Cooperative action of antioxidant defense systems in *Drosophila*

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Molecular oxygen is key to aerobic life but is also converted into cytotoxic byproducts referred to as reactive oxygen species (ROS) [1]. Intracellular defense systems that protect cells from ROS-induced damage include glutathione reductase (GR), thioredoxin reductase (TrxR), superoxide dismutase (Sod), and catalase (Cat) [2]. Sod and Cat constitute an evolutionary conserved ROS defense system against superoxide; Sod converts superoxide anions to H_2O_2, and Cat prevents free hydroxyl radical formation by breaking down H_2O_2 into oxygen and water [2]. As a consequence, they are important effectors in the life span determination of the fly *Drosophila* [3–7]. ROS defense by TrxR and GR is more indirect. They transfer reducing equivalents from NADPH to thioredoxin (Trx) and glutathione disulfide (GSSG), respectively, resulting in $Trx(SH)_2$ and glutathione (GSH), which act as effective intracellular antioxidants [2, 8]. TrxR and GR were found to be molecularly conserved [9]. However, the single GR homolog of *Drosophila* [10, 11] specifies TrxR activity [12], which compensates for the absence of a true GR system for recycling GSH [12]. We show that TrxR null mutations reduce the capacity to adequately protect cells from cytotoxic damage, resulting in larval death, whereas mutations causing reduced TrxR activity affect pupal eclosion and cause a severe reduction of the adult life span. We also provide genetic evidence for a functional interaction between TrxR, Sod1, and Cat, indicating that the burden of ROS metabolism in *Drosophila* is shared by the two defense systems.

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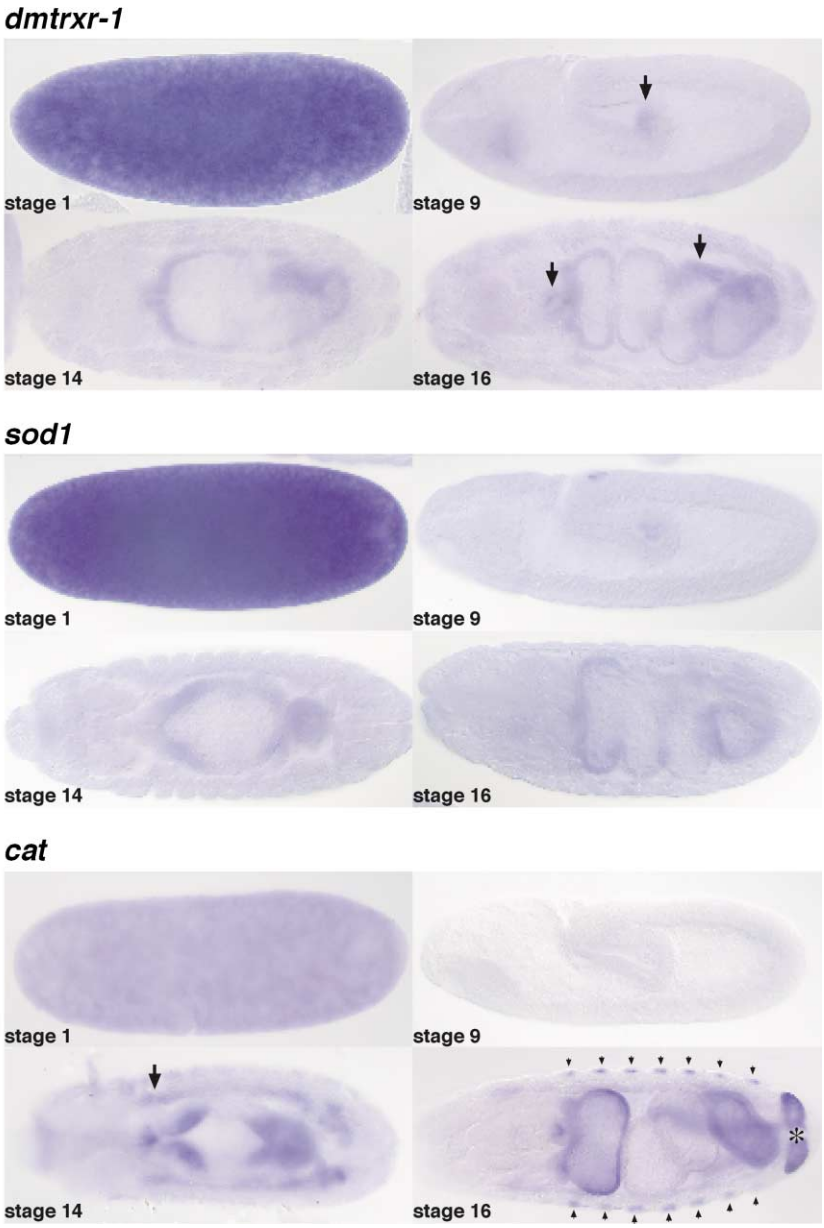
Results and discussion

