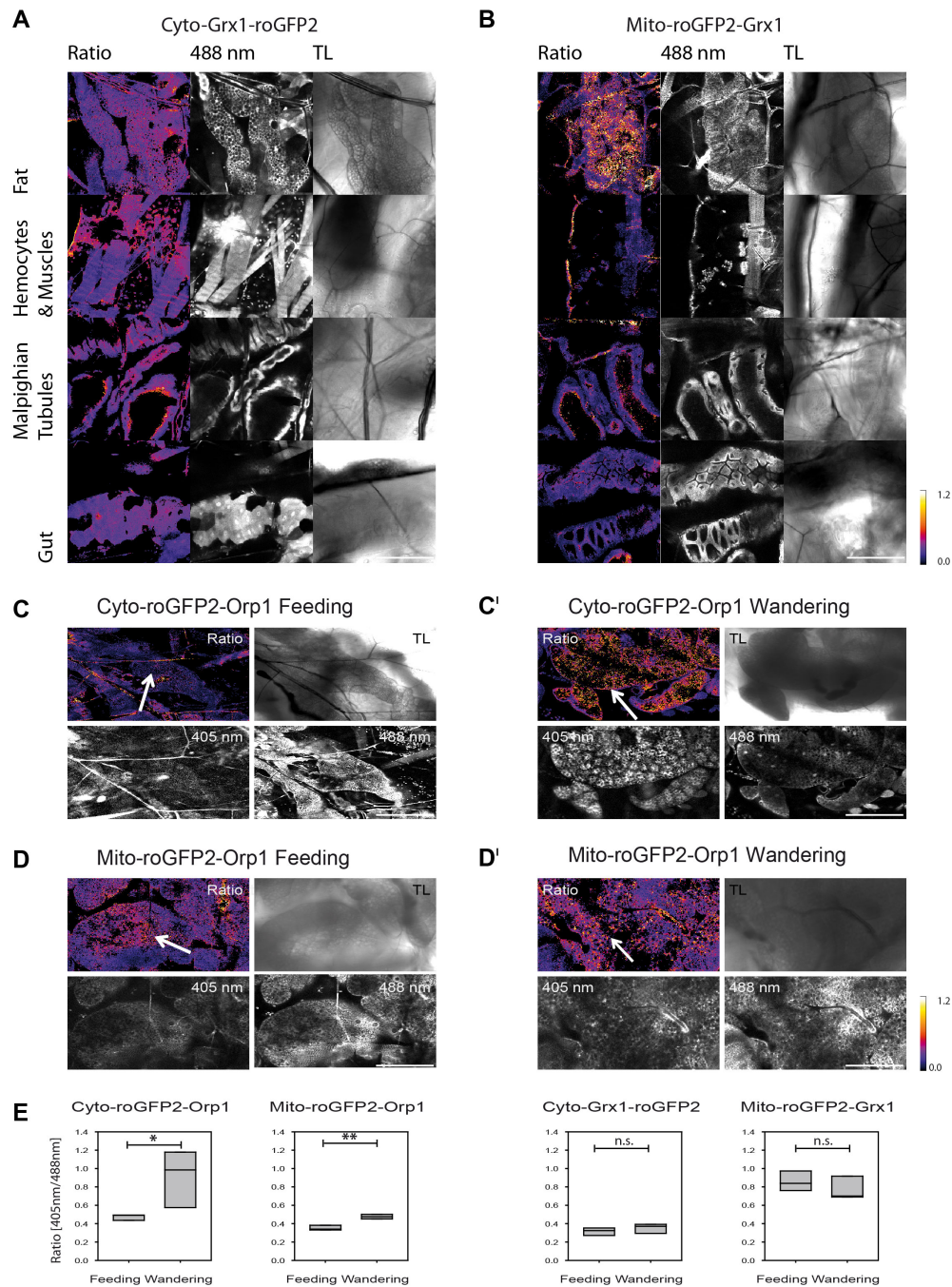


Figure S2: Representative images for the identification of tissues in intact third instar larvae. Representative images of tissues expressing either cyto-Grx1-roGFP2 (A) or mito-roGFP2-Grx1 (B): fat tissue, hemocytes and muscles, Malpighian tubules and gut. Left panels: 405 nm/488 nm ratio; middle panels: 488 nm excitation single channel; right panels: transmitted light (TL). Scale bars: 300 μ M. Anterior adipose tissue in feeding (C, D) and wandering larvae (C', D') was imaged for cytosolic (C, C') and mitochondrial H_2O_2 responses (D, D'). Fat tissue is indicated by arrows. Upper left panels: 405 nm/488 nm ratio, lower panels: 405 nm and 488 nm excitation single channels; upper right panels: transmitted light (TL). Scale bars: 120 μ M. (E) Quantification using 3 wandering and 3 feeding larvae per sensor. Boxes: lower/upper quartile. * $p < 0.05$, ** $p < 0.01$.

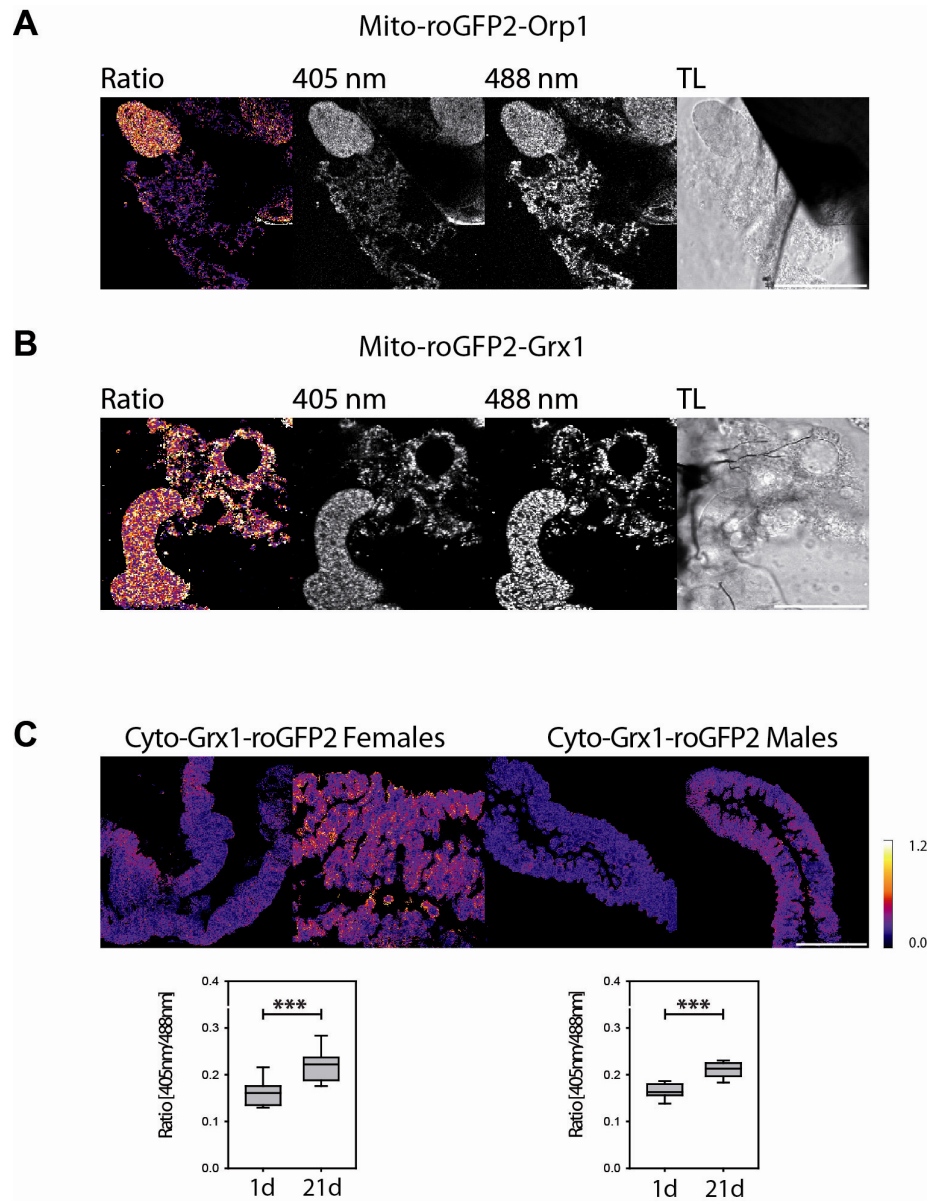


Figure S3: Distinct redox states in two types of abdominal fat tissue with different morphology (A, B) and a weak age-related increase in cytosolic E_{GSH} in gut enterocytes (C). Representative images of dissected adult fly fat tissue expressing mito-roGFP2-Orp1 (A) or mito-roGFP2-Grx1 (B). Left panels: 405 nm/488 nm ratio; middle left panels: 405 nm excitation single channel; middle right panels: 488 nm excitation single channel; right panels: transmitted light (TL). Scale bars: 75 μ M. Comparison of midgut enterocytes between young (1 d) and old flies (21 d) expressing cyto-Grx1-roGFP2 (C). An age-dependent increase in cytosolic E_{GSH} is evident for both