A putative GR-coding gene of *Drosophila* was previously identified by comparative genome sequence analysis and named *gr* [10]. However, subsequent biochemical characterization of the gene product revealed that it exhibits TrxR instead of GR activity; therefore, the gene was renamed *dmtrxr-1* [12]. This finding, in conjunction with the apparent absence of a bona fide GR in *Drosophila*, suggests that, in flies, the recycling of GSH from GSSH is carried out principally by the thioredoxin system rather than by GR [12]. If TrxR plays the dominant role in GSH recycling, we would predict that genetic impairment of this thiol-based GSH recycling system would have serious debilitating consequences on the organism under normoxic conditions. To test this prediction, we generated and analyzed mutations in *dmtrxr-1*. We also used a loss-of-function *dmtrxr-1* mutation to determine the functional relationship between the Trx/TrxR system and the Sod1/Cat system of ROS metabolism; mutations of *sod1* and *cat* were shown previously to produce complex mutant phenotypes including a severe reduction of the life span [3, 4].

In order to visualize the predominant sites of expression of genes specifying the two antioxidant defense systems, we performed in situ hybridization of antisense RNA probes for *dmtrxr-1*, *sod1*, and *cat* to whole-mount preparations of embryos at different stages of development [13]. Figure 1 shows that the different transcripts are expressed maternally and that they are highly enriched in overlapping spatial patterns throughout embryogenesis, including the developing and mature midgut. *dmtrxr-1* and *sod1* are coexpressed in the germline progenitor cells, whereas *cat* is expressed in the fat bodies and the oenocytes (Figure 1c), which are thought to be involved in cuticle secretion [14, 15], detoxification [16], and pheromone production [17]. Thus, the key components of the two different ROS defense systems appear to be enriched in coextensive, spatially restricted patterns during embryogenesis. Sites of high expression include the gut and germ cells, both of which may require special protection against destructive oxidants.

The *dmtrxr-1* gene is located at polytene chromosome position 7D on the X chromosome [10]. In order to obtain mutants for the gene, we performed P element insertion mutagenesis screens [18], resulting in four independent single P element insertions (Figure 2a). Since all four insertion mutations caused the same hypomorphic mutant phenotype (see below), we continued to work with the semilethal P element insertion line *l(1)G0481*, termed *dmtrxr-1*^{481} mutation. Remobilization of the inserted P