females (left) and males (right). Quantification (bottom panels) using 15 flies per age and gender. Boxes: lower/upper quartile, whiskers: 5th/95th percentile. *** $p < 0.001$. The false colour scale applies to all six ratio images. Scale bars: 150 μ M.

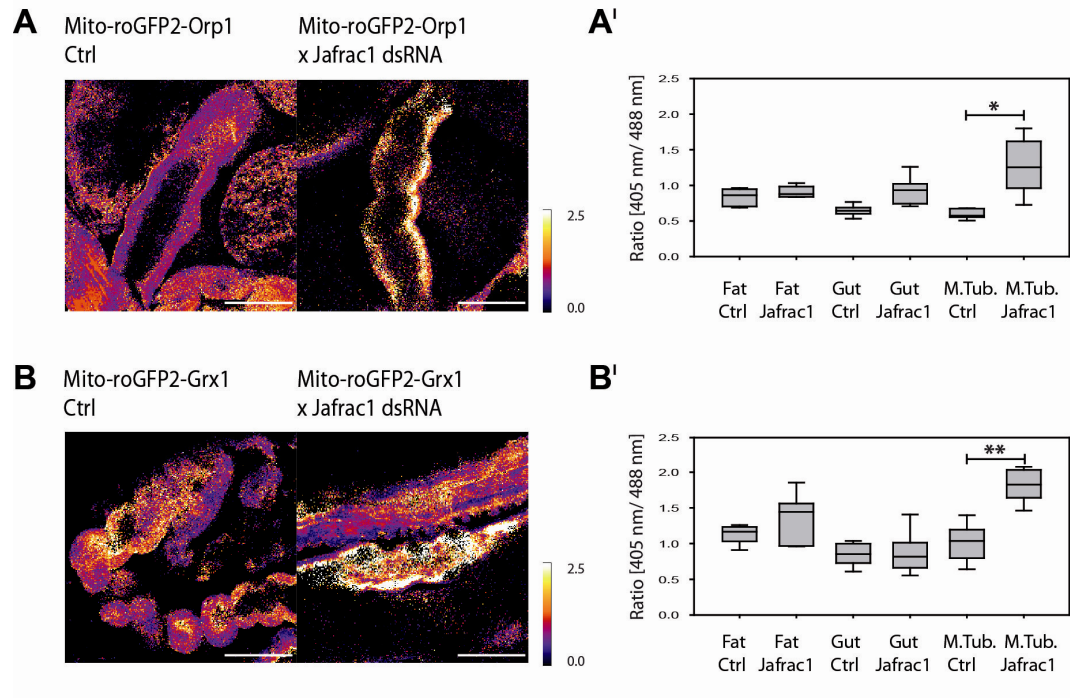


Figure S4: Jafrac1 silencing mediates an increase in mitochondrial oxidation in Malpighian tubules. Live imaging of mito-roGFP2-Orp1 (A) and mito-roGFP2-Grx1 (B) in Malpighian tubules (M. Tub.) of third-instar larvae. Scale bars: 75 μ M. Quantification (A', B') for three different tissues using 5 larvae per condition. Boxes: lower/upper quartile, whiskers: 5th/95th percentile. * $p < 0.05$, ** $p < 0.01$.

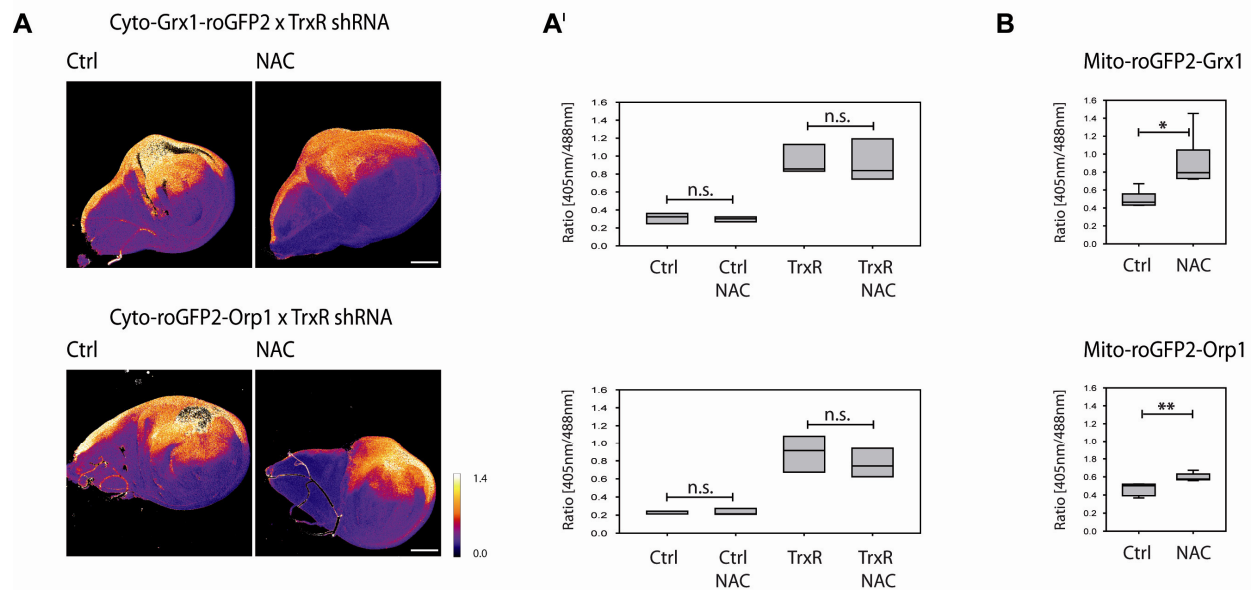


Figure S5: Influence of NAC on larvae and adult flies. (A, A') NAC feeding (1 mg/mL) does not counteract oxidative processes in the wing disc as mediated by silencing of TrxR. Wing discs expressing cyto-Grx1-roGFP2 (A, top) and cyto-roGFP2-Orp1 (A, bottom) together with TrxR shRNA under the engrailed promoter. The false colour scale applies to all four ratio images. Scale bars: 150 μ M. Quantification using 3 wing discs per condition (A'). Boxes: lower/upper quartile. (B) NAC feeding (1 mg/mL) for 48 h increases mitochondrial GSSG (top) and H_2O_2 (bottom) in the Malpighian tubules of adult flies. Quantification using 5 flies per condition. Boxes: lower/upper quartile, whiskers: 5th/95th percentile. * $p < 0.05$, ** $p < 0.01$.