Figure 1

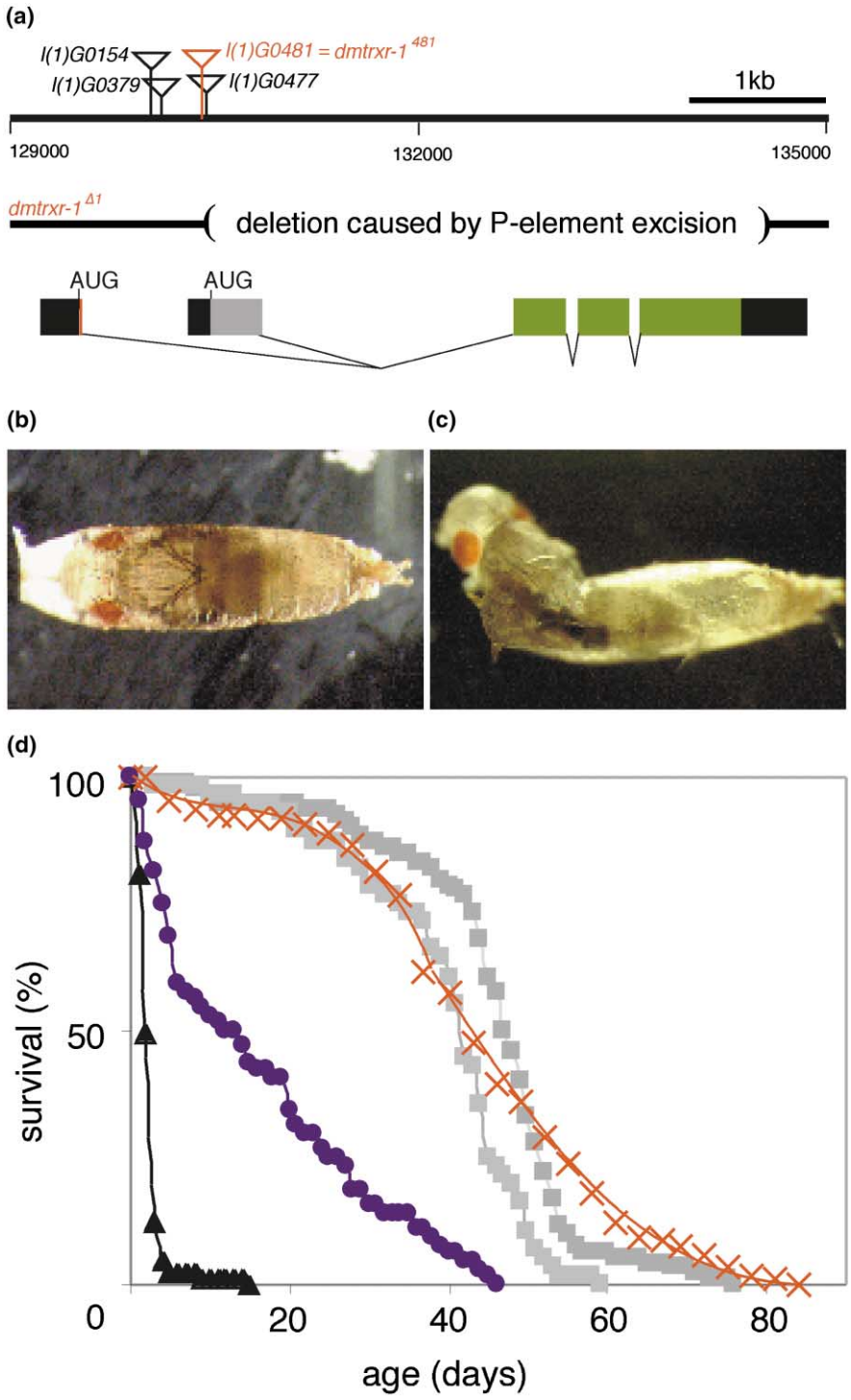


Expression of *dmtrxr-1*, *sod1*, and *cat* transcripts during *Drosophila* embryogenesis as revealed by RNA in situ hybridization to whole-mount preparations [13]. The top rows in (a)–(c) show lateral views of the embryos, anterior is left, and dorsal is top; stages according to [28]. **(a)** *dmtrxr-1* expression showing that the transcript is provided maternally (stage 1); zygotic expression occurs in germ cell progenitors (stage 9; arrow), the developing midgut (stages 14, 16), hindgut, and proventriculus (stage 16; arrows). **(b)** Sod1 transcripts are provided maternally (stage 1) and continue to accumulate in essentially the same spatial and temporal patterns as DmTrxR-1 transcripts. **(c)** Cat transcripts are provided in low amounts maternally (stage 1). Zygotic expression occurs in the same portions of the gut as observed with *dmtrxr-1* and *sod1*, but not in the germ cell precursors. Additional sites of *cat* expression are the fat bodies (stage 14; arrow), oenocytes (stage 16; arrowheads), and anal pads (stage 16; asterisk).

element caused a reversion to wild-type, indicating that the insertion was the cause of the mutant phenotype (see below). Furthermore, imprecise P element excisions caused small deletions within the *dmtrxr-1* open reading frame, resulting in *dmtrxr-1* lack-of-function alleles such as *dmtrxr-1*$^{\Delta 1}$ (Figure 2a).