Supplemental Experimental Procedures

Cloning of biosensor constructs

Constructs for the *in vivo* inducible expression of redox biosensors were generated by cloning cyto-Grx1-roGFP2, mito-roGFP2-Grx1, cyto-roGFP2-Orp and mito-roGFP2-Orp into pUAST. For stable ubiquitous expression of these biosensors, the open reading frames were cloned into the pCasPer4 vector containing an alphaTub84B promoter and SV40 polyA ('pCasPer4:Tub'). Full sequences are available upon demand. *pUAST-cyto-Grx1-roGFP2*: The roGFP2-Grx1 coding sequence was amplified from pLPCX-Grx1-roGFP2 (Gutscher et al., 2008) (forward primer: 5'-CCCTCTAGAAGATCT-3'; reverse primer: 5'-CCCGGTACCTTACTTGTACAGCTCGTC-3') and inserted into pUAST using BglII and KpnI. *pUAST-mito-roGFP2-Grx1*: The mito-Grx1-roGFP2 coding sequence was amplified from pLPCX-mito-Grx1-roGFP2 (Gutscher et al., 2008) (forward primer: 5'-CCCAGATCTATGGCCTCCACTCGTG-3'; reverse primer: 5'-CCCGGTACCTTACTTGTACAGCTCGTC-3') and inserted into the vector pBIND using BglII and KpnI. The mitochondrial targeting sequence from *Neurospora crassa* ATP synthase (NC) was exchanged for the mitochondrial targeting sequence from human cytochrome c oxidase 8 (COX8) (forward primer: 5'-GATCTATGTCCGTCCTGACGCCGCTGCTGCGGGCTTGACAGGCTCGGCCGGCGGCTCCCAGTGCCGCGCGCCAAGATCCATTCGTTGCCGCCGG-3'; Reverse primer: 5'-GATCCCGGCGGCAACGAATGGATCTTGGCGCGCGGCACTGGGAGCCGCCGGGCGGAGCCTGTCAAGCCCCGCAGCAGCAGCGGCGTCAGGACGGACATA-3') using BglII and BamHI. The roGFP2-Grx1 coding sequence was amplified from pBINAR-roGFP2-Grx1 (forward primer: 5'-CCCGGATCCGTGAGCAAGGGCGAG-3'; reverse primer 5'-CCCGGTACCCTGCAGAGCTCCAATCT-3'). Grx1-roGFP2 in pBIND was exchanged for roGFP2-Grx1 using BamHI and KpnI. The resulting COX8-roGFP2-Grx1 coding sequence was then transferred to pUAST using BglII and KpnI. *pCasPer-cyto-Grx1-roGFP2*: the cyto-Grx1-roGFP2 coding sequence was amplified from pUAST-cyto-Grx1-roGFP2 (forward primer: 5'-CCCGCGGCCGCATGGCTCAAGAGTTTGTG-3'; reverse primer: 5'-CCCTCTAGA TTA CT TGTACAGCTCGTC-3'). The sequence was inserted into pCasPer4:Tub using NotI and XbaI. *pCasPer-mito-roGFP2-Grx1*: the coding sequence of mito-roGFP2-Grx1 was amplified from pUAST-mito-roGFP2-roGrx1. (forward primer: 5'-CCC GCGGCCGCATGTCCGTCCTGACGC-3'; reverse primer: 5'-CCCTCTAGA CTGAGAGCTCCAATCT-3'). The sequence was inserted into pCasPer4:Tub using NotI and XbaI. *PCasPer-cyto-roGFP2-Orp1*: The roGFP2-Orp1 coding sequence was amplified from pLPCX-roGFP2-Orp1 (Gutscher et al., 2009) (forward primer: 5'-CCCGCGGCCGCATGGTGAGCAAGGGCG-3'; reverse primer: 5'-CCCCTCGAG CTATTCCACCTCTTTCAA-3') and inserted into pCasPer4:Tub using NotI and XhoI. *pCasPer-mito-roGFP2-Orp1*: The roGFP2-Orp1 coding sequence was amplified from pCasPer-

cyto-roGFP2-Orp1 (forward primer: 5'-CCCGGATCCGTGAGCAAGGGCGAG-3'; reverse primer: 5'-CCCTCTAGA CTATTCCACCTCTTTCAAA-3'). RoGFP2-Orp1 was exchanged for roGFP2-Grx1 in pCasPeR-mito-roGFP2-Grx1 by using BamHI and XbaI.

Technical information relating to roGFP2-based redox probes

pH-insensitivity of the fluorescence ratio

The ratiometric probe response is considered pH-independent because ambient pH does not significantly influence the measured fluorescence ratio (405 nm/488 nm) in the physiologically relevant pH range between 5.5 and 8.5. Thus, pH alterations within the physiological range are not expected to cause roGFP2 responses that could be mistaken for redox changes. The pH independency of the roGFP2 fluorescence ratio 405 nm/488 nm was initially documented by James Remington (United States Patent No. US 7,015,310 B2, Mar. 21, 2006), and was subsequently confirmed by others (Schwarzlander et al., 2008). The pH-stability of the fluorescent ratio is a basic feature of roGFP2 and therefore also applies to the fusion proteins. This was also shown experimentally (Gutscher et al., 2008) (see Supplementary Figure 2 therein). pH-insensitivity of the fluorescence ratio distinguishes roGFP2 from ratiometric redox probes based on cpYFP: e.g. cpYFP itself, claimed to be a ratiometric probe for superoxide (Wang et al., 2008), and HyPer which is a ratiometric probe for hydrogen peroxide based on cpYFP (Belousov et al., 2006). It has been found that the cpYFP fluorescence ratio responds sensitively to pH (Schwarzlander et al., 2011). The pH sensitivity of the HyPer fluorescence ratio has also been reported (Supplementary Figure 1 of Belousov et al., 2006). In fact, a HyPer mutant with inactivated H₂O₂-sensing domain (C199S) is now used as a ratiometric pH probe (named 'synthetic pH sensor' = SypHer) (Poburko et al., 2011).

Another pH-related aspect is the fact that a pH change always entails a shift of thiol-disulfide equilibria. Ratiometric probes that do not directly respond to ambient pH (as described above), may nevertheless sense redox changes that are caused by a pH change (e.g. a shift in the thiol-disulfide equilibrium of GSSG/GSH). However, both theoretical considerations and experiments show that roGFPs do not respond to pH-induced shifts of thiol-disulfide equilibria, because the mid-point potential of the roGFP2 thiol-disulfide redox pair shifts in concert with the mid-point potential of the equilibrating thiol-disulfide redox pair (Meyer and Dick, 2010).

Finally, pH changes are expected to influence the kinetics of disulfide exchange. Generally a higher pH will favor thiol deprotonation, and therefore reactivity. For roGFPs these kinetic differences only appear to be relevant on very short time scales (Schwarzlander et al., 2008) and are not expected to play a significant role for the organismal mapping of redox differences and changes.

In practice, for the measurement of fluorescence ratios and for the determination of the degree of oxidation (OxD), pH is neither expected to cause artifacts nor does it enter into calculations. Nevertheless, to calculate absolute E_{GSH} values from OxD requires the Nernst equation and thus needs a pH correction term should there be a deviation from standard conditions. Our recent review (Meyer and Dick, 2010) covers these and other probe related considerations in more detail.

Oxidation and reduction of the Orp1-based redox probe

Oxidant receptor peroxidase 1 (Orp1) is well characterized as a sensitive and specific H_2O_2 sensor protein by which yeast cells regulate their transcriptional response to H_2O_2 . The physiological function of Orp1 is to convert H_2O_2 into a disulfide bond and to transmit that disulfide bond to a specific recipient protein, the transcription factor Yap1, which in the roGFP2-Orp1 probe is functionally replaced by the fluorescent reporter roGFP2, see Gutscher et al., 2009. In yeast, Orp1 appears to contribute to H_2O_2 tolerance only as a sensor and not as a H_2O_2 -scavenging peroxidase (Toledano et al., 2004). The oxidation of Orp1 by H_2O_2 is expected to be highly efficient, considering second order rate constants typically exhibited by thiol-based Gpx family members, and the ~ 1.6 V potential difference (+1350 mV H_2O_2/H_2O vs. -315 mV Orp1[S-S]/Orp1[SH/SH]). The high efficiency of Orp1-mediated roGFP2 oxidation has been documented by *in vitro* experiments showing almost stoichiometric conversion of H_2O_2 into roGFP2 disulfide bonds (Gutscher et al., 2009). This suggests that reductive systems may only become effective in reducing roGFP2-Orp1 when H_2O_2 levels fall below a certain (low) threshold. Reduction of roGFP2-Orp1 takes place within minutes after a H_2O_2 bolus has been metabolized, and the kinetics of reduction is similar to the HyPer probe (compare Figure 5B in Gutscher et al., 2009), which is thought to be reduced by glutathione (Meyer and Dick, 2010). However, a role for the thioredoxin system is possible as well, as in yeast the Orp1 disulfide bond is known to be susceptible to reduction by thioredoxin.

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Seeing the Light: Probing ROS In Vivo Using Redox GFP

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Reactive oxygen species (ROS) dictate biological outcomes and are linked with myriad pathologies. However, measuring ROS in vivo remains a major obstacle in the field. Here, Albrecht et al. (2011) demonstrate the efficacy of redox-sensitive GFP in measuring glutathione redox state and H₂O₂ levels of tissues in *Drosophila*.

Biology at its core is based on making observations. The ability to detect a phenomenon with appropriate sensitivity and precision is crucial to testing a hypothesis. The study of reactive oxygen species (ROS) has been hampered by the inability to appropriately detect specific types of ROS. Since ROS have been implicated in multiple pathologies such as aging, cancer, diabetes, and neurodegeneration, as well as physiological process such as oxygen sensing, it remains critical that ROS levels be accurately detected (Finkel, 2011). Mitochondrial electron transport chain and NADPH oxidases are the major sources of superoxide (O₂^{•−}) generation within cells (Hamanaka and Chandel, 2010). Superoxide is rapidly converted to hydrogen peroxide (H₂O₂) by superoxide dismutases (SODs). H₂O₂ is converted into water by catalase, glutathione peroxidases (GPxs), and peroxiredoxins (Figure 1). Glutathione peroxidases reduce H₂O₂ to water by oxidizing GSH to GSSG. NADPH is utilized by glutathione reductase to convert GSSG to GSH. Historically, ROS have been implicated as inducers of lipid, protein, and DNA damage resulting in cellular toxicity. However, it is increasingly appreciated that low levels of H₂O₂ can participate in cellular signaling to maintain homeostasis while higher levels of H₂O₂ and hydroxyl radical (•OH) participate in cellular damage (Janssen-Heininger et al., 2008). The compartmentalization of H₂O₂ within cells is also likely to dictate biological outcomes. Thus, sensitive and precise measurement of H₂O₂ levels in vivo is essential to examining the role