The *dmtrxr-1* mutants present a set of complex phenotypes. Embryonic development was normal, and virtually all *dmtrxr-1*$^{\Delta 1}$ mutant larvae hatched. About 70% of these larvae survive to the first instar stage, only to die as second instar larvae without showing morphologically discernible phenotypes. This pattern of mortality suggests that the viability of *dmtrxr-1*$^{\Delta 1}$ mutants as embryos and first larval instars depends on TrxR function supplied by maternally-derived *dmtrxr-1* transcripts (see above). In contrast, all *dmtrxr-1*^{481} mutants develop into third instar larvae and 75% become pupae. Most *dmtrxr-1*^{481} mutant individuals die as normal-appearing pharate adults in the pupal cases (Figure 2b) or during the process of eclosion (Figure 2c). Only about 20% eclose into normal-appearing adults, the majority of which die within 2–3 days (Figure 2d; also

Figure 2



The *Drosophila* gene *dmtrxr-1*, P element insertion mutants, and their effect on pupal eclosion and life span. **(a)** A physical map of the *dmtrxr-1* locus, the P-lacW insertion sites [29], the two alternatively spliced transcripts (4 exons each; boxes), and the location within AE003443 DNA (in 7D18–20 of the X chromosome [30], as revealed by plasmid rescue of P element adjacent fragments and sequencing of both genomic and cDNAs [31]). Note the P element insertion *l(1)G0481* that represents the *dmtrxr-1*^{481} allele and the lack-of-function mutation *dmtrxr-1*$^{\Delta 1}$. The two transcripts code for different 5′ regions, resulting in different amino-terminal ends of the deduced protein (red and gray boxes, respectively). Remobilization of the P elements caused reversion of the mutant phenotype to wild-type, indicating that the insertion is the cause of the mutant phenotype [32]. **(b)** About 75% of the hemizygous *dmtrxr-1*^{481} males die as pharate adults or **(c)** during eclosion (see Table 1). **(d)** The life span of wild-type (red crosses) and hemizygous *dmtrxr-1*^{481} males (black triangles), hemizygous *dmtrxr-1*^{481} males ubiquitously expressing the biochemically characterized TrxR cDNA [12] (gray squares), as well as hemizygous *dmtrxr-1*^{481} males ubiquitously expressing *cat* cDNA (blue circles). Note that the life span of the eclosed hemizygous *dmtrxr-1*^{481} males is severely reduced (black triangles) and that their shortened life span was rescued in response to transgene-dependent TrxR (light and dark gray boxes represent two independent insertion sites on the second chromosome). Transgene-derived ubiquitous TrxR expression was achieved by the Gal4/UAS system [20] using the *act5C-Gal4* driver [21] in combination with *UASdmtrxr-1* cDNA (genotype: *dmtrxr-1*^{481}*/Y;; UASdmtrxr-1/+;; act5C-Gal4/+*). Cat expression was achieved with the same driver in combination with *UAScat* (genotype: *dmtrxr-1*^{481}*/Y;; UAScat/+;; act5C-Gal4/+*). *dmtrxr-1* cDNA (LD21729 [33]) was subcloned into the PUAST vector using XhoI and BglII sites; PUAS*dmtrxr-1* was used for transformation of *w*^{1} flies as described [34].

see below). Despite their shortened life span, adult mutant flies are able to mate and give progeny. No significant difference in pupal mortality and shortened adult life span was observed between homozygous *dmtrxr-1*^{481} females and hemizygous *dmtrxr-1*^{481} males (data not shown). These results suggest that the *dmtrxr-1*$^{\Delta 1}$ mutation is a null allele, whereas the *dmtrxr-1*^{481} mutation provides sufficient residual TrxR activity for incomplete development into pupae and even a few short-lived adults. To demonstrate that these effects are indeed caused by impaired activity of

Table 1

Effects of altered *sod* and *cat* expression on hemizygous *dmtrxr-1*^{481} males.