of H₂O₂ in physiological or pathological processes. In this issue of *Cell Metabolism*, Albrecht et al. (2011) assessed GSSG/GSH ratio and H₂O₂ levels in *Drosophila* tissues by using redox-sensitive GFPs (roGFPs) targeted to either cytosol or mitochondria.

In the past two decades, fluorescent dyes have been widely utilized to measure H₂O₂ levels in vitro. However, these dyes have several limitations, since their signal is dependent on cellular uptake and can undergo photosensitized oxidation (Murphy et al., 2011). Markers of oxidative damage such as the detection of 8-hydroxyguanine are often utilized to detect oxidative stress in vivo. Measurement of the glutathione redox state (GSH/GSSG ratio) from tissues or cells is the other major technique to detect ROS levels. However, redox state of glutathione is not always reflective of H₂O₂ levels and varies within different compartments of the cell. Previously, Remington and colleagues developed a redox-sensitive GFP (roGFP) that makes it possible to decipher GSSG/GSH and H₂O₂ levels in different compartments within the cells (Hanson et al., 2004). RoGFPs are engineered to have two cysteine residues on their surfaces, and the oxidation of the cysteines determines the fluorescence property of the protein. An EGFP variant referred as roGFP2 has also been engineered to be redox sensitive. These are pH-insensitive, ratiometric, redox-sensitive fluorescent probes. RoGFP2 can be linked to glutaredoxin (Grx) or thiol peroxidase (Orp1) to increase sensitivity for measuring glutathione redox state or

H₂O₂ levels, respectively (Meyer and Dick, 2010) (Figure 1).

Albrecht et al. (2011) assessed GSSG/GSH and H₂O₂ levels in *Drosophila* tissues and whole body by generating four different transgenic flies containing roGFP2-Grx or roGFP2-Orp1 targeted to either the cytosol or mitochondrial matrix. A major obstacle in using fluorescence imaging in vivo is that not all parts of *Drosophila* are optically accessible. To capture redox state of the roGFP probes immediately after dissection, they developed a method whereby the tissues are exposed to the fast-acting, tissue-permeating alkylating agent, N-ethyl maleimide (NEM), followed by paraformaldehyde (PFA) fixation. NEM prevents thiol oxidation of the probe during tissue preparation and PFA fixation. The four different roGFP transgenic flies demonstrate both tissue-specific and cellular compartment-specific changes in glutathione redox state and H₂O₂ levels. For example, silencing of mitochondrial complex I subunit ND42 led to an increase in GSSG in the mitochondria but not the cytosol. The ubiquitous silencing of Jafra1, the major peroxiredoxin in *Drosophila*, increased both cytosolic H₂O₂ levels and GSSG only in certain tissues e.g., Malpighian tubules. These findings highlight the importance of combining genetic interventions that modulate ROS with proper tools to measure glutathione redox state or H₂O₂ levels.

A major question in the aging field is whether H₂O₂ levels increase or decrease during aging. Albrecht et al. (2011) decided to tackle this by examining H₂O₂