Genotype			X chromosome: + n	X chromosome: + (%)	X chromosome: *dmtrxr-1*^{481} n	X chromosome: *dmtrxr-1*^{481} (%)
X/Y ; + / + ; + / *TM3*			456[a]	(100)	117	(25.6)
X/Y ; + / + ; *sod*^{n108} / *TM3*			628[a]	(100)	15	(2.4)
X/Y ; + / + ; *cat*^{n1} / *TM3*			497[a]	(100)	47	(9.5)
X/Y ; + / + ; *act Gal4* / +			540[b]	(100)	93	(17.2)
X/Y ; *UASdmtrxr-1* / + ; *act Gal4* / +			346[b]	(100)	427	(123)
X/Y ; *UAShsod1* / + ; *act Gal4* / +			337[b]	(100)	9	(2.7)
X/Y ; *UAScat* / + ; *act Gal4* / +			232[b]	(100)	82	(35.3)

[a] X = *white*.
[b] X = *FM6*.
Hemizygous *dmtrxr-1*^{481} males derived from either w^1, *dmtrxr-1*^{481}/w^1;;***/TM3 × w^1/Y;;***/TM3 or w^1, *dmtrxr-1*^{481}/*FM6*;***/*** × *y*, w^1/*Y;;act5C-Gal4/TM6B* parents (chromosome *** is indicated). Eclosion rate of males containing a *dmtrxr-1* wild-type X chromosome (100%) relative to males bearing the *dmtrxr-1*^{481} mutant X chromosome in an otherwise identical genetic background. Balancer chromosomes and mutations are described [35].

TrxR-1, we expressed the biochemically characterized TrxR-1 enzyme [12] from an *actin*-Gal4/UAS-driven cDNA-containing transgene [20] in the *dmtrxr-1*^{481} mutants. The transgene-bearing hemizygous *dmtrxr-1*^{481} males develop into normal, healthy-looking adults. Irrespective of the chromosomal location of the *actin*-Gal4-driven transgene, which provides ubiquitous and constitutive TrxR-1 activity [21], the life span of the rescued flies is in the range of the wild-type life span (Figure 2d).

The results obtained with the hypomorphic allele, *dmtrxr-1*^{481}, suggest that impairment of TrxR activity has severe consequences on pupal mortality and adult life span similar to those reported earlier for mutants affecting the Sod/Cat-dependent ROS defense system [3, 4, 19] (see above). Because a functional reduction of each of the two antioxidant defense systems confers a similar visible mutant phenotype, we sought to determine by genetic means whether the two systems cooperate in vivo. We generated genetic strains containing the *dmtrxr-1*^{481}-bearing X chromosome in different genetic backgrounds that include heterozygosity for null mutations of the *sod1* and *cat* genes, respectively (Table 1). The scoring of the mutant effect was based on the frequency of eclosed adult males relative to the eclosion of males that contain the corresponding wild-type X chromosome. Table 1 shows that, depending on the chromosomal background, the eclosion rate of hemizygous *dmtrxr-1*^{481} individuals varied between 17.2% and 25.6% as compared to *dmtrxr-1*$^{+}$ siblings. Similarly, only 27% of homozygous *sod1*^{n108} mutant individuals eclose (data not shown), and, as observed with hemizygous *dmtrxr-1*^{481} individuals, the majority of the eclosed *sod1*^{n108} mutant flies dies within 2–3 days [3]. In contrast, *sod1*^{n108} heterozygotes eclose normally into adults with a normal life span [3]. However, only 2.4% ($p < 0.001$; chi-square analysis) of heterozygous *sod1*^{n108}, hemizygous *dmtrxr-1*^{481} mutant individuals eclose (Table 1). The significantly lower eclosion rate indicates that a partial reduction of *sod1* activity in *dmtrxr-1* mutants impacts pupal eclosion even more severely than either the complete absence of *sod1* or the partial reduction of *dmtrxr-1* activity alone. Likewise, only 9.5% ($p < 0.001$; Table 1) of heterozygous *cat*^{n1}, hemizygous *dmtrxr-1*^{481} mutant individuals eclose. Furthermore, hemizygous *dmtrxr-1*^{481} males, which are also homozygous for *cat*^{n1} or *sod1*^{n108}, die as pharate adults and never reach adulthood. This indicates that the double mutant defects are dependent on the gene dose of *cat* and *sod1* in conjunction with *dmtrxr-1*^{481}. Virtually the same result was obtained with different *sod1* and *cat* alleles generated in different chromosomal backgrounds, indicating that the observed interactions with *dmtrxr-1*^{481} are neither allele-specific nor background-dependent effects. Collectively, these results indicate that the Sod1/Cat and the Trx/TrxR systems share the burden of ROS defense. It should be noted that another important biological reductant in *Drosophila*, namely urate, plays an important antioxidant role in the crisis of oxidative stress that has been suggested to occur during late metamorphosis and eclosion [22].

In addition to mutant analysis, we also performed gain-of-function studies to express human Sod1, previously shown to substitute for the lack of *sod1* activity in the fly [6] (data not shown), and *Drosophila* Cat in hemizygous

dmtrxr-1 males. Sod1 and Cat were expressed from UAS-cDNA-containing transgenes in response to a Gal4 driver under the control of the constitutively active *actin* promoter [21]. Overexpression of human Sod1 reduced the frequency of pupal eclosure from 17.2% to 2.7% ($p < 0.001$; Table 1). This result is counterintuitive and shows that, in the absence of TrxR activity conferred by *dmtrxr-1* [12], enhanced levels of Sod1 are deleterious. This finding is consistent with a previous report showing that enhanced levels of Sod1 are to some extent deleterious even to wild-type flies [23]. A possible explanation for this phenomenon is that, in the presence of excess Sod1, superoxide is converted to H_2O_2 more rapidly than can be metabolized by Cat, which leads in turn to the increased generation of an H_2O_2-dependent hydroxyl radical [24]. Alternatively, or in addition, the effects of SOD1 overexpression may involve the bicarbonate-dependent peroxidase activity of Sod1 [25, 26].

In contrast, transgene-mediated augmentation of Cat increases the eclosion rate of hemizygous *dmtrxr-1* mutants 2-fold (35.3%; $p < 0.001$; Table 1). Presumably, increased Cat activity partially rescues the reduced eclosion rate of *dmtrxr-1* mutants by compensating for the loss of *dmtrxr-1* activity. In addition, the life span of the Cat-overexpressing hemizygous *dmtrxr-1* mutant flies is extended. Figure 2d shows that 40% of such flies survive 15 days after eclosion, by which time all hemizygous *dmtrxr-1* mutant flies have died. Thus, *cat* overexpression can partially compensate at the organismic level for the loss of *dmtrxr-1* activity. Interestingly, it was recently shown in yeast that many H_2O_2-inducible genes are upregulated in a thioredoxin reductase mutant (Δ*trr1)* [27].