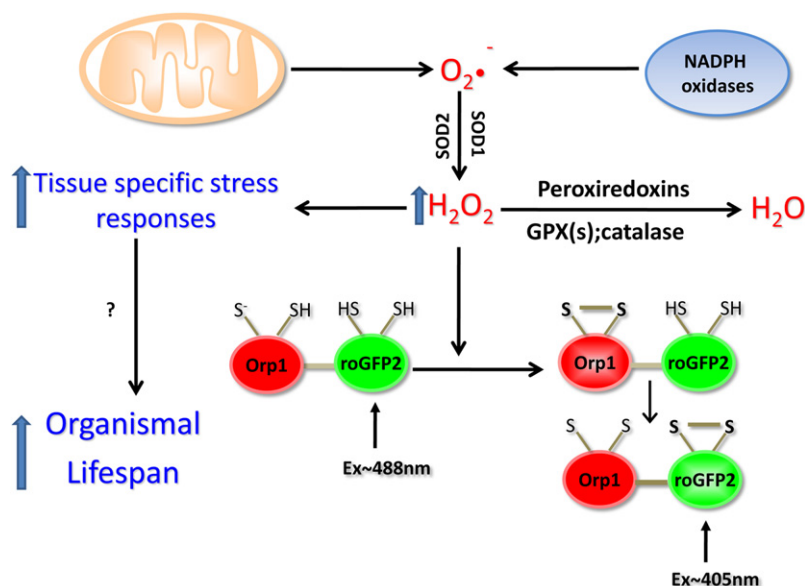


Figure 1. Redox-Sensitive GFPs Detect H₂O₂

Mitochondrial electron transport chain and NADPH oxidases can generate superoxide, which is converted into H₂O₂ by SOD1 or SOD2. H₂O₂ is detoxified into H₂O by glutathione peroxidases (GPXs), catalase, and peroxiredoxins. H₂O₂ can activate stress responses by initiating cellular signaling through oxidizing cysteine residues of protein(s), resulting in either activation or inactivation of the protein. RoGFP2 linked to Orp1 can be used to detect H₂O₂ levels. This probe can be localized to different intracellular compartments, including the mitochondrial matrix. H₂O₂ can oxidize the cysteine residues of Orp1, resulting in a disulfide bridge. Subsequently, thiol-disulfide exchange occurs between Orp1 and roGFP2 cysteines, resulting in the formation of the roGFP2 disulfide bridge. The reduced roGFP2 exhibits excitation around 488. Upon oxidation, roGFP2 gains excitability at 405 nm and loses excitability at 488. Albrecht et al. (2011) observed that an increase in organismal life span of *Drosophila* correlated with an increase in H₂O₂ levels in certain tissues. It is tempting to speculate that the increase in H₂O₂ levels will activate stress response in certain tissues that contribute to the fitness of the organism.

levels in the gut enterocytes of *Drosophila*, the most prominent site of age-related oxidation. They observed that females had higher levels of cytosolic H₂O₂ in the gut enterocytes than males. The females had a median life span 12 days longer than males. Furthermore, extension of life span in females by a mutation in the insulin/IGF signaling pathway was associated with increased cytosolic H₂O₂ levels in the gut. Although these findings do not establish causality that an increase in H₂O₂ results in an increase in life span, they are consistent with an emerging idea across multiple species that an increase in H₂O₂ levels correlates with longevity (Lapointe and Hekimi, 2010). A recent report in *Cell Metabolism* demonstrated in *Drosophila*

that dietary restriction (DR), known to increase life span, did not alter mitochondrial matrix H₂O₂ levels in vivo using a ratiometric mass spectrometry redox-sensitive probe (Cochemé et al., 2011). However, this study did not examine H₂O₂ levels in cytosol. It will be of interest to utilize these transgenic roGFP flies to assess whether DR also alters GSSG/GSH and H₂O₂ levels in the cytosol and mitochondrial matrix of specific tissues (Figure 1).

In establishing whether H₂O₂ is causal in biological processes, the field has been hampered by use of antioxidants such as N-acetylcysteine (NAC), which does not directly scavenge H₂O₂ and can also reduce disulfide bonds within proteins. Albrecht et al. report that NAC unexpect-

edly increased mitochondrial H₂O₂ levels in various tissues. Thus, the assumption that NAC is a pan-antioxidant should be treated with caution. NAC is a precursor to glutathione synthesis and is likely beneficial only in conditions where GSH is severely depleted. The recent development of mitochondrial-targeted antioxidants (Murphy and Smith, 2007) coupled with the use of roGFPs will be pivotal in determining whether mitochondrial oxidant production is causal in physiological or pathophysiological processes. Finally, the exciting findings of Albrecht et al. set the precedent for the development of transgenic mice containing roGFP2-Grx or roGFP2-Orp1 targeted to either the cytosol or mitochondrial matrix, thus enhancing our understanding the role of H₂O₂ in the context of signaling associated with normal physiology and pathologies associated with diseases.

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