In contrast to bacteria, worms, and mammals [12], thiol-based intracellular antioxidants of *Drosophila* and possibly other insects are generated by a single enzyme system in which TrxR plays a key role in maintaining intracellular redox homeostasis [12]. The results presented here show that genetically impairing the biochemically characterized TrxR activity [12] does not interfere with morphological aspects of fly development, but diminishes the viability of the organism, ostensibly due to unbalanced redox homeostasis of the organism. The similar phenotype of *sod1* and *dmtrxr-1* mutants, the coextensive tissue distribution of enhanced expression of the genes in the embryo, and the genetic interactions between components of the Trx/TrxR- and Sod/Cat-dependent ROS defense systems indicate that the two antioxidant defense systems can act in concert and can partially compensate for each other, at least in organismic terms. This study reveals a functional interaction between the well-established Sod1/Cat enzymatic system [2] and the newly described $Trx(SH)_2$/GSH thiol-based antioxidant system in a comprehensive system of ROS defense metabolism in *Drosophila*. Genetic intervention in this integrated ROS defense system can have far-reaching effects on both preadult development and on adult life span.

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References

1. Raha S, Robinson BH: **Mitochondria, oxygen free radicals, disease and aging.** *Trends Biochem Sci* 2000, **25:**502-508.
2. Halliwell B, Gutteridge JM: *Free Radicals in Biology and Medicine*, 3rd edn. Oxford: Oxford University Press; 1999.
3. Phillips JP, Campbell SD, Michaud D, Charbonneau M, Hilliker AJ: **Null mutation of copper/zinc superoxide dismutase in *Drosophila* confers hypersensitivity to paraquat and reduced longevity.** *Proc Natl Acad Sci USA* 1989, **86:**2761-2765.
4. Griswold CM, Matthews AL, Bewley KE, Mahaffey JW: **Molecular characterization and rescue of acatalasemic mutants of *Drosophila melanogaster.*** *Genetics* 1993, **134:**781-788.
5. Orr WC, Sohal RS: **Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster.*** *Science* 1994, **263:**1128-1130.
6. Parkes TL, Elia AJ, Dickinson D, Hilliker AJ, Phillips JP, Boulianne GL: **Extension of *Drosophila* lifespan by overexpression of human SOD1 in motor neurons.** *Nat Genet* 1998, **19:**171-174.
7. Sun J, Tower J: **FLP recombinase-mediated induction of Cu/Zn-superoxide dismutase transgene expression can extend the life span of adult *Drosophila melanogaster* flies.** *Mol Cell Biol* 1999, **19:**216-228.
8. Carmel-Harel O, Storz G: **Roles of the glutathione- and thioredoxin-dependent systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress.** *Ann Rev Microbiol* 2000, **54:**439-461.
9. Williams CH, Arscott LD, Muller S, Lennon BW, Ludwig ML, Wang PF, *et al.*: **Thioredoxin reductase two modes of catalysis have evolved.** *Eur J Biochem* 2000, **267:**6110-6117.
10. Candas M, Sohal RS, Radyuk SN, Klichko VI, Orr WC: **Molecular organization of the glutathione reductase gene in *Drosophila melanogaster.*** *Arch Biochem Biophys* 1997, **33:**323-334.
11. Mockett RJ, Sohal RS, Orr WC: **Overexpression of glutathione reductase extends survival in transgenic *Drosophila melanogaster* under hyperoxia but not normoxia.** *FASEB J* 1999, **13:**1733-1742.
12. Kanzok SM, Fechner A, Bauer H, Ulschmid JK, Muller HM, Botella-Munoz J, *et al.*: **Substitution of the thioredoxin system for glutathione reductase in *Drosophila* blastoderm.** *Nature* 1995, **376:**253-256.
13. Rivera-Pomar R, Lu X, Perrimon N, Taubert H, Jäckle H: **Activation of posterior gap gene expression in the *Drosophila* blastoderm.** *Nature* 1995, **376:**253-256.
14. Rinterknecht E, Matz G: **Oenocyte differentiation correlated with the formation of ectodermal coating in the embryo of a cockroach.** *Tissue Cell* 1983, **15:**375-390.
15. Schal C, Sevala VL, Young HP, Bachmann JAS: **Sites of synthesis and transport pathways of insect hydrocarbons – cuticle and ovary as target tissues.** *American Zoologist* 1998, **38:**382-393.
16. Ruiz de Mena I, Fernandez-Moreno MA, Bornstein B, Kaguni LS, Garesse R: **Structure and regulated expression of the delta-aminolevulinate synthase gene from *Drosophila melanogaster.*** *J Biol Chem* 1999, **274:**37321-37328.
17. Ferveur JF, Savarit F, O'Kane CJ, Sureau G, Greenspan RJ, Jallon JM: **Genetic feminization of pheromones and its behavioral consequences in *Drosophila* males.** *Science* 1997, **276:**1555-1558.
18. Cooley L, Kelley R, Spradling A: **Insertional mutagenesis of the *Drosophila* genome with single P elements.** *Science* 1988, **239:**1121-1128.
19. Parkes TL, Kirby K, Phillips JP, Hilliker AJ: **Transgenic analysis of the cSOD-null phenotypic syndrome in *Drosophila.*** *Genome* 1998, **41:**642-651.
20. Brand AH, Perrimon N: **Targeted gene expression as a means**

of altering cell fates and generating dominant phenotypes. *Development* 1993, **118:**401-415.
21. Gonzalez-Gaitan M, Jäckle H: **Tip cell-derived RTK signaling initiates cell movements in the *Drosophila* stomatogastric nervous system anlage.** *EMBO J* 2000, **1:**366-371.
22. Hilliker AJ, Duyf B, Evans D, Phillips JP: **Urate-null rosy mutants of *Drosophila melanogaster* are hypersensitive to oxygen stress.** *Proc Natl Acad Sci USA* 1992, **89:**4343-4347.
23. Reveillaud I, Niedzwiecki A, Bensch KG, Fleming JE: **Expression of bovine superoxide dismutase in *Drosophila melanogaster* augments resistance of oxidative stress.** *Mol Cell Biol* 1991, **11:**632-640.
24. Schonbaum GR, Chance B: **Catalase.** In *The Enzymes*, Vol. XIII, 3rd edn. Edited by Boyer PD. New York: Academic Press; 1976:363-408.
25. Liochev SI, Fridovich I: **On the role of bicarbonate in peroxidations catalyzed by Cu,Zn superoxide dismutase.** *Free Rad Biol Med* 1999, **27:**1444-1447.
26. Goss SP, Singh RJ, Kalyanaraman B: **Bicarbonate enhances the peroxidase activity of Cu,Zn-superoxide dismutase. Role of carbonate anion radical.** *J Biol Chem* 1999, **274:**28233-28239.
27. Carmel-Harel O, Stearman R, Gasch AP, Botstein D, Brown PO, Storz G: **Role of thioredoxin reductase in the Yap1p-dependent response to oxidative stress in *Saccharomyces cerevisiae.*** *Mol Microbiol* 2001, **39:**595-605.
28. Campos-Ortega JA, Hartenstein V: *The Embryonic Development of* Drosophila melonogaster. Heidelberg: Springer Verlag; 1985.
29. Bier E, Vaessin H, Shepherd S, Lee K, McCall K, Barbel S, *et al.*: **Searching for pattern and mutation in the *Drosophila* genome with a P-lacZ vector.** *Genes Dev* 1989, **3:**1273-1287.
30. Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PD, *et al.*: **The genome sequence of *Drosophila melanogaster*.** *Science* 2000, **287:**185-195.
31. Pirrotta V: **Cloning *Drosophila* genes.** In *Drosophila, a Practical Approach.* Edited by Roberts DR. Oxford: IRL Press; 1986:83-110.
32. Karpen GH, Spradling JC: **Analysis of subtelomeric heterochromatin in the *Drosophila* minichromosome Dp1187 by single P element insertional mutagenesis.** *Genetics* 1992, **132:**737-753.
33. Rubin GM, Hong L, Brokstein P, Evans-Holm M, Frise E, Stapleton M, *et al.*: **A *Drosophila* complementary DNA resource.** *Science* 2000, **287:**2222-2224.
34. Rubin GM, Spradling AC: **Genetic transformation of *Drosophila* with transposable element vectors.** *Science* 1982, **218:**348-353.
35. Lindsley DL, Zimm GG: *The Genome of* Drosophila melanogaster. London: Academic Press; 1992.